DYNAMIC MEASUREMENTS OF THE
REACTION OF SOLVATED PROTON
WITH ENERGY CONSERVING PROTEINS

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**ABSTRACT**

In the present study we employed the solvated proton as a gauge particle to probe the surface of proteins, with the purpose of characterizing the conformational changes associated with the function of energy conserving proteins. The measurements were carried out using the Laser Induced Proton Pulse technique, where a short laser pulse is used to perturb the acid-base equilibria in aqueous solution. The monitoring system consisted of time resolved spectroscopic measurements, following the absorbancies of the pyrane anion generated by the laser pulse in the bulk, and of a pH indicator attached to the protein under study. A rigorous kinetic analysis was carried out to determine rate constants of proton-transfer reactions which could reconstruct the whole set of observed signals by a single set of rate constants. A mathematical evaluation of the analytic procedure is given in Appendix A.

A preliminary step in this study was to reconstruct the reversible protonation of the surface group of cytochrome c. The protein was subjected to a brief proton pulse and the signals were analyzed to determine how many of the protein’s proton binding sites reacted with the discharged protons during the 1 µs perturbation; what the rate constants and the pK values of the reacting sites are, and to what extent the redox structure of the heme-iron affects the above parameters. The results of the analysis indicated high accuracy up to the point of identifying enhanced freedom of motion in certain sections of the protein (residues 15-44), in accord with the studies of Calvert et. al. [1].

Based on the established validity of the measurements, we investigated the proton binding capacity of the cytoplasmic surface of bacteriorhodopsin, comparing the properties of the (unphotolysed) BR state with that of the late M intermediate, whose physiological function is to pick free proton from the cytoplasmic matrix. The analysis indicates that the M state, either of the D96N mutant or the triple mutant (D96G/F171C/F219L), exhibits an enhanced proton binding capacity with respect to that of the BR state of the native protein. Comparison between the results of the kinetic analysis and inter-residues distances [2] indicated a correlation between the enhanced proton binding capacity and the rearrangement of the residues during the BR → M transition.

The fast proton binding capacity of the bacteriorhodopsin surface is in apparent contradiction with the slow equilibration of the BR surface, after the reformation of the protonated Schiff base (M state) with pH indicators in the bulk. On the basis of kinetics analysis, we concluded that the rate-limiting step is a propagation of ‘proton deficiency’
from the carboxylate of D96 to the surface of the protein. The slowness of the process implies that, in contrast to surface reactions, the propagation inside the protein calls for a coordinated conformation transition. Even replacement of a remote residue like K30, not apparently associated with the trajectory, can slow down the depletion propagation.

The main focus of this work was centered on providing an insight into the mechanism of the co-transport of lactose and proton by lac permease and that of the proton transfer through the membranal domain of the transhydrogenase.

The studies with lac permease focused on two aspects. The first was a direct measurement of the rate of proton entry into the substrate-binding site, using a fluorescein maleimide bound to C148 (at the substrate binding site) mutants of a cysteine less protein. It was demonstrated that the accessibility of the site to the bulk varies with the pH. In the low pH regime (pH ≤ 7.1), the site is well exposed, while in the range of pH ≥ 7.4, the site is constricted and the carboxylate moiety of E126 is very close to the bound dye. We suggest that the replacement of the protein-lactose interactions by the hydration of the substrate, after binding of a proton, is the driving force that expels the substrate of the site.

The inner space of the lac permease was also probed by a free diffusing proton, detecting the fine properties of the water molecule in the protein. The pyranine was adsorbed to the protein (at low ionic strength), at a site that is not the substrate binding one, but structurally coupled with it. Upon photo excitation, the dye generates an ion pair of excited pyranine anion and free diffusing proton, and time resolved fluorescence measurements followed its diffusion in the micro-cavity. The analysis indicated that the inner space of the cavity is characterized by intensive interaction of the water molecule with the protein site, leading to diminished activity (a_water = 0.71) of water molecules. When substrate is added to the complex, the dye is retained in the site but is better solvated. These two lines of research indicate that the lac permease is a flexible protein that undergoes a reversible cycle of solvation and replacement of the substrate-water interactions by substrate-protein interactions.

The last protein to be studied was the transhydrogenase. The enzyme was labeled by fluorescein either at the periplasmic or the cytoplasmic orifice of the proton-conducting channel. The analysis indicates that both sites are enriched with carboxylate and histidine clusters that enhance the delivery of proton to the channel. Detailed analysis also revealed a proton-binding site with relatively high pH that is in high connectivity with the dye, either when it is bound to the periplasmic or cytoplasmic surface. This site is proposed to be the
inner space of the proton-conducting channel. When the rate constants characterizing the reactivity of both sides of the enzyme with free proton are combined by a mathematical model, the short proton pulse is stretched over time and the release of proton on the cytoplasmic surface is comparable with the time constant of the hydride transfer between the two nucleotides.

To sum up, the Laser Induced Proton Pulse technique was shown to detect both small and large scale configurational changes and can be added to many other experimental techniques applied for mapping domains in proteins, besides characterizing protein conformational changes.
ABBREVIATION:

ΦOH: 8-hydroxy-pyrene-1,3,6, trisulfonate: pyranine
ΦΟ⁻: pyranine anion
Flu: fluorescein
EC: extracellular
CP: cytoplasmic
BR: bactiorhodopsine
SB: Schiff base
PM: purple membrane
LM: lauryl maltoside
Lac permease: lactose permease
TDG: β, galactopyranosyl 1-thio-galactopyranoside
PE: phosphatidyl ethanolamine
PG: phosphatidyl glycerol
NEM: N-ethylmaleimide
PCMB: p-Chloro mercurio benzoat
INTRODUCTION

Proteins are the most complex nano-scale structures and are characterized by sharp dielectric boundaries and strong electrostatic forces. The combination of these features with the flexibility of structure renders enzymes to be the most efficient catalytic structures in nature. The structural flexibility of protein enables them to respond to perturbing forces by altering their conformation. Thus, modulation of the protein’s charge by a redox reaction, uptake of a charged substrate, or passage of ion through the protein, will impose a set of novel electrostatic interactions for which the protein will compensate by changing its conformation or dissipating the extra energy by interacting with its environment like binding/release of a proton (proton pumping), transforming the structure of a substrate (generation of chemical energy) or perturbing the potential energy of solutes separated by an impermeable membrane (active transport). According to this concept, the elasticity of the protein is instrumental in the convergence of one form of energy (such as electrostatic potential) into other forms (chemical bond synthesis, active transport, or redox reactions). In the present study we shall investigate some of these features by using the free diffusing proton as a gauge particle for probing the surface of energy conserving proteins during conformation changes.

The passage of proton through proteins is common to most membranal energy-conserving enzymes. While the routes of proton passage differ among the various proteins, the mechanism of proton propagation is based on the same chemical-physical principles. Due to its high charge density and solvation energy, a proton will not be readily inserted into the low dielectric constant matrix of a protein unless there is appropriate compensation by some structural rearrangement, or it is covalently bound to an anionic site. The stabilization of the proton in the protein is only the first step in the pumping mechanism; the proton has to re-dissociate and react with one or many sites until it crosses the protein and is released to the bulk on the other side. In the present research we shall regard proton pumping as a sequence of dissociation, diffusion and rebinding steps. The proton progresses through a sequence of dissociation/association steps, where the protein and water molecules function as a solvent that lowers the energy penalty associated with the generation of ions in the
protein. The propagation of the proton in the protein is a random walk between the temporary proton binding sites that make the conducting path that is biased by the intra-protein electrostatic potential. In the present work we wanted to elaborate on the coupling of the mechanism of proton transfer within proteins with their biological activity. In accordance with this mechanism, the research focused on the mechanism of the partial events: the dissociation, diffusion and forces that convert a channel into an efficient pump.

Naturally, the most suitable probe reaction for the proton-pumping protein will be the proton transfer reaction. In the present study we describe how the proton transfer reaction was applied for the study of structural organization of defined domains on the surface of some proteins. In the first step, the sensitivity of the probing reaction was evaluated by measuring how it is affected by minute modification of a small globular soluble protein, like changing the redox state of the iron atom in cytochrome c from the ferric to the ferrous state. Once the sensitivity of the monitoring system and the accuracy of the kinetic analysis were established, we tested two proteins that utilize the $\Delta \mu H^+$ as a driving force for their catalysis. One is the lac permease and the other was the transhydrogenase from *E. coli*. For the study of conformational changes associated with proton transfer we used the bacteriorhodopsin, a well-known proton pumping protein with a highly resolved structure.

**THE LASER-INDUCED PROTON PULSE**

The basic reaction, common to all our studies, is the excitation of aromatic alcohols (ΦOH) to their first electronic singlet state. Following excitation, the depletion of electron density between the oxygen and hydrogen atoms leads to proton dissociation and an ion pair (ΦO*+H+) is formed. The dissociation dynamics are measured by following the fluorescence of the excited dye molecule with the time window comparable to the relaxation-dissociation reaction. The dissociation step is associated either with the appearance of the red-shifted fluorescence of the ΦO* state and the decay of the ΦOH* emission. The proton is released at close proximity to the anion, thus its initial dispersion in the bulk is inherently non-homogeneous, and the probability of finding the proton near the excited anion is higher than that averaged
for the whole space of the solution. For this reason the dynamics of the system are best described by the geminate recombination formalism of Agmon as detailed below.

Ultra-fast (femto-second to nanosecond) spectroscopic measurements reveal the most initial events: the dissociation of the covalent bonds, the solvation of the photoproducts and their diffusion in the solvent. The dynamics of these events is described by a Brownian motion in a non-homogeneous electric field as formulated by the time-dependent Debye-Smoluchowski equation [3-5]. The mathematical model of Agmon, which implements the Debye-Smoluchowski equation for a Brownian motion propagation of a charged particle, is an extremely versatile one. Initially fast dissociation kinetics were used to measure the rate constants of proton dissociation and correlate them with the physical chemical properties of aqueous solution [6] [7]. Later, Agmon applied the Debye-Smoluchowski equation for describing the geminate recombination in terms of a Brownian diffusion under electrostatic potential gradient [4, 8] and the analysis of the measured signals became a straightforward operation so that many systems could be investigated: Concentrated salt solution [9-11], mixed solution [12] Solid-water interface [13], solvation dynamics [14, 15].

The essence of the ultra-fast kinetics is the corollary between the short observation time and the size of the space probed by the proton. Within the few nanoseconds observation, the proton cannot diffuse more than a few nm around the parent molecule. The brief observation period allows scanning domains no larger than an active site of an enzyme [16-20]. Accordingly, the time resolved fluorescence decay was also used to measure the physico-chemical properties of microscopic cavities defined by lipids or protein matrices: the inter-membranal aqueous phase between phospholipids membranes [21, 22], the microcavities of apomyoglobin and the PhoE channel [18, 19] and mitochondrial preparations [23, 24]. These experiments determined the chemical potential of the water in the measured spaces and the diffusion coefficient of the proton.

In parallel with the ultra-fast experiments, in this study we have been using the proton pulse as a general perturbation of acid base equilibria in aqueous solutions. In these experiments a proton emitter is added to a protein solution and excited by a nanosecond laser pulse. The discharged protons are detected by the transient absorbencies of the proton emitter anion, which quantify the proton-balance in the bulk of the solution, while the reaction of protons at the protein’s surface are monitored by the state of protonation of pH indicators bound to proteins [23, 25, 26]
The Laser Induced Proton Pulse was also used for selective acidification on one side of a phospholipid membrane and to measure the mechanism of ion transport by ionophores [27-29].

The release of protons into aqueous solution initiates a large number of simultaneous proton transfer reactions having a diffusion-controlled rate constant. The kinetic analysis of the transients is treated as a sum of many simultaneous parallel reactions. The reconstruction of the observed dynamics is attained by numeric integration of rate equations that account for all proton transfer reactions, which take place within the perturbed space. Each reactant is coupled with all other proton-binding sites by chemical reactions that were converted into the rate constants of the differential rate equation [30]. These equations determine how the velocity of each reaction is affected by all other reactants. The numeric integration of these equations produces the temporal protonation of every reactant during the course of the observation. The kinetic analysis of the results consists of treating the rate constants as adjustable parameters. A systematic search over the parameter space is carried out looking for a combination of rate constants that will reproduce, with high accuracy, a large number of experimental observations gathered under a wide range of initial conditions.

Transient absorbance measurements were carried out with systems of increasing complexity, beginning with binary mixtures of the proton emitter and pH indicator, through micellar models, ending with a protein like bacteriorhodopsin [31-36], cytochrome c oxidase [25] and cytochrome c [37]. The versatility of proton transfer measurements is evidenced by the variety of systems that were subjected to analysis; membrane surfaces [24, 27, 38], protein-water interfaces [17, 25, 32-34, 39, 40], the mechanism of ion transport by ionophores [28, 29] and the gramicidin channel [41].

THE KINETIC PROPERTIES OF PROTON TRANSFER REACTIONS

The solvated protons are involved in all biochemical-catalytic reactions and have a special role in the energy-conservation processes of the biosphere. From a biophysical point of view, these processes are based on the same mechanism: the proton progresses through a sequence of dissociation-association steps where the protein and water molecules function as a solvent that lowers the energy penalty associated with the generation of ions in the protein.
The rate constant of diffusion-controlled reaction is given by the Debye-Smoluchowski equation, which correlates between well-defined molecular physical parameters and the rate of collision [16, 30, 38, 42]:

\[
\text{Eq. 1.1} \quad k_{dc} = \left[ \frac{4\pi N}{1000} \sum_{D} \cdot R_0 \right] \cdot \left[ \frac{\delta}{\exp(\delta) - 1} \right] \times \left[ \exp\left(\frac{\delta R_0}{1 + \kappa R_0}\right) \right]
\]

The first term of the equation defines the rate of collision between reactants in the absence of electrostatic interactions. The parameters appearing in the first term are \(N\) (Avogadro number), \(\sum D\) (sum of diffusion coefficient of the reactants), \(\kappa\) is the Debye length and \(R_0\) (collision distance). For a free diffusing proton \(D_{H^+} = 9.3 \times 10^{-5}\) cm\(^2\)/s and \(R_0 \approx 6\) Å, the resulting rate of encounter between \(H^+\) and the uncharged target is about \(4 \times 10^{10}\) M\(^{-1}\)s\(^{-1}\).

The other terms in Eq.1.1 account for the electrostatic interaction between \(H^+\) and the acceptor. The intensity of the interaction is expressed by the term \(\delta\), which is the ratio between the Coulomb cage radius \(R_c\) and \(R_0\). The former is the distance at which the electrostatic interaction, whether attractive to or repulsive between the reactants, equals the thermal energy (\(e_o\) is the electronic charge (4.8 \(\times\) \(10^{-10}\) e.s.u) and \(k_B\) is the Boltzmann constant).

\[R_c = |Z_1Z_2|e^2/\varepsilon k_BT\]

At room temperature and for \(|Z_1Z_2|=1\), the value of \(R_c\) is 7 Å. (\(R_c\) is also referred to in some texts as the Debye radius or Onsagar length.) The nature of the electrostatic interaction is given by the sign of \(\delta\), which is equal to that of the charge product \((Z_1Z_2)\). Attractive interactions will have a negative \(\delta\) value, while repulsive forces are equated with a positive value of \(\delta\).

Once a proton (or other charged particle) penetrates the Coulomb cage, its diffusion is biased by the electrostatic interaction. If the interaction is attractive (\(\delta<0\)), the ions will collide in less than 1 ns. Repulsive interactions (\(\delta>0\)) will cause the particle to be deflected and the probability of encounter will decrease. The relationship between \(\delta\) and the rate of the reaction is given by the term \((\delta/)(\exp(\delta)-1))\). A strong repulsive potential, \(\delta>1\), shrinks the expression very rapidly. On the other hand, for attractive potential, the expression approaches linearity with \(\delta\) for \(\delta<-3\). Thus, a very positive acceptor can effectively avoid protonation, while realistic negative charges cannot increase the rate of protonation, even by a factor of 10.
The last term in Eq. 1.1 accounts for the effect of the ionic strength on the rate of reaction between charged particles. It combines $\delta$, the radius of encounter ($R_o$) and the Debye length ($\kappa=3.3 \times 10^{-7} (I)^{1/2}$ (in cm$^{-1}$ units), where $I$ is the ionic strength of the solution) into an expression which suppresses the intensity of the electrostatic interaction. The larger the charge of the acceptor, the more susceptible it will be to screening by ions in the solution. Under physiological conditions, in the presence of about 10mM buffer or substrate as in many biochemical reaction mixtures, a single charge anion will react with a proton at a rate of about $(1-4) \times 10^{10}$ M$^{-1}$s$^{-1}$. Rate constants that are lower than these values suggest some structural peculiarities of the proton-binding site.

**THE DISSOCIATION OF A PROTON**

The dissociation reaction is the first step in any proton transfer reaction, whether it proceeds in aqueous solution or inside a protein. It calls for collaboration between an acid molecule and the solvent, since it is actually a proton transfer from an acidic moiety (AH) to the solvent. The dynamic aspects of the dissociation reaction depend on the nature of the reactants.

Under conditions where the acceptor availability is not rate limiting ($a_{water}=1.0$), the rate is controlled by the thermodynamic stability of the HA. A reversible one-step dissociation will fit the free energy relationship between the rate constant (association/dissociation) and the thermodynamic constant: $k_d=k_aK_d$

As in most cases, the binding of proton ($k_a$) is a diffusion-controlled reaction, and its value will vary over a rather narrow range. Consequently, the rate constant of proton dissociation will be directly proportional to the pK. The rate of proton dissociation varies over a huge range: a strong acid will surrender its protons to the solvating water within a few pico-seconds, while the H$_2$O molecule will dissociate spontaneously (to H$^+$ and OH$^-$) only once in 9h. Still, these two reactions are basically similar: a transfer of H$^+$ from the potential donor well to that provided by a pre-existing, short life time, configuration of water molecules that serve as an acceptor. Consequently, we must distinguish between the properties of the two partners, the acid and the solvent. As the acidic moiety dissociates into an ion pair, the electrostatic energy of the system increases. Thus, the solvation of the products should be energetically sufficient to compensate for the incremental self-energy of the system.
For a proton dissociating at the protein’s surface, there are ample water molecules to stabilize the products. Inside a protein, both the intra-protein water molecules and the protein itself should function as part of the stabilizing system.

**PROTON UPTAKE BY THE PROTEIN’S SURFACE**

The rates measured for the protonation of monensin imbedded in phospholipid membranes and for the reaction of phosphatidyl serine with free protons are $1 \cdot 10^{10} \text{ M}^{-1}\text{s}^{-1}$ [43], while the entry port of the gramicidin channel reacts with free protons at $1.5 \cdot 10^{10} \text{ M}^{-1}\text{s}^{-1}$ [41]. These rate constants are compatible with the Debye-Smoluchowski equation. Still, in some cases, like the cytoplasmic side of BR, the rate constants were found to be as high as $5.5 \cdot 10^{10} \text{ M}^{-1}\text{s}^{-1}$ [33-36], which is higher than the Debye-Smoluchowski prediction. This implies that the carboxylates on these enzymes are in close proximity that increases their electrostatic attraction and enlarges their probability of protonation.

In contrast with the fast reactions that were recorded for cytochrome c oxidase or bacteriorhodopsin, the reprotonation of the surface of lac permease or transhydrogenase was much slower. This indicates that both enzymes had a poor capacity to compete for free protons. These proteins’ surfaces are kinetically inefficient proton acceptors, in accordance with their physiological function that is limited by the substrate binding/release dynamics instead of the proton limitation as noticed for the catalytic cycle of cytochrome oxidase.

It is of interest to note that proteins, like cytochrome c oxidases or reaction center, that have to generate a $\Delta \mu H^+$ operate under high flux conditions, developed a system that enhances their reaction with bulk protons [25] (Y. Marantz, Ph.D. thesis). In contrast, the surfaces of transhydrogenase and lac permease are kinetically inefficient proton acceptors, which is in accordance with their physiological function that is limited by the substrate binding/release dynamics.

At present, we have limited information for statistical correlation between the physiological function of a protein and its capacity to react with protons in the bulk. Yet, a comparative study with bacteriorhodopsin (see chapter 2a) reveals that an enhanced reactivity with protons is well correlated with physiological demands.
PROTON EXCHANGE BETWEEN ADJACENT GROUPS

A proton, just released from an acidic moiety, cannot disperse into the bulk of the solution without mounting over the electrostatic barrier of the anion's attraction. The size of the barrier is given by the attractive electrostatic potential that decays with the distance, reaching the value of the thermal energy at the distance given by the Coulomb cage radius. The electrostatic force causes the proton to slide backwards with the reformation of the covalent bond, a process called geminate recombination [3, 19, 44]. The dissociation of an acidic moiety is a series of numerous dissociation recombination attempts, each leading to an increment of the dissociated pair populations [16, 39, 40]. Yet, as the time interval between proton dissociation and subsequent recombination is a few ns or less, the equilibrium is established very rapidly. The high efficiency of the geminate recombination suggests that, for two sites that are within the R_C distance, a proton released from one will have a high probability to react with the next, with minimal loss due to dispersion to the bulk [38-40]. The rates of proton transfer to a nearby site was measured as the rate of deprotonation of an excited pyranine molecule in concentrated solution (up to 8M) of acceptor, such as acetate, and was found to be as high as $1 \cdot 10^{12} \text{M}^{-1}\text{sec}^{-1}$ [45].

The carboxylate and histidine residues of a protein are rigidly fixed on its rigid scaffolding and cannot freely diffuse one with respect to the other. Still, these residues can rapidly exchange protons amongst them at a very fast rate. The probability and the rate of proton exchange between surface sites is a function of the nearest approach between them and the frequency at which they reach that transient configuration. The reactions under these conditions are reminiscent of proton dissociation in water, except that the acceptor is not the water molecules but the local proton acceptor that acts as the solvent. When the local transient conditions are favorable, the proton transfer reaction to the acceptor can be faster than the dissociation reaction, especially in a micro-cavity where the activity of the water can be small [45]. Studies with model systems established that the rate constant of proton exchange between sites anchored on a rigid body could be expressed by a 'virtual second order rate constant' [32] and their values can be as high as $10^{12}$. Due to the virtuality of the concentration term used in the calculation, the $\text{M}^{-1}\text{s}^{-1}$ unit cannot be assigned to these rate constants. In the present text, all virtual second order rate constants are printed in *italics*. 
The primary calibration of the virtual second order reaction was attained by studying the velocity of protonation of the chromophoric site of fluorescein derivatives, where additional proton binding sites were attached to the dye’s skeleton [32]. The rates of the reaction of the dye with free protons were found to be within the limits set by the Debye-Smoluchowski equation for diffusion controlled reactions. However, the rate constants for proton exchange between the sites on the dye molecule were exceptionally high, as much as $10^{12}$ M$^{-1}$s$^{-1}$. Such high rates imply that the reaction mechanism is not a collision between two dye molecules but rather a sequential dissociation of the proton from one site and rebinding by the next site, on the very same molecule before the proton is dispersed in the bulk. Thus, the virtual rate constant reflects the same considerations that were discussed in the above section, using terminology of chemical kinetics. Comparison of virtual second order reaction between sites on the fluorescein skeleton revealed a wide variation in the rate constants. This variation indicates that other terms, like solvation shell and local electrostatic potential, also affect the measured rate constants [32].

As a rule, when two residues exchange protons at a rate of $10^{12}$, the two sites are separated by either one or two water molecules, which facilitate rapid proton exchange between them. When the virtual rate constant is $\sim 10^9$, the two sites are 10-15 Å apart, or a positive charge is inserted into the proton transfer trajectory. A slower virtual rate constant implies that the mechanism of the reaction is dissociation, followed by random diffusion until the proton encounter with the acceptor site.

**ELECTROSTATIC PROPERTIES OF PROTEIN**

The electrostatic forces within a protein are made by the partial charges of intra-protein residues, surface charges, dipoles of the peptide bonds and the external field. The low dielectric constant of the protein, together with the shape of the intra-protein cavities and the short distances, also contribute to the electrostatic potential and cause its gradient to be extremely intensive [46]. For example, when the pyranine anion is inserted into the heme binding site of apomyoglobin, its charge is neutralized by the intra-cavity residues to $Z_{\text{net}}=\pm 1$ [19]. Still its Coulomb cage radius (the distance where the electrostatic potential of the proton is equal to the thermal energy) was measured as $\sim 70$ Å, meaning that its electrostatic field expands out of the cavity.

The low dielectric constant of the protein matrix allows the electrostatic potential to propagate over a long distance, coupling the charge translocation with pK
shifts at remote sites. The best-studied example of that long-range coupling is the linkage of electron and proton transfer in the QA and QB domains of the bacterial reaction center [47-52]. Following photo-excitation, the special pair of the reaction center ejects an electron into the cytoplasmic side of the protein, forming the first stable reduction QA \(^{-}\). The one electron reduced quinone has to be stabilized in order to prevent rapid recombination of the electron with the oxidized special pair. This stabilization is gained by raising the pK of surface groups leading to their incremental protonation. Proton uptake measurements carried on several mutants of the reaction center showed that even groups located 17 Å from the QA \(^{-}\) site, such as GluL212, are influenced by the extra charge and participate in its stabilization [53]. The reaction of the proton binding sites in the QA and QB domain, with protons coming from the bulk, partially compensates for the electron charge on the quinone. Similar processes were measured with the stabilization of the reduced QB.

The pK shifts, caused by the appearance of the electron charge on the quinone, are difficult to measure. Such pK shifts, theoretically predicted on the basis of electrostatic calculations, impose technical limitations especially when the responding residues have a pK in the region where the protein is no longer stable. Monitoring a pK shift of a carboxylate residue, with a pK in the range of 4 to 5, calls for lowering the pH of the solution to a point where the protein might denaturate. Even if the protein can survive the treatment, the gradual protonation of the protein during its acidification affects the total charge and alters the pK, which is the very same parameter that has to be measured [54, 55]. To measure the effect of charge increment on the pK of surface groups, the measurements should be done on the time scale of the protonation events (µsec), hence the relationship between the protonation/deprotonation events can establish the pK of the reacting groups. In this thesis, changes in the charge distribution or exposure of a protonable site, appearing as a pK shift in cytochrome c, will be demonstrated utilizing the Laser Induced Proton Pulse method.

**OBJECTIVES AND SIGNIFICANCE**

The long-term goal of the project is to probe the surface of enzymes involved in the chemiosmotic mechanism through the application of the kinetic analysis of proton transfer reactions between residues on their surface. For this purpose we defined the following goals of the research:
• To study the reaction of bulk protons to the surface of proteins, with emphasis on those utilize the potential of the proton gradient (ΔµH⁺).
• To employ the free diffusing proton as a gauge particle for quantitating the intra-protein electric fields with special emphasis on energy transforming sites.
• To follow the proton movement through the proton’s pathway in the proteins.
• To search for kinetic characterizations of the different proteins in order to deduce common features and their biological implications.
• To utilize ultra-fast proton transfer reactions for probing the physical-chemical properties of microscopic biological structures.

Two proteins, whose structures are known up to a resolution of 1.5-1.8Å, were used as a model: cytochrome c and bacteriorhodopsin. Cytochrome c was our test case to discover the extent of the accuracy of the methodology and was used for probing conformational change caused by inserting negative charge to the protein. The bacteriorhodopsin was used for two purposes: 1) Probing the change in the proton binding capacity of the cytoplasmic surface after discharging of the proton to the extracellular side. 2) Timing of the propagation of conformational changes after the deprotonation of D96.

The other two investigated enzymes, lac permease and transhydrogenase, were selected as representative enzymes that couple cross-membranal proton transfer with energy-dependent active transport or with uphill electron transfer reactions. An understanding of the molecular basis of these biological processes requires the investigation of structure-function relations of the proteins. Both enzymes are crystallization-resistant and their proposed structure is not based on diffraction measurements, but was deduced through analysis of biochemical reactions (indeed the proposed structures have been changed during the period of this work). For these reasons, we used the rate of proton transfer between a pH indicator attached to a defined residue and its nearby sites, as a measure of local organization over small domains on the two proteins.
APPLICATION OF KINETIC MEASUREMENTS FOR THE STUDY OF PROTON DRIVEN ENZYMES

In the first experimental chapter of this study we tested the reliability of our measuring system by quantitating the proton binding capacity of cytochrome c in its ferric and ferrous states. As will be shown, the charge of the iron atom affects the proton binding capacity within the range of significance of the detection limit.

In chapters 2a and 2b, we investigated the proton transfer system of the cytoplasmic surface of bacteriorhodopsin. At first we compared the rate of proton delivery to the most-basic unoccupied residue on the cytoplasmic surface and noticed an enhancement of the proton collecting antenna properties of the protein when it was transformed from the resting state (BR state) to the deprotonation Schiff-base configuration (M state). In the other study with bacteriorhodopsin, carried out in collaboration with N. Dencher, we made a kinetic comparison between the rate of protonation of the Schiff-base vs. the rate of surface protonation and concluded that the compensation of the missing proton on the charged D96 inside the protein is rather slow. The mechanism of this reaction can be represent by a ‘hole propagation’ from residue D96 up to the first element of the antenna (D38), and due to some mutations, can take hundreds of milliseconds. This rate is orders of magnitude slower than the equilibration between the bulk and the protein’s surface. The kinetic measurements were corroborated by the available structural information of the bacteriorhodopsin.

Both the other proteins we have studied (lac permease and the transhydrogenase) are ‘slow turn over’ enzymes, where the rate-limiting step is the binding-release of substrate. The experimental algorithm was to label each enzyme, at a selected site, by fluorescein maleimide and to monitor the rate constants controlling the delivery of protons to the site. In the case of lac permease, the site under study was the substrate-binding cavity. The transhydrogenase was labeled by the indicator either at the entry or the exit ports of the proton-conducting channel.

The slow turnover rate of these enzymes ensured that a fast proton pulse would record the state of the enzymes without affecting their configurations. The studies with lac permease demonstrated that, by the application of a proton pulse, we could resolve two fundamental states of the protein. In one state, which is dominating at pH \( \geq 7.4 \), the protein is in a constricted state with strong interaction between E126 and the substrate, or the indicator bound to the same site. At pH < 7.1 the enzyme is in a relaxed state, and the bound dye is well exposed to the bulk (chapter 3a).
Supplementary measurements, carried out at the sub-nanosecond time scale, indicated a significant variation in the solvation of intra-cavity spaces in the protein that are associated with the catalytic cycle (chapter 3b).

The studies with the transhydrogenase revealed a significant difference between the properties of the entry ports or the two faces of the enzyme (chapter 4). The extra-cellular surface is well exposed, while in the periplasmic side the channel’s opening is masked by the two lobes of domains I and II, which reduce the accessibility of protons. Yet, it was shown that both orifices are histidine-rich domains, which resemble what was recently proven to be crucial at the entry of the proton pathways in the photosynthetic reaction center (at the Q_B proton uptake site) [52, 56, 57], bc1 complex from bovine heart [58] and cytochrome c oxidase [59]. The kinetic analysis also revealed that both of the channel ports are in equilibrium with a common proton-binding site that has a high pK and no accessibility to ions and bulk protons. The possibility that this common reactant represents the proton-binding site of the channel itself will be discussed.
**METHODOLOGY**

**THE PROTON PULSE TECHNIQUE**

The experimental observation consists of a delta-function perturbation of the acid-base equilibrium in the solution, attained by a UV laser pulse (355 nm, 3 ns FWHM, 1.4-2.1 mJ/pulse operating at 10Hz) that excites the pyranine to its first electronic singlet state. In its excited state, the pK drops from pK₀=8.0 to pK* =1.4 and the hydroxyl’s proton dissociates with a time constant of ~120 ps. Following the relaxation of the dye to the ground state, the system is poised in a temporary state of disequilibrium, where both free protons and φOH concentrations are above the equilibrium level, while the φOH population is transiently depleted. This initial perturbation propagates to all other proton-binding sites present in the pulsed solution through a diffusion-controlled reaction with the photo-dissociation products (H⁺ and φO⁻). The response of the system to the perturbation proceeds through many parallel pathways, where the velocity of each reaction is determined by the concentrations of the reactants and the respective rate constants. The kinetic and stoichiometric coupling between all reactants implies that a follow-up of one reactant would yield information concerning the state of protonation of all the others. The relaxation is completed when the φOH population reaches its pre-pulse concentration.

Proteins, as polyelectrolytes, are thermodynamically coupled and the protonation of any site can affect the pK accessibility of other sites [60]. Thus, the experiments were carried out under the limitation that the incremental proton concentration was smaller than the total protein concentration, ensuring that each protein molecule reacts with no more than a single proton. Consequently, when a protein is challenged by a proton pulse, its thermodynamic and kinetic characteristics would not vary during the reaction time.

In the main experiments (chapters 3a, 4), a pH indicator molecule, fluorescein-5-maleimide, was covalently attached to a cysteine residue of the protein. A simultaneous monitoring of the two chromophores state of protonation (pyranine in the bulk and fluorescein at the protein) was performed.
KINETIC MEASUREMENTS

The reaction is monitored by a probing Ar laser beam that crosses the observation cell, perpendicular to the excitation beam. The sample was placed in a four-face quartz cuvette and continuously stirred, while the pH of the solution was constantly monitored by a pH electrode. The excitation beam of the Nd: Yag laser (Surelite II (Continuum) operating at 10Hz and delivering pulses with energy of 1.2-2.3 mJ/pulse, 2 ns FWHM that were collimated to an area of ~4x10 mm) and the monitoring beam of a CW Argon laser (Innova 90, Coherent) crossed each other. The transient absorbencies were monitored at two wavelengths: 458 nm, where the pyranine anion has an extinction coefficient of 24000 M⁻¹ cm⁻¹, and at 496 nm, where the fluorescein has its maximum absorbance. During its protonation, the absorbance of fluorescein at 496 nm is not totally bleached and the differential extinction coefficient (basic minus acidic) of fluorescein is \( \Delta \varepsilon \{ \text{Flu} \} \text{basic} - \text{acidic} \} = 50000 \text{ M}^{-1} \text{ cm}^{-1} \) \cite{33, 35}. The conversion of the recorded signals to concentration accounted for the spectral contribution of each dye at the absorbance of the other at the wavelength where it was monitored. The correction values are as follows: the contribution of the fluorescein to the absorbance of the pyranine is \( \Delta \varepsilon \{ \text{Flu} \} \text{basic} - \text{acidic} \} 458 \text{ nm} = 5000 \text{ M}^{-1} \text{ cm}^{-1} \), while the pyranine’s absorbance at the wavelength where fluorescein was monitored was \( \Delta \varepsilon \{ \text{Py} \} \text{basic} - \text{acidic} \} 496 \text{ nm} = 1440 \text{ M}^{-1} \text{ cm}^{-1} \).

A train of excitation pulses irradiated the sample and care was taken to maintain the pH, during the observation period, within ±0.05 units from the initial value. After each measurement, the pH was varied by increments of 0.1-0.3 pH unit through the addition of submicroliter aliquots of NaOH or HCl (10 mM) and the measurements at the two wavelengths were repeated. The pH used was limited to 6.0 as the lower value and 8.25 as the upper one. At the lower end of this range, the enzymes became unstable and tended to precipitate. Measurements at the higher end of the range were limited by the ground state dissociation of the pyranine (pK = 7.7), which depleted the ground state population of \( \phi \text{OH} \) to a level too low to perturb the system.

Each sample was subjected to 1000 laser pulses (1.6 mJ/pulse at a repetition rate of 10 Hz) and the transient absorbencies were measured using a Tektronix TDS
540 digital oscilloscope. The signals were stored as a vector of 15000 data points with a temporal resolution of 2-20 ns/data point. The signals (1000 in most cases) were averaged and subjected to kinetic analysis as described in the text. For a more detailed description see [25, 32, 35, 37, 61].

**KINETIC ANALYSIS**

The response of the reaction system to the pH perturbation is the summation of many parallel, tightly coupled reversible reactions that fall into two categories. The first category is the diffusion-controlled reaction of the proton (or the pyranine anion) with the protein-bound proton binding sites; the rate constants of these reactions are given in M$^{-1}$s$^{-1}$ units. The second mechanism is a proton exchange between the fixed proton binding sites of the protein, which proceed in a local environment where the density of the reactants, separated from each other by 3-10Å units, is comparable to that of a homogeneous solution having a concentration of 1-4M. This environment is better conceived as a reaction in a solid- or semi-solid matrix, where the proton binding sites are practically immobile and only the small ions; H$^+$ and $\phi$O$^-$, diffuse between them. The rate constants of these proton transfer reactions are defined as virtual second order reactions, and printed in italics with no unit notation.

The reaction system consists of a soluble protein or micellar dispersion of the protein or purple membranes and pyranine. For membranal protein both sides of the protein are equally accessible to protons and the pyranine anion. Thus, whether the enzyme was labeled on the cytoplasmic surface or the periplasmic one, both sides are protonated by the same mechanism. Accordingly, the reconstruction of the pyranine signals should always be made by the same set of parameters. The fluorescein traces are more sensitive to the local conditions, as only residues with ~15-20 Å from the chromophore will affect the kinetics of the bound indicator.

For the purpose of analysis, all proton transfer reactions taking place within the perturbed space were defined and linked by equilibrium and rate equation. In principle, each protein proton-binding site should be individually expressed, but this requirement is some times too demanding. In the analysis of lac permease and transhydrogenase, the systems were simplified by grouping the reactive residues into subpopulations, each characterized by average equilibria and rate constants. The equilibria were converted into a set of coupled, first order, non-linear differential rate
equations that complied with the detailed balance principle and the conservation of mass [7, 26, 32]. The integration of these equations over time is an in silico reconstruction of the chemical reactions. With the right selection of rate constants, the integration is a reconstruction of the observed dynamics. This mode of analysis has the same rational as the deduction of the structural model from the x-ray diffraction pattern, where a number of independent observations are reconstructed by a single set of diffracting elements placed at defined coordinates. In the same way, all the independently measured kinetic tracings that had been recorded were simulated by a single set of rate constants. To increase the accuracy of the reconstruction, the signals were analyzed in pairs, each pair consisting of the pyranine and fluorescein signals measured at the same pH value [25, 32]. The reconstruction of the two signals is a summation of all parallel reactions that link the transient protonation of the moieties in the bulk, on the protein’s surface, the two dye molecules and the protonable residues located inside the cavity. The mode of analysis and its results will be described in the text while the rate constants are summarized in the tables.

**NUMERIC RECONSTRUCTION OF THE MEASURED TRANSIENTS**

The manifold shapes of the relaxation dynamics can be reconstructed by a set of coupled non-linear, first order, differential rate equations that comply with the detailed balance principle [16, 39, 40, 62]. The equations account for all proton transfer reactions between each component \( \{R_i\} \) and each of the other proton binding sites \( \{R_j\} \) present in the system as given by Eq. 1.2.

\[
\text{Eq.1.2} \quad \frac{dR_i}{dt} = k_{\text{diss}} [RH_i] - k_{\text{as}} [R_i] [H^+] + \Sigma k_{ji} [RH_i] [R_j] - \Sigma k_{ji} [R_i] [RH_j]
\]

The term \( k_{\text{diss}} \) corresponds with the rate constant of the acid dissociation of \( RH_i \); \( k_{\text{as}} \) is the re-protonation of \( R_i \); \( \Sigma k_{ji} \) \( \{RH_i\} \{R_j\} \) is the sum of all reactions where \( \{RH_i\} \) functions as a proton donor to other proton binding sites, and \( \Sigma k_{ji} \) \( \{R_i\} \{RH_j\} \) is the sum of the back-reaction where \( \{R_i\} \) is the acceptor with respect to all other proton binding sites. The same equations, with the appropriate rate constants, are given for each reactant in the system, and are linked in a way that ensures the mass conservation law. All proton-binding sites were assigned rate constants for their reactions with free protons, freely diffusing-pyranine anion and proton exchange.
reactions with all other proton-binding sites. The distinction between the sites located on the periplasmic vs. the cytoplasmic side was attained by setting \((k_{j/i}=0)\) for all proton exchange between groups located on opposite faces of the enzyme.

Numeric integration of the whole set of the equations is the mathematical equivalent of the chemical reaction. When the equations are supplied with the correct values of the rate constants, the computer-generated dynamics will reconstruct the measured reactions. The reconstruction of a single pair of measured dynamics has a large leeway in the selection of rate constants. Yet, as the number of independent, non-identical signals increases, the solutions converge to a single set of rate constants that solves all experimental results.

Until now we could verify the singularity of the solution only in limited cases where an exhaustive search (using the MIGRAD program) over the whole parameter space was executed, yielding the gratifying conclusion that the system is indeed solved by a single set of parameters (Yam, Ph.D. thesis). In more complex systems even such a brute force approach is beyond reach.

To overcome this limitation, we have collaborated with Dr. G. Fibich from the School of Mathematics, Tel Aviv University. This joint research employs a “genetic algorithm” for systematic search over the whole parameter space with 12 independent adjustable parameters, indicating that the system converges to a single global minimum. This part of the work is described in appendix A, which appears as a manuscript to be submitted to the Proc. Nat. Acad. Sci. U.S.A.

**RECONSTRUCTION OF PROTONATION TRANSIENTS OF RESIDUES THAT ARE NOT DIRECTLY OBSERVED**

The detailed balance principle used in the kinetic analysis of the signals consists of accurate ‘book-keeping’ of all protons released from the excited proton emitter. Thus, when the amount of protons missing from the emitter exceeds those detected by the indicator, plus the free proton in solution, it is taken as evidence that the unaccounted for protons are bound to the various proton binding sites present in the system, either as free diffusing buffers or reactive residues on the protein’s surface.

The accuracy of this conjuncture was tested long ago [61, 63-65] by measuring the effect of small buffer molecules on the dynamics of various proton emitters and
indicators. Later the analysis was extended to reconstruct the protonation dynamics of pure proteins and membranes in solutions [7, 17, 23, 24, 26, 66, 67].

In the present study, we shall use this procedure to evaluate the total proton binding capacity of a protein molecule by reconstructing the protonation dynamics of the pyranine anion and, through the analysis of the bound indicator dynamics, to deduce the capacity of residues, close to the indicator, to function as intermediate proton binding sites that affect the measured dynamics of the indicator.
RESULTS AND DISCUSSION

Chapter 1: Gauging of cytochrome c structural fluctuation by time-resolved proton pulse

INTRODUCTION

The stabilization of a polypeptide chain, in the form of a native folded protein, is attained by a variety of electrostatic and hydrophobic interactions. Consequently, the charging of a protein by redox reaction should be compensated for by some structural rearrangements in order to re-minimize its total energy. These rearrangements have been studied intensively with cytochrome c as a model. Cytochrome c is a small globular protein, with a well-defined, three-dimensional structure, both in its reduced and oxidized states. The well recognized spectral properties of the heme moiety and the suitability of the protein for investigation by advanced physical methods [1, 68-74] have made this protein a preferred model. In all these studies, attention was focused on the vicinity of the redox center of the protein, while the variations at the protein’s surface were not amenable for quantitation. Calvert and coworkers [1] noticed that the solution structure of cytochrome c, isolated from 10 species, varied with the redox state. Most of the conformational changes were located at the residues 10 to 49, which are not stabilized by an $\alpha$ helix structure. Vanderkooi and her coworkers [73, 74] tried to correlate these structural changes with pK shifts using IR spectroscopy but the changes were below the level of detection.

The surface of a protein can be directly probed by monitoring its state of protonation; any change in the charge distribution or exposure of a protonable site will appear as a pK shift. Steady state measurements, where all surface groups are simultaneously titrated, fail to reveal the individual properties of each surface group. During the acidimetric titration, due to the variation of the protein’s charge, the pK of all groups is interdependent. Thus, the ideal method for probing the effect of charging a protein on the reactivity of its surface groups should employ only a single proton that reacts, at random, with one of the proton binding sites. This goal can be attained only in the time-resolved domain, where the protein’s solution is subjected to a short
pulse acidification, while the distribution of protons between the bulk and the protein are continuously monitored. In the present study, we employed the Laser Induced Proton Pulse for monitoring the reversible protonation of cytochrome c in the time-resolved domain [30, 75]. This mode of observation yields the rate constants of proton transfer reaction among the surface groups. Comparison of the rate constants measured with the reduced and oxidized protein revealed conformational changes that were too fast to be detected by time averaging methods.

The subject described in chapter 1 was published in the Israel Journal of Chemistry, 1999, 39, 439-445 [37].

MATERIALS AND METHODS

Oxidation / Reduction of cytochrome c

The redox state of cytochrome c was controlled by the following procedure: 3 mM of Ferricyanide as oxidant or 3 mM of ascorbic acid as reductant were added to 1 mM of the protein for one minute. The mixture was passed through a PD-10 column (Pharmacia) and the protein fraction was collected and immediately subjected to kinetic measurements.

Kinetic measurements

The reduced (or oxidized) cytochrome c was diluted in a solution of 100 mM NaCl containing 20µM pyranine. The pyranine-protein solution was placed in a closed continuous-flow system that was flushed by a stream of argon (15 ml/min). The pH of the solution was continuously monitored and injection of small quantities of HCl or NaOH maintained the pH at the desired level. The flow system pumped the solution into a four-face quartz cuvette at a rate ensuring a total replacement of the solution within the irradiated space during the time intervals (2 Hz) between the laser flashes. The excitation beam of the Nd-Yag laser (\(\lambda=335\) nm, 2 ns FWHM 1.6 mJ/pulse) irradiated an area of 10x2.5 mm, which was also probed by the monitoring beam. The transient absorbencies, following the excitation pulse, were monitored by a probing beam of an Argon laser (\(\lambda=458\)nm) crossing the cell perpendicular to the excitation beam. The data acquisition averaging and analysis were carried out as described in Materials and Methods.
RESULTS AND DISCUSSION

Protonation dynamics of oxidized and reduced tuna heart cytochrome c

The reprotonation dynamics of pyranine anion in the presence of oxidized and reduced tuna cytochrome c are shown in Figure 1.1. Though the measurements were carried out under almost identical conditions, the reprotonation of the pyranine in the presence of the reduced protein exhibits a larger and longer ‘tail’ than the oxidized one. On the other hand, when the same experiment was conducted with horse cytochrome c (data not shown) the curves of the two redox states were identical within the range of the experimental noise. Thus, the difference noticed with the tuna cytochrome c is a real observation, and the lack of difference in the case of horse protein will be discussed later.

To quantify the difference between the dynamics measured with oxidized and reduced protein, a kinetic analysis of the two sets of experimental results was performed. As explained in Materials and Methods above, the first step in the reconstruction of the experimental signals is finding the best model describing the proteins. This model is largely based on the known structure of the protein. The tuna cytochrome c has one histidine that is not bound to the heme and nine carboxylic groups divided in five regions: (Asp26, Glu69 and Glu90); (Asp2, Asp93, Glu66) as two distinct clusters and another 3 solitary moieties.

Figure 1.1: The effect of the redox state of tuna heart cytochrome c on its protonation dynamics. The reaction mixture contained: pyranine (20µM) (I=100mM at pH 7.4) with the addition of 10µM reduced and oxidized tuna cytochrome c. The pyranine-protein solution was excited by laser pulses and the transient absorption at 458 nm was monitored as described in Materials and Methods. The continuous lines superpositioned over the experimental curves are numeric simulations of the dynamics using the rate constants listed in Table 1.1.
The reconstruction of the observed dynamics in a system where all nine carboxylates react with H\(^+\) within the observation time generated a transient buffer capacity that was too high to fit the observed signals. In order to fit the signals, the number of reactive carboxylates had to be reduced to five. On the basis of these arguments, we propose that in the dynamics measurements not all carboxylates are equally reactive with solutes in the bulk. When the observation is limited by the duration of the proton pulse, the number of reacting sites can be smaller than the sum of all proton-binding sites. Each of the five reactive carboxylates was assigned with appropriate rate constants of protonation, dissociation, and collisional proton transfer with the pyranine anion and virtual second order rate constants for a proton exchange reaction with all other surface groups. The concentrations of the proton binding sites were related with the known concentration of the protein, while the rate constants were taken as adjustable parameters. A thorough search in the parameter space yielded a single set of rate constants that generated the fitted lines shown in Figure 1.1. To verify that the rate constants are a general property of the system, the experiments were repeated at the same pH, using a wide range of cytochrome c concentrations (4.5 to 22.5 \(\mu\)M). All measured dynamics were simulated by a unique set of rate constants. The accuracy of the fitting was as good as shown in Figure 1.1.

The measurements of the protonation dynamics of the tuna cytochrome c (reduced and oxidized) were repeated at three different pH values (at varying cytochrome c concentrations) and the results of the rigorous analysis are summed up in Table 1.1.

**Evaluation of the rate constants**

The second order rate constants of protonation of the five carboxylates of the tuna protein (which were arbitrarily marked A, B, C, D, and E) are essentially the same and can be represented by a typical rate constant of \(k = 1 \cdot 10^{10} \text{ M}^{-1}\text{s}^{-1}\). This rate constant corresponds with the kinetic properties that were found for well-exposed carboxylate moieties unaffected by the presence of a nearby charge. The protonation of the single histidine moiety (His26) has a rate constant of \(5 \cdot 10^9 \text{ M}^{-1}\text{s}^{-1}\). This rate constant is within the range of that predicted by the Debye-Smoluchowski equation for the reaction between free proton and an uncharged group on the protein’s surface.

The virtual second order rate constants of proton transfer between the surface groups of all but one proton exchange reaction are smaller than \(10^9\). Based on
accumulated experience with model compounds [32], these values suggest that, on
dissociation from these sites, the protons are released to the bulk with a low
probability for preferential uptake by a nearby acceptor. The rate of proton transfer
between the carboxylate E and His26 was significantly faster than all other reactions
between the fixed proton binding sites. Moreover, this rate constant varied with the
redox state of the cytochrome c: \( k = 2 \cdot 10^{10} \) for the oxidized protein and \( 1 \cdot 10^{11} \) for the
reduced one. Such high values imply that the carboxylate moiety is so close to the
histidine that the released proton is within a reaction sphere of the histidine. The only
carboxylate located close to His26 is Glu44, and we identified carboxylate E that
residue.

**The effect of the pH on the reactivity of the surface groups**

The average charge of a protein is a function of the pH. During an acidimetric
titration, the total charge of the surface varies with the pH and, as the charge density
varies, the pK of all surface groups is shifted. This coupling was first quantified by
the classical Kirkwood-Tanford model [76] that approximated the protein as a sphere
evenly covered by fixed-point charges. This model predicted that all sites would be
affected to the same extent by any change in a charge on the surface of the protein. In
a more realistic presentation of the protein, the degree of coupling depends on the
shape of the protein and the distance between the interacting sites.

The pK shifts of surface groups are technically difficult to detect. For
example, determination of the pK of carboxylate moieties by acidimetric titration
requires lowering the pH of the solution below pH =5, where most proteins become
grossly unstable. In addition, during the acidification, all proton-binding sites react
and the total charge of the protein becomes more positive. Thus, by equilibrium
measurements, the pK of all sites is thermodynamically coupled and shifted with the
progression of the titration. This limitation is avoided by the proton pulse experiment.
During the pulse, the amount of released protons is less than the total protein
concentration; thus no more than one proton may interact with each protein molecule.
As a result, the measurement alters the charge of the protein at the site where the
proton had reacted. The measured kinetics are a summation of all possible sites of
protonation, yet the kinetic analysis discriminates between them by assigning
individual rate constants to each detectable site. Thus, the pK values determined this
way are not affected by the electrostatic coupling as in equilibrium measurements.
Table 1.1: The kinetic and thermodynamic parameters that reconstruct the transient protonation of tuna cytochrome c in 100 mM NaCl solution.

<table>
<thead>
<tr>
<th></th>
<th>Reduced cytochrome c</th>
<th>Oxidized cytochrome c</th>
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<tbody>
<tr>
<td></td>
<td>PH = 6.8</td>
<td>PH = 7.4</td>
</tr>
<tr>
<td>COO⁻ (A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pK</td>
<td>4.12</td>
<td>4.45</td>
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</table>

The shaded rows highlight parameters that vary with the pH or the redox state of the protein.
The virtual second order rate constants are printed in italics. The pK values are accurate within 0.05, while the rate constants were determined with accuracy of ±8%.
For a protein like cytochrome c, where the properties of its surface group vary with the redox state, it is not surprising that the initial pH also affects the kinetic and thermodynamic parameters of the surface groups. As summarized in Table 1.1, in the two-redox states, all carboxylates raise their pK values when the pH is higher, indicating that the sites are electrically coupled. Yet the effect is site-selective; some carboxylates are more affected than others. The effect of the redox state on the pK values indicates that oxidation downshifts the pK values, not to the same extent for all the carboxylates, but in accordance with the intimate environment around each site.

In contrast with the electrostatic shift measured for the carboxylates, the pK of the histidine moiety slightly decreases with the pH. The inverse direction of the change implies the presence of another mode of regulation, such as a conformational change where, at the low pH range, the un-protonated histidine assumes a more hydrophobic environment.

The rate of protonation of His26 is constant at all measured pH values and independent of the redox state of the protein. On the other hand, the reaction between the protonated histidine and the pyranine anion vary with the pH, in general agreement with the Kirkwood-Tanford model. Considering the Z=-4 charge of the pyranine anion, the repulsive electrostatic forces between the pyranine and the nearby Glu44 are more intensive than the Glu-H⁺ interactions. The proton transfer from the histidine to the pyranine is less sensitive to the pH in the oxidized state of the protein. This relaxed dependence may be a reflection of the increased distance between the His26 and the Glu44, as derived from the slower virtual second order reaction between the two sites.

The effect of the redox state on the dynamics

The oxidation of the heme had a very localized effect on the pK of the surface groups. Sites A, B and C exhibit lower pK values in the oxidized cytochrome c, while the pK of the sites D, E and His26 were practically insensitive to the redox state. Similarly, the rate constants of protonation of the surface group were found to be unaffected by the redox state. Of special interest is the observation that oxidized protein differs from the reduced one in the rate of proton transfer between His26 and Glu44. The virtual rate constant of the reduced protein was \( k = 10 \cdot 10^{10} \) while for the oxidized protein, the rate was four times smaller, \( k = 2.5 \cdot 10^{10} \). Such changes in the rate
of reaction imply that the protonic connectivity between the two sites had been changed. A four-fold decrease in the rate of proton transfer between two adjacent sites can be due to enlargement of the separation distance between the sites or to some rearrangement of the solvation shell of the reactants. Still, on examining the protein’s crystal structure in its two redox states, no such changes were noticed. The three dimensional model of the protein (having a resolution of 1.5 and 1.8 Å for the reduced and oxidized states, respectively [68]), does not reveal any structural alteration in the Glu44-His26 region. The distances between the nitrogen atom of the imidazol ring of His26 and the carboxylate of Glu44 are 8.1 and 7.6 Å for the oxidized and the reduced states. Thus, we cannot attribute the four-fold modulation of the rate constant to any time independent conformation of the protein that is dominant enough to be expressed in the average crystal structure of cytochrome c.

The high-resolution methods, like X-ray diffraction measurements are slow scanning procedures and reveal only the time averaged dominant conformations. The fast proton transfer probing of the present study amplifies those conformations that have the highest rate of proton transfer, even if they contribute only a minor fraction to the conformations population. Thus the faster rate of proton exchange implies that the flexibility of the Glu44 residue varies with the redox state of cytochrome c. When reduced, the probability that thermal fluctuations will bring Glu44-H⁺ into contact with His26 is higher than for the oxidized protein. Examination of the crystal structure data supports our conclusion. The R-value [68] of the oxygen atoms of the carboxylate moiety of Glu44 is significantly larger in the reduced state of the protein, while the imidazole ring has about the same values in both redox states. Thus, the kinetic analysis sharpens the structural understanding of the protein by providing direct evidence that could not have been deduced by the slow scanning methods.

Figure 1.2: Structure of tuna cytochrome c. This picture is based on the X-ray structure (PDB entry: 5cyt) of Takano [68]. In this presentation Glu44 is in red, and His26 is in cyan. The heme groups and the Fe ion are highlighted.
The redox-coupled alterations of the proton binding dynamics were observed with the tuna protein but not with the horse one, even though the experimental quality of the two sets of measurements was comparable. The absence of the redox effect implies that the horse protein has a more rigid structure and does not exhibit the conformational fluctuations attributed to the tuna cytochrome c. Calvert and coworkers [1] used FTIR spectroscopy to quantitate the fraction of the unordered structure of cytochrome c in solution. On the basis of the shifts in the second derivatives of the spectra, they concluded that the tuna protein has a substantial fraction (23%) of unordered structure, while the horse protein has only 13% of unordered structure. Apparently the variation in the level of the unordered state, in the 19-44 domain, makes the difference between the effect of the redox state on the dynamics and reactivity of the various members of the cytochrome c family.

CONCLUDING REMARKS

A protein in solution is a flexible structure and has a large population of conformations that differ by a few kT units. Within these manifold states, there are those where the momentary connectivity between two sites or residues is much higher than the average value. These conformations might be totally lost with respect to their contribution to the average state of the protein, but due to their inherently lowered activation energy, may provide the main catalytic pathway. In the present study, we employed a high time-resolving pulse protonation observation that revealed enhanced proton exchange dynamics between two sites with no corollary with the averaged structure of the protein.

Time-resolved monitoring of reversible protonation of a protein’s solution reveals its temporal proton binding capacity. Each of the proton binding sites reacts with the protons with its own time constant and surrenders the proton to the solution or to the pyranine anion, according to a precise pattern controlled by concentrations and rate constants. Qualitative conclusions can be obtained even by the shape of the curves, but upon detailed kinetic analysis that sums up all modes of interactions, the
precise rate constants of the overall reactions can be dissected into individual, partial reactions.

The charging of the heme residue of cytochrome c causes well-defined changes in its structure, and most of them are located near the heme moiety itself. Yet, as shown in the present study, some of the structural modulations affect the interaction of the side chains with each other and with the solvent. Interestingly, we noticed that most changes were located in a well-defined region: the exposure of the imidazole ring of His26 to the bulk and its connectivity with Glu44.

Bulk-surface proton transfer is an essential element in all electron transfer enzymatic reactions. The redistribution of charges on the protein following the redox reaction calls for a rapid stabilization, which in most cases is assisted by proton uptake from the bulk. Two types of proton binding sites are involved in that process: the carboxylates of the surface that are the first to react, but retain the protonated state for a short time, and the medium pK moieties (like histidine) that ensure the long time stability of the protein. As shown in this study, the two types of reactants can interact with each other by channeling protons among them, in a way that is very structurally dependent.

It is our conclusion that new, exciting information is still hidden within the structure of a protein, and is not attainable by slow, time-averaging observations. The utilization of the proton transfer as a gauge reaction will open new avenues of research, especially when transient states are under investigation.
Chapter 2: Bacteriorhodopsin

Bacteriorhodopsin (BR) is the best-understood ion-transport-protein and has become a paradigm for membrane proteins in general and transporters in particular. Models of high resolution up to 1.55Å (for review: [77, 78]) and the development of a transformation system are the basis of the investigations on structure and function relationship of the light driven proton pump BR.

Figure 2.1: Structure of bacteriorhodopsin. This picture is based on the X-ray structure (PDB entry: 1C3W). The protein is represented by its Cα chain with a detailed location of the retinal molecule (violet), D36, D38, D96, D212, D85, E194, E204 and inner water molecules (blue).

The protein is a seven transmembranal α helices protein, which spontaneously forms patches of two-dimensional crystal in the bacterial inner membrane. These patches are referred to as the purple membrane (PM). The helices are arranged in an arc-like structure model and tightly surround the chromophore; as a retinal molecule that forms a Schiff base (SB) with a conserved lysine (K216) on helix G. Figure 2.1 shows the cross section of BR with the residues important for proton transfer. The retinal molecule separates the cytoplasmic (CP) half channel from the extracellular (EC) one. The channel is lined by amino acids crucial for efficient proton transport by BR (especially D96 and D38 in the cytoplasmic half channel and D85, D212, R82, E194 and E204 in the extracellular one).
The maximum absorption of the retinal molecule is tuned by its geometry, the protonation state of the SB, and its precise electrostatic interaction with the surrounding charges (D85, D212 and R82) and dipoles. Absorption of a photon by the retinal molecule initiates a catalytic cycle that leads to vectorial transport of a proton out of the cell. The intermediates of the cycle were defined on the basis of time-resolved absorption spectroscopy and were termed: $J \rightarrow K \rightarrow KL \rightarrow L \rightarrow M_1 \rightarrow M_2 \rightarrow N$ and O. Three pairs of reversible events control the catalytic cycle: isomerisation, proton transport and accessibility changes (switch). Their sequential order ensures the vectoriality of the proton pumping reaction. The retinal molecule first photoisomerized from all-trans to a 13-cis configuration followed by a proton transfer from the Schiff base to the proton acceptor D85 [79, 80]. It is accompanied by a proton released to the bulk phase by the ‘releasing dyad’ (E194, E204) [81, 82]. The SB changes its accessibility from the extracellular to the intracellular half channel [83-87], and then regains its proton from D96 in the cytoplasmic channel [79, 88]. The last stage of the photocycle is the retinal’s reisomerization and its back switches to the extracellular half channel.

A major, light-induced conformational change in the transmembranal regions of BR during the course of the photocycle was evidenced by combination of neutron [89], X-ray [90, 91] and electron diffraction studies [92-98] as well as infrared spectroscopy [99-101]. The structures of the initial state and the early intermediates (K, L and M_1) are well approximated by one protein conformation, while the structures of the later intermediates (M_2, N and O) are well approximated by the other protein conformations. This conformational change between M_1 and M_2 states makes an important contribution towards efficiently switching proton accessibility of the Schiff base from the extracellular side to the cytoplasmatic side of the membrane [95]. Subramanian et al (1999) [95] reported projection structural changes in wild-type BR and the D96G mutant trapped to 10 or 20 ms after illumination at 5ºC. Based on these projections, it was concluded that the main structural change in the photocycles of both proteins was largely localized to the central four helices lining the proton channel (F, G, B and C). The main feature of the structural change is ordering of helix G at the cytoplasmic end and an outward tilt of helix F, with Pro186 likely to serve as a ‘hinge’ residue.
Chapter 2a: Gauging of the cytoplasmic surface of the M state of bacteriorhodopsin by a proton pulse

INTRODUCTION

The best observed feature of the photocycle is the protonation state of the Schiff base and its absorption spectrum. In the protonated state, the retinal molecule has a maximal absorption at \(\lambda \geq 570\) nm and varies with the state of protonation of residues like D85 and D212 that are close enough to modulate the energy of the electronic transition of the chromophore. Residues that are more than 8-10 Å from the Schiff base (for example D96) are too remote, and their state of protonation is deduced indirectly. In the deprotonated state of the Schiff base, the retinal molecule has a maximal absorbance at \(~410\) nm and the spectral difference between the various conformations of the M state is very small [102-105].

In contrast with the state of protonation of the Schiff base, conformation of the protein is more difficult to deduce and progress was gained by a variety of physical methods such as FTIR [99, 106-110], electron spine resonance [111-116] proton pulse [31, 33-36] and structural studies [78, 90, 93, 117-123]. Yet, even to date, some interconnecting loops, especially of the cytoplasmic surface, are poorly resolved. The last 16-18 residues at the C’-terminal end of the protein are probably too loose to attain a coherent conformation during crystallization and have not yet been resolved. The major conformation transition associated with the photocycle coincides with the M\(_1\) to the M\(_2\) step, where helices G and F exercise a transmembranal motion with enhanced hydration of the protein [115, 124-128]. This structural transition is possible only in the presence of free water molecules [122]; at a relative humidity below 75%, (e.g. 57%) the protein can release the Schiff base proton to D85, but the conformational change associated with the M\(_2\) formation does not materialize [124].

The mechanism of proton transfer reactions between the Schiff base and its immediate acceptor (D85) and donor (D96) are well recognized, as the reaction is spectrally detectable. The proton transfer from the Schiff base to D85 is the first step of the proton pumping machinery and appears as the replacement of the BR spectrum by that of the deprotonated one of the M state. In a similar mode, the re-protonation of
the Schiff base by the proton from D96 marks the M decay. Besides these two events, the intra-protein proton transfer is less recognized. The neutralization of the negative charge of D85 drives a sequence of pK shifts leading to release of a proton from the proton releasing domain, associated with E204, E194 and water molecules in their neighbourhood [111, 112, 129-135]. The re-protonation of D96 consists of the later steps of the photocycle [104, 136, 137] in a reaction, which has surprisingly slow dynamics and is regulated by the chemical potential of the water in the reaction system. The protonation of D96 necessitates proton transfer from the nearest carboxylate, D38, which in the BR state is some 7.5Å apart, while in the M state it is even larger (~11 Å) [78, 82, 113, 138-141]. The effect of this translocation on the surface protonable group is the subject of this chapter.

Following the re-protonation of D96, the protein reacts with the bulk phase and the process is commonly recorded as deprotonation of a pH indicator that is either soluble or bound to the protein [33, 35, 36, 142-144]. The re-protonation of the protein’s surface with the bulk proceeds through diffusion controlled reactions, either with free proton or with protonated buffer molecules in the bulk. The mechanism was intensively investigated in our laboratory by Sharron Checover (Ph.D. thesis) [36, 145], who used the Laser Induced Proton Pulse technique for resolving the proton transfer reactions on the protein’s surface. In these experiments, the purple membrane suspension was subjected to rapid successive proton pulses, and the rate of proton transfer between the protein’s surface and the pyranine anion was monitored in the time resolved domain. The rigorous analysis of the signals yielded a set of rate constants indicating that the BR state of the protein is characterized by a proton-collecting antenna located on its cytoplasmic surface. The elements of the antenna system consist of three carboxylates (D104, E161 and E234) that form a tight cluster with a rather high pK (5.5) that reacts with free proton at a rate constant of $k=5.5 \times 10^{10} \text{M}^{-1}\text{s}^{-1}$. This rate constant is significantly larger than that of an isolated carboxylate on a low dielectric surface, and indicates a merging of the Coulomb cages of more than two carboxylates. The cluster is close enough to D36 and D38 to serve as a local proton reservoir that delivers the proton with a virtual second order reaction of $k>10^{10}$ [32]. As was revealed by the studies of Hubbell and coworkers [116] and Rodig and Sieber [106], the late M state of the protein exhibits a new organization of the surface groups. Thus, it is of interest to find out whether the proton collecting function of the surface is different in the M state.
For this purpose, in the present study we employed two mutants, D96N and the triple mutant (D96G/F171C/F219L). The first one can be readily pumped into the late M state (M$_N$ or M$_2$) by constant illumination [95, 105, 146-151]. The triple mutant has a lower tendency to accumulate in the M state (Tittor and Oesterhelt, unpublished results) and unless the actinic light is very intensive, the protein retains its BR state of the chromophore while its structure resembles the late M configuration [95].

The measurements described in the present study indicate that the late M configuration of the protein exhibits a proton collecting system that differs in detail, but not in functionality, from that observed for the BR state of the protein.

**MATERIALS AND METHODS**

**Mutagenesis and expression**

The protein used in the present study was a generous gift of D. Oesterhelt (Max-Planck-Institute, Martinsried, Germany). Site-specific mutants of bacteriorhodopsin were prepared according to Ferrando [152]. Mutagenesis was followed by transformation and homologous expression in *Halobacterium salinarum* strains HN5 and L33, with the help of the shuttle-plasmid pEF 191. Mutated proteins were isolated as purple membrane sheets according to Oesterhelt and Stoeckenius [153].

**Kinetic measurements**

Kinetic measurements were carried out as described in Methodology and [33, 35, 36]. The reaction mixture was constantly mixed by a small magnetic disk and its pH was monitored. In cases where the pH was drifting, small aliquots of HCl or NaOH were added to maintain the pH within ±0.05 units of the initial value.

The sample was irradiated by the third harmonic frequency of a Nd/Yag laser $\lambda$=335 nm 1.5-1.8 mJ/pulse at a rate of 10 Hz. The probing light was the 458 nm band (for monitoring the pyranine) or the 528 nm band (for monitoring the photon triggered M$\rightarrow$BR transition) of a CW Ar laser. The extinction coefficients were 24000 and 35000 M$^{-1}$cm$^{-1}$, respectively.

**Preparation of sample for measurements:** The purple membranes were suspended in 30 ml of unbuffered 150mM NaCl solution and spun down by
centrifugation. The process was repeated 3 times to remove all buffers from the reaction mixture. The final suspended membrane was supplemented by pyranine, placed in the measuring cuvette and purged by water-saturated N₂ gas for 15 min before initiation of the experiment. The purging continued throughout the whole experimental period. Because the measured kinetics are very sensitive to the presence of buffer, care was taken to correct for the residual concentration of bicarbonate that survives the purging procedure. For this purpose, a pyranine solution in 150 mM NaCl was treated by the same procedure, and through its kinetic analysis the residual content of dissolved CO₂ was determined. The same concentration of CO₂ was assumed to be present in the membrane suspension.

**Analysis** of the signal was carried out as described in Methodology and [36] and the kinetic parameters used to reconstruct the WT dynamics are given in Table 2a.1.

**Table 2a.1:** The kinetic properties of the proton binding sites of the BR state of the WT bacteriorhodopsin (Checover, Ph.D. thesis). The listed values reconstruct 14 independent measurements that were carried out at varying pH values 6.4 ≤ pH ≤ 8.1 with 6.4 µM protein, 45.1 µM pyranine and 150 mM NaCl.

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<tr>
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<td>≥1 10⁻¹⁰</td>
<td>&lt; 10⁻⁸</td>
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<td>0.5±0.3 10⁻¹⁰</td>
<td>-</td>
<td>9±2 10⁻⁹</td>
</tr>
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</table>

* n corresponds with the number of carboxylates that are characterized by the same parameters. The rate constants are given in M⁻¹s⁻¹ except for the virtual second order reaction whose values are printed in italics.

**RESULTS AND DISCUSSION**

**Kinetics of relaxation of the pyranine signals**

The rate limiting step of the D96N photocycle is the re-protonation of the Schiff base, leading to accumulation of the M state during continuous illumination. Time resolved measurements, using either FTIR or Spin labelling techniques [106, 116, 146, 154] have indicated that the accumulated state of the protein is characterized by a special conformation which appears at a slower rate than the formation of the absorbance of the deprotonation Schiff base; thus a special
conformation state had been assigned to the deprotonation form accumulated under these conditions and was termed $M_N$. In the present study, the accumulation of this state was ensured by illuminating the measuring cell by a green ($\lambda \geq 520$ nm) light source, which caused a total bleaching of the 568 nm absorbance of the solution with subsequent accumulation of absorbance at 410 nm. As the kinetic measurements were carried out with unbuffered solution, it was readily noticed that, when the light was turned on, the solution exhibited a small but resistant acidification (0.1-0.05 pH unit) that was perfectly reversible when it was turned off. The probing laser beam also caused similar acidification. Yet, to ascertain a constant level of $M$ state accumulation, the steady state was maintained by the white light source.

**Figure 2a.1:** The re-protonation kinetics of pyranine following pulse dissociation. The experiments were carried out in an unbuffered solution containing 150 mM NaCl, 23 µM pyranine pH=7.2. The reaction mixture was probed by a CW beam of Ar laser ($\lambda = 458$ nm, main Panel, or 528 nm, the inset) and excited by a train of short laser pulses ($\lambda = 355$ nm, 1.6 mj/pulse at a rate of 10Hz). The solution was purged by water-saturated N$_2$ and the pH was maintained constant by small aliquots of acid or base added during the observation time.

Curve Py was measured in the absence of any addition and corresponds with the re-protonation of the incremental pyranine anion generated by the photo dissociation of the dye. Curve D96N was measured under the same conditions in the presence of 6.3 µM of D96N that was irradiated by a focused beam of a 100W lamp ($\lambda \geq 520$ nm). Curve WT is the reconstruction of the WT protein by the parameters listed in Table 2a.1, under the precise experimental conditions of curve Py. The inset depicts the regeneration dynamics of the BR state caused by the excitation of the $M$ state chromophore by the laser pulse.

Figure 2a.1 depicts three relaxation dynamics; the steepest one (labelled as Py) was measured with pyranine dissolved in 150 mM NaCl and is characterized by a typical non-exponential relaxation curve. The initial phase is rapid but after ~5-7 µs the reaction becomes much slower. The fast phase corresponds with the diffusion-controlled reaction between the pyranine anion and the free proton. The slower phase is due to the presence of a marginal concentration of bicarbonate that traps some of
the released protons and transfers it to the pyranine anion in a collisional reaction. The curve labelled D96N was recorded in the same solution when supplemented by 7.3 µM of D96N-mutated BR. In this experiment, the fast initial relaxation is almost missing, indicating that the proton binding sites on the protein’s surface effectively compete with the pyranine anion for the free proton. The slower phase corresponds with a mixed relaxation process where some of the protein bound protons are released to the bulk by a dissociation reaction, while those attached to more basic proton binding sites are transferred to the dye by collisional proton transfer. For a detailed discussion of the mechanism see [36]. The curve labelled WT in Figure 2a.1 is a reconstruction of the pyranine signal using the same initial conditions and parameters of the WT protein as listed in Table 2a.1. It is clear that the retaining power of the BR state of the WT protein is significantly larger than that of the mutant.

The buffer capacity of a protein, determined by proton pulse measurements, is a function of the rate constant at which the surface proton binding sites react with free protons and their pK values, which are parameters determined by kinetic analysis. Because of the suspected difference between the buffer capacity of the WT and the mutated protein, it was imperative to determine the fraction of the BR initial state that was regenerated by photons absorbed by the M state [86, 155-157]. Accordingly, the measurements were repeated using a measuring beam of λ=528 nm, where the M to BR-states transition is characterized by a differential extinction coefficient of ∆ε(BR-M) = 35000 M⁻¹cm⁻¹ that was calculated from the spectra difference of D96N in its BR and M states [158]. The recorded optical transient corresponded with 0.2±0.04 µM of BR generated by the exciting laser pulse. In the range 6.7 ≤ pH ≤ 8.7, the amount of the regenerated BR initial state was independent of the pre-pulse pH, and of the presence of pyranine in the reaction mixture. This amount was roughly ~5% of the total protein concentration and constant for the BR concentrations used in the present experiment. The regeneration of the BR state had a rise time of τ ~300-400 ns. A millisecond follow-up of the absorbance at 528 nm indicated that the increment of BR was constant in time and was removed from the observation space through dilution by the magnetic stirring. Because of the rather small increment of the BR initial state, its contribution to the buffer capacity of the system was not included in the kinetic analysis.
Kinetic analysis

The kinetic analysis of the signal consists of in silico reconstruction of the observed signal, where the input system is a set of coupled differential rate equations that corresponds to all proton transfer reactions preceding in the reaction space. The rate constants of all proton transfer reactions are the adjustable parameters [16, 29, 30, 36, 61, 159, 160].

The reconstruction of the measured signal was initially attempted by the parameters of the WT protein whose cytoplasmic surface is characterized by four reactive elements (see Table 2a.1): 1) The D38 carboxylate, which is partially exposed to the bulk and functions as the most basic, unoccupied proton binding site on the surface. 2) The carboxylate of residue D36 that is fully exposed to the bulk. Due to the proximity between the two residues (5.1 Å), D36 functions as an efficient proton donor to D38 with a very fast virtual second order rate constant. 3) A cluster of three carboxylates (D104, E161 and E234 [36]) that are close enough to function as a single, proton-attractive site that delivers proton to D36, and D38. 4) The fourth reactive element of the surface is a carboxylate located on the extracellular surface of the protein. Its contribution to the dynamics is rather small and will not be discussed in the present study. All other bulk accessible residues of the protein make a negligible contribution to the dynamics, probably because their pK values are sufficiently low (pK ≤4). Such groups, once protonated, retain the bound proton for a time frame shorter than 1µs [16], which is too short to modulate the magnitude and shape of the pyranine re-protonation dynamics.

As seen in Figure 2a.1, curves D96N and WT, the solution suitable for the BR state of the WT protein was inadequate for the reconstruction of the transient measured with the D96N-M state protein. A further attempt to reconstruct the signals of the D96N-M state protein was based on the reaction pathway of the BR-WT system (cluster→D36→D38), while modulating the pK values and the rate constants of the proton transfer reactions. As shown in Figure 2a.2, Panel A, neither the modulation of the pK of the cluster (Panel A), its rate of reaction with free proton (Panel B) nor the pK of D38 (Panel C) could retrace the signal measured with the D96N-M state. Modulation of the number of carboxylates (Panel D), which make up the cluster was also insufficient to reconstruct the D96N-M state dynamics. These attempts indicate that a different pathway of proton transfer reactions characterizes the dynamics.
measured with the D96N-M state. Considering that the motion of the G and F helices affected the surface organization of the M structure, a new solution was looked for.

**Figure 2a.2:** Comparison between the D96N-M state signal and reconstructed curves generated by the WT parameters. The experimental signal was measured at pH=6.97 and the reconstructed by the reaction pathway of the BR state of the WT protein with modification of the parameters. Panel A depicts the reconstructed dynamics when the pK of the cytoplasmic cluster is varied (6.0, 5.7 and 5.4; lines 1, 2 and 3, respectively). In Panel B the rate constant of protonation of the cytoplasmic cluster varied (10^10, 2 10^10 and 6 10^10 M^-1s^-1). In Panel C the pK of the most-basic unoccupied base, identified with D38, was varied from 6, 6.5 and 7. Panel D demonstrates how the number of carboxylates in the cluster affects the shape of the reconstruction curve: one carboxylate (line 1) three carboxylates (line 2) and five (line 3).

Instead of carrying out a *de novo* search over the whole parameter space, we adopted a strategy of correlating the kinetic analysis with the published structure of the cytoplasmic surface of the M state of the protein. Of the available M state PDB files (1C8S; 1DZE; 1F4Z; 1CWQ), we preferred that of Sass [2] (1CWQ) as it revealed almost all residues on the cytoplasmic surface, except those from 240 to 247 (at the tip of the C-terminal domain).

Figure 2a.3 depicts the cytoplasmic face of the M structure of bacteriorhodopsin. In this figure, D38 is coloured in yellow and its carboxylate carbon, like that of all other marked carboxylates, is emphasized in black. D38 is inserted in a hydrophobic pocket that is emphasized in white and its oxygen atom of
the carboxylate moiety is 4 Å from the nitrogen atom of K41 (~1 Å closer than in the BR state of the WT protein). This proximity might stabilize the structure through a salt bridge. There are two proton conducting tracks leading to D38.

Figure 2a.3: The cytoplasmic surface of the M state model of bacteriorhodopsin (PDB file 1CWQ, Chain B). Residue D38 is coloured in yellow; D36 and D237 are in orange; D102 and E232 are in green and residues E234 and E161 are in cyan. For clarity, the carboxylate’s carbon atom of these residues is marked in black. Residues P37 and F42 and I99 are coloured in white. For the distance between the residues in the BR and the M state, see Table 2a.2.

One pathway, coloured in orange, consists of D36, which is 6.9 Å from the carboxylate of D38 and is adjacent to the E237 carboxylate (4.4 Å apart). The separation between the carboxylates along this pathway is comparable with the width of one or two solvation layers. Such proximity is suitable to sustain a fast proton transfer reaction, as was noticed between the fluorescein proton binding sites [32]. The second proton-conducting track, coloured in green, consists of D102 and E232. The D38-D102 separation (carboxylate to carboxylate) is 7.7 Å (practically the same distance as in the BR state of the WT). The D102 and E232 carboxylates are some 4.0 Å apart. The cyan coloured carboxylates (E161 and E234) seem to form another proton attractive pair that are 6.2 Å apart but are not well connected to D38. The distances between these residues in the BR and the M state, based on PDB file 1CWQ, is given in Table 2a.2. Comparison of the compiled data indicates that, in the
M state, the carboxylates appear in a more condensed configuration, that enhances the probability of efficient proton transfer between the nearby sites [32].

**Table 2a.2: The distances between the carboxylate residues involved in the proton collecting function of the cytoplasmic surface of Bacteriorhodopsin. The values were measured from the structure of Sass et. al. [99].**

<table>
<thead>
<tr>
<th>Residue pair</th>
<th>M state (Å)</th>
<th>BR state (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D38/D96</td>
<td>10.2</td>
<td>10.9</td>
</tr>
<tr>
<td>D36/D38</td>
<td>6.9</td>
<td>8.2</td>
</tr>
<tr>
<td>D36/D237</td>
<td>4.4</td>
<td>2.6</td>
</tr>
<tr>
<td>D38/E232</td>
<td>6.9</td>
<td>9.4</td>
</tr>
<tr>
<td>D38/D102</td>
<td>7.7</td>
<td>6.3</td>
</tr>
<tr>
<td>D102/E232</td>
<td>4.0</td>
<td>4.8</td>
</tr>
</tbody>
</table>

The reconstruction of the transient measurements, recorded with the D96N-M state protein, was carried out in compliance with the structural features. The most basic, unoccupied proton-binding site of the protein, whose reaction with free pyranine is extremely slow, was identified as D38, confirming results of previous studies [31, 33-36]. The two proton conducting tracks, D102+E232 and D36+E237, were allowed to have a pK value that ranged from 4 to 7 and their rate constant of proton exchange with D38 was set to vary from $10^8$ to $10^{12}$. The upper limit corresponds with proton transfer between well-connected sites, where the donor and acceptor may share a common water molecule in their solvation shell. The lower value does not imply any connectivity between the reactants. The rate of the protonation of the two tracks by free protons was allowed to vary from $5\cdot10^5$ to $5\cdot10^{10}$ M$^{-1}$s$^{-1}$, a range that extends from above the maximal rate according to the Debye-Smoluchowski equation, down to ~1% of this value. Within this parameter space we looked for a set of parameters that could reconstruct all experimental recordings with no systematic deviations.

The kinetic analysis of a complex system, such as a protein, necessitates that all protein molecules will be in the same conformation, and only one proton will probe the protein’s surface. Under these restrictions, the response of the protein is stochastic; the initial state of all protein molecules is the same, and there is no cross correlation between the molecules. Exposing a protein molecule to more than one proton implies that the first proton will affect the reactivity of the protein with the
second. In the same sense, when the protein population exists in more than one state of protonation, there is a possibility that the analysis will yield average values, which are specific for the composition of the population at the pH of measurements. For this reason, the analysis is initiated by reconstructing the signals measured at the high end of the pH range, assuming that above pH=8, all surface carboxylates will be ionized while the more basic residues, like the lysine and tyrosine moieties, will be in their fully protonated state. Indeed, in the high range 6.9 ≤ pH ≤ 8.25, a single set of parameters was obtained which accurately reconstructed all signals gathered in that range. On lowering of the pH of the measurements, some of the carboxylates are protonated and the homogeneity of the protein population is lost. Indeed, below pH=6.9 the consistency of the solution was lost, indicating that we have a mixture of states that varies their proportion with the pH. Therefore no attempts were made to investigate the kinetics at pH values lower than 6.9.

Table 2a.3: The kinetic parameters characterizing the proton transfer reactions on the cytoplasmic surface of the mutated bacteriorhodopsin (D96N) in its M state. The listed values reconstruct 25 independent measurements that were carried out at varying pH values 6.9 ≤ pH ≤ 8.25 with varying protein and pyranine concentrations and 150 mM NaCl.

<table>
<thead>
<tr>
<th></th>
<th>N°</th>
<th>pK</th>
<th>X′ + H+</th>
<th>XH+ ΦO-</th>
<th>XH +D38</th>
<th>XH+track 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>D38</td>
<td>1</td>
<td>6.4</td>
<td>0.1± 0.03 10^10</td>
<td>k&lt;10^-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.3</td>
<td>2.5 ± 0.4 10^10</td>
<td>0.9 ±0.2 10^10</td>
<td>1±0.2 10^10</td>
<td>-</td>
</tr>
<tr>
<td>track 2</td>
<td>2</td>
<td>4.8</td>
<td>2.5 ± 0.5 10^10</td>
<td>0.9±0.25 10^10</td>
<td>2±0.2 10^10</td>
<td>1 10^-</td>
</tr>
<tr>
<td>COO- ext</td>
<td>1</td>
<td>5.1</td>
<td>0.5±0.1 10^10</td>
<td>5.± 1 10^10</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* n corresponds with the number of carboxylates that are characterized by the same parameters. The rate constants are given in M^-1 s^-1 except for the virtual second order reaction whose values are printed in italics.

The set of parameters that accurately reconstructed 25 independent experimental records, measured with the D96N mutant protein, at a wide range of pH values and protein concentrations, is given in Table 2a.3. The quality of the fitting is depicted in Figure 2a.4. The two panels depict the reconstructed dynamics at two extreme pH values. Panel A corresponds with a measurement carried out at pH=6.97, where the pyranine is below its pK value and most of it is in the ΦOH state, ensuring a high yield of released protons. At this pH, the carboxylate of D38 is largely deprotonated and functions as the unoccupied proton-binding site on the protein with the highest pK. The experimental signals, together with the reconstructed curve, are
presented at two levels of time resolution: 250 µs (main panel) and 50 µs (inset). Panel B depicts the reconstruction of the signal measured at pH=8.25, where the dissociation of the ΦΟΗ in the ground state lowers the magnitude of the perturbation and accelerates the relaxation due to the higher abundance of the pyranine anion. Accordingly, the signals differ in amplitude and relaxation time, yet both of them and the 21 signals measured in the intermediate pH range and at different protein/pyranine ratios, are reconstructed by the same set of parameters (Table 2a.3).

**Figure 2a.4:** Reconstruction of the relaxation of the pyranine signals measured with D96N-M state at the extreme pH values where the kinetic parameters are still pH independent. Panel A depicts the reconstruction at pH=6.97 at 250 µs and 50 µs (inset). Panel B depicts the reconstruction of the signal measured at pH=8.25 by the very same rate constants.

The parameters listed in Table 2a.3 constitute the minimal system capable of reconstructing all experimental observations. A higher number of reactive residues will increase the complexity of the system, with no gain in the accuracy of the reconstruction or the understanding of the system.

The unoccupied proton binding site with the highest pK has a value of 6.4; its rate constant for the reaction with free protons is ~10% of the rate of a diffusion controlled reaction and it is practically inaccessible to pyranine anion. Based on these properties, we identify it with D38. The other proton binding sites are two pairs of
carboxylates having pK values of 5.3 and 4.8, defined as tracks 1 and 2, respectively. Both tracks have comparable rate constants for reaction with free protons and pyranine anions, but differ in the rate constant for proton transfer to D38. According to the rate constants, track 2 is better connected with D38, but considering the same minimal distance between D38 and D36 and D232 respectively, there is no way to identify the tracks with the residues.

**The mechanism of surface proton transfer**

Figure 2a.5 depicts the reconstructed time evolution of the protonated state of the proton binding sites whose parameters are given in Table 2a.3. Both tracks, 1 and 2, react with free protons at the same rate constants, yet as the rate of proton exchange between track 2 and D38 is faster than the other, and the ΔpK values also favour track 2 as a donor, the depletion of the protonated state of track 2 is faster and its maximal amplitude is smaller.

![Figure 2a.5](image)

**Figure 2a.5:** The reconstructed time evolution of the protonated state of the proton binding sites ascribed to the D96N-M state protein. Curve A is the experimental signal together with the reconstruction pyranine dynamics. Curves B and C correspond with the transient protonation of tracks 1 and 2, respectively, and curve D is the protonation of D38. Note how the rise of curve D is coupled with the decay of curves B and C.

The accumulation of protons on track 1 has the same initial velocity but its deprotonation is almost twice as slow. The combination of the two accessory systems leads to protonation of D38 within ~20 µs, even though by itself this residue is hardly
accessible to the bulk; its rate of reaction with free proton is \(\sim 10\%\) of the rate predicted by the Debye-Smoluchowski equation and pyranine hardly accepts protons from D38 (see Table 2a.3). The limited access of pyranine to the opening of the proton-conducting channel also extends the dwell time of protons on D38 up to \(\tau \sim 100\mu s\). The combination of a fast protonation of D38 and limited accessibility to soluble proton acceptors ensures efficient delivery of protons to the proton-conducting channel.

The protonation dynamics of the triple mutant

The triple mutant (D96G/F171C/F219L; [95]) is characterized by a ground state structure that resembles the late M state of the WT bacteriorrhodopsin [95]. The intensity of light source used in the present study did not produce an appreciable transient absorbance at 410 nm and it was assumed that the protein mostly remained in its ground state. The triple mutant membranes were suspended in 150 mM NaCl and, in the presence of 23 \(\mu M\) pyranine, subjected to proton pulse experiments, but without background illumination. The transient formation of the pyranine anion, its subsequent re-protonation and the reconstructed curves are depicted in Figure 2a.6.

![Figure 2a.6](image-url)

**Figure 2a.6:** Experimental signal of the triple mutant and its reconstructed dynamics. The measurements were carried out in unbuffered, 150 mM NaCl containing 3.5 \(\mu M\) protein, 15.3 \(\mu M\) pyranine at pH=7.86. Curve A depicts the experimental signal and its reconstruction, while curves B, C and D reconstruct the transient protonation of track 1, track 2 and D38 respectively.

The strategy of analysis was to start the process at high pH values and to proceed towards lower values, until the pH in which the independence of the
parameters was lost. In the range $7.7 \leq \text{pH} \leq 8.1$, a single set of parameters (listed in Table 2a.4) that fitted 14 independently measured signals was obtained. Below pH=7.7, the set of parameters became pH-dependent and continued to vary down to pH=6.2. Therefore no attempt was made to analyse the variable range of the solution.

Table 2a.4: The kinetic characteristics of the proton binding sites on the cytoplasmic surface of the triple mutant of bacteriorhodopsin. The listed values reconstruct 25 independent measurements that were carried out at varying pH values $7.7 \leq \text{pH} \leq 8.1$ with varying protein and pyranine concentrations and 150 mM NaCl.

<table>
<thead>
<tr>
<th>n</th>
<th>pK</th>
<th>X+H+</th>
<th>XH+ΦO-</th>
<th>XH+D38</th>
</tr>
</thead>
<tbody>
<tr>
<td>D38</td>
<td>1</td>
<td>6.9</td>
<td>$10^{10}$</td>
<td>k&lt; $10^{7}$</td>
</tr>
<tr>
<td>track 1</td>
<td>2</td>
<td>4.9</td>
<td>$4.0 \pm 0.5 \times 10^{10}$</td>
<td>$8.5 \pm 0.5 \times 10^{9}$</td>
</tr>
<tr>
<td>track 2</td>
<td>3</td>
<td>5.6</td>
<td>$4.5 \pm 0.5 \times 10^{10}$</td>
<td>$8.5 \pm 0.5 \times 10^{9}$</td>
</tr>
<tr>
<td>COO$_{\text{ext}}$</td>
<td>1</td>
<td>5.1</td>
<td>$0.5 \pm 0.1 \times 10^{10}$</td>
<td>$0.5 \pm 0.1 \times 10^{10}$</td>
</tr>
</tbody>
</table>

* n corresponds with the number of carboxylates that are characterized by the same parameters. The rate constants are given in $M^{-1}s^{-1}$ except for the virtual second order reaction, the values of which are printed in italics.

The pH-independent solution of the triple mutant system is similar in nature to that of the D96N-M state protein. There is one residue that functions as the most basic, unoccupied proton binding site, and this residue is poorly exposed to the bulk. Besides, there are two proton-conducting tracks leading to it. The difference between the triple mutant and the D96N-M state is in the pK values and the rate constants of the proton transfer reactions. The pK of the most basic, unoccupied proton-binding site of the triple mutant is higher than that of the D96N-M state, and the two tracks are more efficient. One is made of two carboxylates that are sufficiently close to form a common target. The second track is made of a cluster of three carboxylates that has even higher proton attractivity, both in pK and in the rate of reaction with free proton. Thus, based on functional analysis, the proton-collecting features of the triple mutant seem to resemble those of the D96N-M state. Considering the observation of Tittor et. al. (submitted) that the triple mutant exists as a mixture of all trans and 13 cis, a detailed structural evaluation of the parameters is premature.
CONCLUDING REMARKS

The structure of two proteins, both considered representatives of the ‘late M state’, was analysed in the present study [2, 95, 139, 161]. In both cases we noticed that the protonation of the carboxylate identified as D38 proceeds through an indirect pathway. The residue itself is partially buried with a limited accessibility of bulk ions (both protons and the pyranine anion) and its protonation is mediated by a fast proton transfer from well-exposed carboxylates.

The proton collecting antenna was first ascribed to the cytoplasmic surface of the BR (initial-) state of the WT protein, but the participating residues, identified by extensive mutation analysis, are different from those functioning in the late M structures. The structural rearrangements in the M state allow for a more efficient protonation of the partially buried residue D38 that is mostly likely the proton donor for D96 at the entrance of the reprotonation channel of bacteriorhodopsin.
Chapter 2b: Slow intra-protein proton transfer reactions in bacteriorhodopsin

INTRODUCTION

The initial events of the photocycle that precede the conformational change of the retinal molecule are extremely fast, and those leading to the proton release last less than 100µs. The steps that follow are much slower; the recovery of the protein back to the BR state takes some few milliseconds. At the cytoplasmic half of the channel, the protonation of the Schiff-base (during the M decay phase) is at the expense of the proton coming from the carboxylate of D96. This is a well-observed step, where the chromophore regains its purple colour. Yet, the re-protonation of the D96 by a proton coming through the cytoplasmic section of the channel is undetected by the electronic spectrum of the chromophore. The re-protonation of D96 can be inferred either by FTIR spectroscopy or by monitoring the proton abstraction from the bulk phase using a pH indicator. As will be shown in the present study, the propagation of the proton depletion (proton-hole), caused by the deprotonation of D96, towards the surface is a rather slow process, lasting up to 1 second in some mutants. Such a slow passage implies that the rate-limiting step of the reaction is a structural fluctuation in the protein, leading through one, or more steps, to the protonation of D96 at the expense of the deprotonation of D38 [162], which is located near the cytoplasmic surface. The re-protonation of D38, as was described in chapter 2a, is mediated through proton transfer reactions on the surface of the protein. Thus, the mechanism of proton delivery to D96 is a summation of many proton transfer reactions both on the surface and through the protein matrix.

In the present chapter we shall exploit the fact that the surface-bulk proton transfer is a microsecond event for deducing the pace of the conformation-dependent proton-hole propagation inside the protein. For this purpose we had used two pH indicators: one is the Schiff-base itself that records its protonation by the D96 carboxylate. The second indicator is water-soluble and its deprotonation corresponds with the time needed for the proton deficiency, created on D96, to propagate to the surface. The indicator of choice for monitoring the dynamics of bulk surface proton
transfer is pyranine (ΦOH) that, due to its negative charge (Z=-3 or -4, depending on its state of protonation), is not adsorbed to the purple membrane (PM).

The mechanism of proton transfer between the protein’s surface and the indicator in the bulk follows two pathways. One mechanism consists of dissociation of the proton from a donor residue and its diffusion-controlled reaction with the various acceptors. Yet, in most cases, the rate-limiting step of this pathway is the dissociation time of the donor residue. The other mechanism, which operates in parallel, is a collisional proton-transfer reaction, where the rate-limiting step is the collision between the reactants before proton transfer takes place [39]. The velocity of this pathway varies with the concentrations of the reactants, their diffusivity and accessibility. In previous studies [36, 145], we had investigated the rate constants of the two pathways and concluded that both mechanisms allow equilibration between the bulk and surface of bacteriorhodopsin at 100 to 200µs. The dominance of one pathway or the other depends on the concentration of the reactants. A high concentration of indicator or buffers present in the reaction system enhances the contribution of the collisional mechanism to the overall rate of reaction.

In the present study, we focused our attention on the last phases of the photocycle. We noticed a slow proton transfer reaction, which was orders of magnitude longer than the bulk surface equilibration time, that was manifested after the chromophore had relaxed to its BR state. This temporal discrepancy indicates that the system should be described in terms of quasi-equilibrium, where the bulk surface reactions are fast but the re-protonation of the protein is delayed due to the slow propagation of the proton-hole to the surface of the protein.

The slow propagation of the proton-hole was detected with the WT protein, but the effect was augmented when some charged surface residues, located far from the proton-pumping channel, were mutated to uncharged ones. These observations indicate that minor modulation of the charge distribution on the exposed loops connecting the cross-membranal helices is sufficient to precipitate aberrations in the conformational changes essential for the normal operation of the photocycle. To validate whether replacement of a charged residue on the loops can alter the structure of the protein, the very same mutants whose photocycle dynamics exhibited a delayed 'proton-hole' propagation were subjected to a brief proton pulse and the microsecond re-protonation dynamics of the pyranine anion were subjected to a rigorous kinetic analysis (Checover Ph.D. thesis). These experiments demonstrate that, in all cases, the
bulk surface proton transfer reactions were never the rate-limiting step in the protein’s re-protonation process.

MATERIALS AND METHODS

The mutated proteins were the generous gift of D. Oesterhelt (Max-Planck-Institute, Martinsried, Germany).

The kinetic measurements of the photocycle were carried out in a logarithmic time base, a device not present in our laboratory. For this reason, the measurements were carried out in collaboration of Prof. Dencher (Department of Biophysics and Biochemistry, Technical University of Darmstadt, Germany), who made the instrument available to us.

The pyranine signal is the difference between traces measured in the absence of buffers minus that measured in the presence of buffers. For this reason the marking on the abscissa is ∆ΔC.

RESULTS

The photocycle of the WT bacteriorhodopsin

The transient formation of the M state (measured at 412nm) and the corresponding bleaching of the BR state (measured at 568nm), as measured for the WT bacteriorhodopsin, are depicted in Figure 2b.1, Panel A (upper and lower curves respectively). The absorption traces associated with the protonation state of the Schiff base exhibit the familiar dynamics features of bacteriorhodopsin: a fast, submicrosecond deprotonation and re-protonation in the τ <100 ms time range. Panel B depicts the transient protonation of the pyranine anion (measured at 459nm), added to the solution as a pH indicator. In the absence of another proton acceptor in the solution, any proton released from the Schiff base should be present either as a free diffusing one, or bound to the dye or the protein’s surface. Yet, as was shown in [31], the contribution of the free protons to the total proton balance is negligible. Comparison between the amount of deprotonated Schiff base formed during the photocycle and the concentration of protonated pyranine indicates that only ~50% of the protons released from the Schiff base reacted with the indicator during the photocycle. The rest of the released protons are attached to the surface of the membrane and are probably bound to the PM’s variety of proton binding sites [36]. Measurements of the same kinetics in the presence of a screening electrolyte (150 mM KCl) (Figure 2b.1, dash curves) demonstrate that the M (and the BR) dynamics
are practically identical to those measured at vanishing salt concentration. The pyranine signal is however much larger, and more than 70% of the released protons were bound to the indicator. Another feature characterizing the dynamics measured in the presence of the screening electrolyte is a misbalance between the re-protonation of the Schiff base and deprotonation of the indicator in the bulk.

**Figure 2b.1**: Flash-induced concentration transients of the ground state and M intermediate of wild-type (WT) bacteriorhodopsin at 568 nm and 412 nm (Panel A, bottom and top curves, respectively) and of pyranine at 459 nm (Panel B); in water (—) and in 150 mM KCl (dash-dotted). All measurements were carried out at pH 7.5. Please note that the pyranine signal measured in the presence of screening electrolyte extends to a longer time frame than the transients measured with the Schiff base.

A comparison between the level of completion of the photocycle, quantitated by the decrement of the absorbance at 568 nm, and the quantity of protonated pyranine demonstrates that the reprotonation of the Schiff base and the deprotonation of the indicator are not coupled. For example, at the 10 ms time point, 0.73 µM of the protein there is still in a non-BR state (both in the absence or presence of KCl), while the amounts of protons stored in the ΦOH are either 0.51 µM or 1.38 µM, in the absence or presence of a screening electrolyte, respectively. Thus, the re-protonation
of the Schiff base (measured at 568nm) and the re-protonation of the protein (evaluated by the quantity of protonated indicator) can proceed at a different pace.

**The effect of replacement of charged surface residues on the photocycle**

Figure 2b.2 (top panel) depicts the photocycle traces of the WT bacteriorhodopsin protein, and of 9 other mutants, in which charged residues, located outside the immediate vicinity of the Schiff base, were replaced by neutral ones. The initial phases of the photocycle, associated with the rise of the M intermediate, are almost identical for all preparations. The M decay curves also vary within a factor of 2, except for two mutants D38C and R227Q, that exhibit a significantly slower M decay.

![Photocycle traces](image)

*Figure 2b.2: Flash-induced concentration transients of the M intermediate at 412 nm (Panel A), and of pyranine at 459 nm (Panel B) of wild-type bacteriorhodopsin and BR mutants E161C, R164C, E166Q, R227Q, K30C, D38C, D104C, D102C, and E166C. All measurements were carried out at pH 7.5.*

The apparent ‘consensus’ between the photocycle traces among the various mutants is lost when the monitored parameter is the transient protonation of the pyranine
(Figure 2b.2, bottom traces). In this case, there is a large diversion between the traces in amplitude, rise time and rate of relaxation to the prepulse state.

Table 2b.1 is the summation of the amplitudes measured for the M formation and the yield of pyranine protonation. The columns denote the maximal amplitude of the M state, the protonated indicator and its normalization with respect to the M state, both in the absence and the presence of screening electrolyte. To assist in the evaluation of the data, the numbers printed in italics represent values that are smaller than those of the WT protein and the numbers in bold are values that exceed the WT value.

Table 2b.1: The yield of protonated pyranine generated during the photocycle and its dependence on the salt concentration in the solution. The normalised yields of protonated indicator are printed in italics for values smaller than the WT and in bold letters for values that exceed the WT. In the last column, the underlined values indicate the mutations where the screening electrolyte lowered the yield of protonated pyranine.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>M µM</th>
<th>ΦOH (KCl=0)</th>
<th>ΦOH/M</th>
<th>ΦOH 150mM KCl</th>
<th>ΦOH/M 150mM KCl</th>
<th>Relative yield of ΦOH high salt/no salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.65</td>
<td>1.9</td>
<td>0.52</td>
<td>2.77</td>
<td>0.76</td>
<td>1.45</td>
</tr>
<tr>
<td>K30C</td>
<td>3.65</td>
<td>1.3</td>
<td>0.35</td>
<td>1.6</td>
<td>0.44</td>
<td>1.23</td>
</tr>
<tr>
<td>D102C</td>
<td>3.7</td>
<td>0.82</td>
<td>0.22</td>
<td>1.7</td>
<td>0.47</td>
<td><strong>2.03</strong></td>
</tr>
<tr>
<td>D104C</td>
<td>3.7</td>
<td>1.6</td>
<td>0.44</td>
<td>1.16</td>
<td>0.31</td>
<td>0.72</td>
</tr>
<tr>
<td>E166C</td>
<td>3.65</td>
<td>1.7</td>
<td>0.45</td>
<td>2.12</td>
<td>0.58</td>
<td>1.24</td>
</tr>
<tr>
<td>E166Q</td>
<td>3.18</td>
<td>0.42</td>
<td>0.13</td>
<td>1.14</td>
<td>0.36</td>
<td>2.7</td>
</tr>
<tr>
<td>R164C</td>
<td>2.93</td>
<td>1.78</td>
<td><strong>0.6</strong></td>
<td>2.3</td>
<td><strong>0.78</strong></td>
<td>1.12</td>
</tr>
<tr>
<td>K159C</td>
<td>3.29</td>
<td>0.6</td>
<td>0.18</td>
<td>1.23</td>
<td>0.37</td>
<td><strong>2.05</strong></td>
</tr>
<tr>
<td>E161C</td>
<td>3.11</td>
<td>2.39</td>
<td><strong>0.78</strong></td>
<td>2.8</td>
<td><strong>0.9</strong></td>
<td>1.16</td>
</tr>
<tr>
<td>D38C</td>
<td>3.28</td>
<td>1.32</td>
<td>0.4</td>
<td>1.86</td>
<td>0.57</td>
<td>1.41</td>
</tr>
<tr>
<td>R227Q</td>
<td>3.25</td>
<td>2.36</td>
<td><strong>0.73</strong></td>
<td>2.12</td>
<td><strong>0.65</strong></td>
<td><strong>0.9</strong></td>
</tr>
</tbody>
</table>

The main effect of the screening electrolyte is an increase in the yield of protonation of the free indicator. The surface of the purple membrane is covered by a large number of proton binding sites, and when a proton is released from the protein, it has a high probability to be taken up by the surface groups [31]. In a solution with a high concentration of screening electrolyte, some of the bound protons are replaced.
by the prevailing cations and the yield of protonated pyranine increases. Examination of the results presented in Table 2b.1 are in qualitative accord with this assumption, but the magnitude of the effect varies with the nature of the mutant, and in a couple of cases (D102C and R227Q, marked by underline in the table) the effect is reversed. Thus, it is clear that the bulk surface proton transfer process is a complex function of the ionic strength, having some aspects associated not only with ionic equilibria but also with structural modulations.

A. Mutation that extended the M state decay

In comparison with the WT-M state decay, the signals measured with D38C and R227Q, are significantly slower (Figure 2b.3, Panel A) and extend well over 10 ms. The slow re-protonation of the Schiff base is paralleled by delayed deprotonation of the pyranine (Figure 2b.3, Panel B).

![Figure 2b.3](image-url): Flash-induced concentration transients of the M intermediate at 412 nm (Panel A, C) and of pyranine at 459 nm (Panel B, D) of wild-type bacteriorhodopsin and BR mutants D38C and R227Q in water (Panel A, B) and 150 mM KCl (Panel C, D). All measurements were carried out at pH 7.5

Addition of the screening electrolyte selectively accelerated the dynamics measured with R227Q (Panels C and D) but not those recorded with D38C. Thus,
even though the two mutants are characterised by similar M decay dynamics, the underlying mechanism seems to differ. The re-protonation of the Schiff base in both cases is coupled in time with the deprotonation of the indicator, and the 50% mark for the relaxation of the M state and the deprotonation of the indicator occurs at the same time point. Thus, even though the photocycles of the two mutants were distinctively modulated, the coupling between the regaining of the protein’s BR state and the re-protonation of the protein is maintained.

### B. Mutation with slow proton-hole propagation

In contrast to the above mutations, where the lengthening of the photocycle is coupled with delay in the re-protonation of the Schiff base, the mutants whose dynamics are presented in Figure 2b.4 exhibit another feature: the re-protonation of the Schiff base precedes in time the deprotonation of the indicator in the bulk.

![Figure 2b.4](image)

**Figure 2b.4:** Flash-induced concentration transients of the M intermediate at 412 nm (Panel A, C) and of pyranine at 459 nm (Panel B, D) of WT and bacteriorhodopsin mutants E161C, D102C, D104C and K30C in water (Panel A, B) and 150 mM KCl (Panel C, D) (pH 7.5). The BR concentration was $C_{\text{wt}} = 0.364 \text{ gl}^{-1}$, $C_{\text{E161C}} = 0.311 \text{ gl}^{-1}$, $C_{\text{D102C}} = 0.072 \text{ gl}^{-1}$, $C_{\text{D104C}} = 0.264 \text{ gl}^{-1}$, $C_{\text{K30C}} = 0.272 \text{ gl}^{-1}$.

The three mutants, K30C, E161C and D104C, exhibit standard M formation dynamics with a slight modulation of the M decay phase, which deviates from the WT curve by less than a factor of 2 and, at the 100 ms time point, the recovery of the BR state is ~98%. The signals measured at low and high concentrations of screening
electrolyte are practically identical. A surprising feature was noted with respect to the dynamics measured for the pyranine; the signals are stretched in time beyond the Schiff base re-protonation. This effect is further amplified in the presence of a screening electrolyte and the deprotonation of the indicator becomes extremely slow and is extending to the 1 sec time frame.

**Figure 2b.5:** Flash-induced concentration transients of the M intermediate at 412 nm (Panel A) and of pyranine at 459 nm (Panel B) of the K30C-mutant of bacteriorhodopsin; in water (---) and in 150 mM KCl (- - -) (pH 7.5). The BR concentration in water and in 150 mM KCl was $C_{K30C} = 0.272 \text{ gl}^{-1}$.

This feature is best demonstrated in Figure 2b.5 that records, on an expanded time scale, the recovery of the BR state of the K30C mutant (Panel A) and the $\Phi_{OH}$ deprotonation dynamics (Panel B). The figure depicts the traces from the 1 ms time point and on. As seen in the figure, the amplitudes of the photocycle intermediates are almost independent of the screening electrolyte, while the salt increases the yield of protonated pyranine. At the 10 ms time point, both at low and high salt concentrations, 1.32$\mu$M of the protein had not regained the BR state. At the same time point, the concentration of the protonated pyranine (in the absence of screening electrolyte) is 0.8 $\mu$M but, at 150 mM KCl, the amount of protons stored in the form of $\Phi_{OH}$ 1.6 $\mu$M exceeds that of the unprotonated Schiff base. Finding in the solution of more protons than the amount needed to re-protonate the Schiff base implies that...
the protein had created an internal ‘deficit’ by abstracting proton from D96, and the compensation of the intra-channel donor is slower than the bulk-surface equilibration time ($\tau \leq 100\mu s$, see Figure 2a.5). The discrepancy between the recovery of the BR state and the deprotonation of the indicator extends to an even longer time frame. It can be seen in panel A that, 100 ms after the excitation, the relaxation of the photocycle is almost over and only 0.2 $\mu M$ of the protein had not regained its BR state, at the same time, the pyranine is still 0.41$\mu M$ above its equilibrium level. This state of transient disequilibrium is noticed even at 400 ms, where 99.95% of the Schiff base had regained the ground state, while the pyranine is 0.1 $\mu M$ above its equilibrium level. These numbers clearly indicate that the Schiff base had been protonated at a rate that exceeds the capacity of the protein to equilibrate with the bathing solution. Considering the fact that, even in the presence of a screening electrolyte, the bulk surface proton transfer gains equilibrium in less than 100$\mu s$ (see figure 2a.5) it is clear that we are observing a delayed proton transfer process inside the protein. The protonated D96 carboxylate donated a proton to the Schiff base but the ‘proton-hole’ is only slowly compensated by the bulk, where the rate-limiting step is the proton transfer from D38 to D96. The slowness of the reaction, especially in the presence of the screening electrolyte, implies that one or, more likely, a combination of structural changes that allow the two residues to exchange proton among them controls it. Once the D38 had transferred its proton to D96, its reaction with the indicator in the bulk is a rapid process lasting a few tens of microseconds. As deduced from these observations, relying on the photo cycle as the only parameter for characterizing normal functioning of bacteriorhodopsin is an insufficient requirement [163]. Not only the photocycle but also the rate of protein-bulk interaction must be measured.

The three mutants E161C D104C and K30C that exhibit a delayed deprotonation of the pyranine differ in their sensitivity to the presence of the screening electrolyte. The signals measured with E161C and K30C are characterized by a slow protonation that is further delayed in the presence of salt. On the other hand, with D104C, the pyranine’s amplitude, in the presence of a screening electrolyte, is smaller than in its absence, and the recovery of the BR state is accelerated in the presence of ionic screening. The dynamics measured with the three mutants exhibit major disequilibrium between the protein and bulk above the 10 ms time point, while as shown in chapter 2a (Figure 2a.1 and 2a.5), the bulk-surface equilibration is established in a fraction of a millisecond. Thus, the later phases of the photocycle
should be considered in terms of quasi-equilibrium. The surface and the bulk are in equilibrium, while the proton-hole in the protein slowly migrates towards the surface, where it is rapidly filled by the solution.

**Figure 2b.6:** Flash-induced concentration transients of the M intermediate at 412 nm (Panel A) and of pyranine at 459 nm (Panel B) of the E166C and E166Q-mutants of bacteriorhodopsin in water and 150 mM KCl (pH 7.5).

**C. Comparative kinetics measurements with E166Q and E166C**

The delayed propagation of the proton-hole to the surface is not only a function of the mutated residue but also depends on the residue to which it was transformed. Two E166C mutants had been tested; the same carboxylate was replaced either by cysteine or glutamine, and the result of the replacement was in contrast with each other (See Figure 2b.6). At 100 ms after the initiation of the photocycle there is still 0.24 µM of protonated indicator, even though the Schiff base had completely regained its BR state. Replacement of the same residue by glutamine (E166Q) affects the bulk surface proton transfer reaction in a different way: the yield of protonated pyranine falls dramatically, and the propagation of the proton-hole to the surface does not exhibit any delay.
DISCUSSION

The present study documents a temporal delay in the re-protonation of the bacteriorhodopsin molecule during the post-N phase of the photocycle, which is a process that can last well beyond the time when the chromophore regains its stable BR state. This delayed reaction is associated with slow proton-hole propagation, presumably from D96 towards the surface. Once the proton-hole has been exposed to the surface, its protonation is achieved within the sub-millisecond time frame.

The slow propagation of the proton-hole was noticed while monitoring a detailed proton balance between two sinks; the protein and the bulk. The state of protonation of the protein was monitored by recording the protonation state of the Schiff base, while the bulk was monitored by quantitation of the decrement protonation of the pyranine molecules. The decay of the M state, formed during the photocycle, indicates that the proton released towards the extracellular side of the proton conducting system had been replaced by that bound to D96. Yet, as this residue is too far from the chromophore to affect its absorption spectrum, we cannot detect its re-protonation except by counting the proton content of the other sink, namely the bulk. The quantitative follow-up of the bulk was attained by the addition of a low concentration of a pH indicator, pyranine, which in this case was the only soluble proton acceptor in the reaction system. Any temporal discrepancy between the two observable moieties implies that the proton-hole made upon removal of a proton from D96 had not been filled by a bulk proton.

The comparison between the two processes indicates that in some cases, even with the WT protein, there is a temporal discrepancy between the amount of reprotonated Schiff base and the protonated pyranine level in the bulk, indicating that the intra-protein proton transfer reactions were not compensated for by bulk-surface proton transfer reactions. The decoupling was noticed to be affected by the presence of a screening electrolyte, which had negligible effects on the photocycle dynamics, but effectively modulated both the amplitude and dynamics of the protonation state of the indicator in the bulk. This effect of the salt could have been accounted for by invoking a general screening effect. However, we had noticed that the electrolyte screening effects varied between the mutants. Thus while, in most cases including the WT protein, the screening electrolyte increased the amplitude of the protonated indicator, the pyranine yield was lowered for the D104C and the R227Q mutants.
What is more, adding a screening electrolyte to the measurements with D104C had lowered the pyranine yield, but enhanced the temporal delay in the propagation of the proton-hole to the surface (see Figure 2b.4). Based on these observations, it is an inevitable conclusion that the rate-limiting step in the re-protonation of the protein is due to slow steps associated with the hole propagation towards the cytoplasmic surface, most probably controlled by the protein dynamics and affected by the protein-solvent interactions.

The modulation of the proton-hole propagation dynamics was seen to be affected by mutations that are not associated with the residues not commonly implicated with the proton pumping mechanism, such as K30, D104, E161, E166 and R164, that are located on the exposed inter-helices loops. In our opinion, this observation emphasised the importance of the loops in the functioning of bacteriorhodopsin as a proton pumping protein.

It is a general concept that the structure of the protein is determined by inter-helices interactions [164], and that the photocycle is coupled with relative motion of the helices [99, 120, 132]. The present data indicate that the interaction between the exposed residues, either one with the other, or even with the solution, can modulate the mechanism or the rate, of the proton-hole propagation inside the protein. This notion was tested by measuring the dynamics of bulk surface proton transfer using the Laser Induced Proton Pulse (Checover Ph.D.). The microsecond resolution of these observations reveals that the replacement of the surface-charged residues indeed led to gross modulation of the distribution of the proton binding sites on the protein’s surface. The functions altered by the mutations were the pK of the surface groups, their rate of reaction with free diffusing ions and even the number of sites that reacted with the solutes in the bulk.
Chapter 3: lactose permease

The lactose permease (lac permease) of *E. coli* is a cytoplasmic membrane protein that catalyzes a stoichiometric symport of galactopyranosides and $\text{H}^+$ [164]. The enzyme has been solubilised, purified to homogeneity, reconstituted into proteoliposomes and shown to act as a monomer [165]. Hydropathy analysis [166] and more sophisticated algorithms indicate that the lac permease contains 12 transmembrane $\alpha$ helices that traverse the membrane in a zigzag fashion connected by relatively hydrophilic loops with both N and C termini on the cytoplasmic face (Figure 3.1) [164, 167].

![Secondary structure model of lac permease](image)
Figure 3.2: Helix packing of the lac permease viewed from the cytoplasmic surface. The positions of the four residues irreplaceable for coupling [Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X) and Glu325 (helix X)] are enlarged and in blue. Positions of the two interacting pairs of Asp-Lys residues [Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X)] are enlarged and in red. Positions of NEM-sensitive Cys replacements are indicated with a small green dot. Positions of residues involved in substrate recognition and/or translocation [Glu126 (enlarged), Arg144 (enlarged), Met145, Cys148, Val264, Gly268 and Asn272] are in yellow. Positions shown in purple are involved in thiol cross-linking. Placement of helices III and IV is based on thiol cross-linking experiments between Cys residues in periplasmic loops and/or transmembrane helices (The figure was taken from Frillingos et. al., [168]).

On the basis of a detailed characterization of mutations of the following residues: Cys148 [169, 170], Glu126 and Arg144 [171-174] and binding studies with a series of substrate analogues [175], the following model (see Figure 3.3) for galactoside binding was postulated [168, 172]: i) One of the guanidino NH$_2$ groups of Arg144 forms an H-bond with the OH group at the C-4 or C-3 position(s) of the galactosyl moiety of the substrate, an interaction that plays a key role in substrate specificity. ii) The other guanidino NH$_2$ group of Arg144 forms a salt bridge with Glu126, and the interaction holds Arg144 and Cys148 in an orientation that allows specific interaction with the galactosyl moiety. One of the oxygen atoms of the carboxylate at position 126 may also act as an H-bond acceptor from the C-6 OH of the galactosyl moiety, and the C-2 OH is also an important determinant. iii) Met145, which is on the same face of helix V as Cys148, is thought to be important in this respect. In addition, other studies [176, 177] indicate that Gly residues in helices IV
and V provide the conformational flexibility that is important for substrate binding and transport. Although major determinants for substrate binding in lac permease lie at the interface between helices IV and V, we found out that it is important to obtain detailed physical properties of this cavity (see chapter 3b and [178]).

![Image](image.png)

**Figure 3.3:** Putative substrate binding site in lac permease. Helices IV (white) and V (green) are shown with Glu126 (green), Arg144 (white), Met145 (orange) and Cys148 (yellow). The galactosyl and glucosyl moieties of lactose are in light and dark blue, respectively. As indicated, one of the guanidino NH2 groups of Arg144 H bonds with the hydroxyl group at the C4 and/or C3 positions of the galactosyl moiety, and the other guanidino NH2 group interacts electrostatically with Glu126 which may also act as an H-bond acceptor from the C6-OH of the galactosyl moiety. Cys148 interacts weakly and hydrophobically with the galactosyl moiety, and Met145 interacts even more weakly with the glucosyl part of lactose (The figure was taken from Frillingos et. al., [168]).

The overall mechanism for lactose/H+ symport involves a minimum of six reaction steps: i) binding of H+ to the lac permease; ii) binding of substrate; iii) selective inversion of the exposure of the bound substrate with respect to the membrane plan; iv) release of substrate; v) release of H+; and vi) return of the unloaded lac permease to its first configuration. In contrast, exchange and counterflow involve steps 2 and 4, which do not involve H+ translocation [179, 180]. In addition to active transport, lactose efflux, exchange, and counterflow are blocked in His322, Arg302 and Glu269 mutants. However, lac permease mutants with neutral replacements for Glu325 are defective in all steps that involve net H+ translocation rather than an effect on translocation across the membrane.
As can be concluded from the recent studies of Kaback and coworkers, when the protein has no bound substrate [181, 182], the enzyme is protonated, and the proton is shared between His322 and Glu269, while Glu325 is charge-paired with Arg302. The substrate is bound with high affinity at the interface between helices IV (Glu126) and V (Arg144 and Cys148) (Figure 3.2), which is accessible from the outside surface of the membrane. Substrate binding induces a conformational change that leads to transfer of the $\text{H}^+$ from His322/Glu269 to Glu325 and reorientation of the binding site of the inner surface, followed by decrease in affinity and dissociation of the substrate. Glu325 is then deprotonated on the inside due to re juxtaposition with Arg302. The His322/Glu269 complex is then reprotonated from the outside surface to initiate the cycle again.

It was shown [181] that lactose accumulation driven by the proton electrochemical gradient is very sensitive to the lipid phase transition, exhibiting a 7-8 fold decrease in $E_a$ above $18^\circ$, the temperature at which the phase transition occurs in the wild-type membrane. In contrast, the effect of the lipid phase transition on equilibrium exchange, a reaction that does not involve proton translocation, exhibits only a 2-3-fold decrease in $E_a$. More importantly, mutant E325A which is specifically defective in all reactions that involve $\text{H}^+$ symport but catalyzes equilibrium exchange and counterflow as well as wild-type exhibits a completely linear Arrhenius plot with respect to equilibrium exchange and an $E_a$ of 9.3 Kcal/mol that approximates that of active transport and efflux above the phase transition. Thus, the conformational change in the lac permease that corresponds to the translocation step in the overall mechanism appears to be completely unaffected by the lipid phase transition. It is reasonable to suggest that the primary effect of the phase transition is on the coupling between lactose and $\text{H}^+$ translocation. These observations should be considered in association with the calculation of Groves et. al., [183] who suggested a link between charge motion within a membrane protein and lateral rearrangement of the lipid matrix surrounding the protein.
Chapter 3a: Probing of the substrate-binding domain of lactose permease by a proton pulse

INTRODUCTION

The catalytic function of lac permease is the combination of two processes [168]. One is the mechanism that converts the $\Delta \mu H^+$ into a substrate driving force by imposing vectoriality on the binding and release of the galactoside. The other process prevents a proton slip during the catalytic cycle; otherwise the enzyme would function as an inherent uncoupler that dissipates the $\Delta \mu H^+$. The large size of the transported substrate, and the strict requirement to maintain a proton leak-proof seal, imply that the protein must undergo extensive conformation transformation [184, 185]. Considering that the transported substrate does not carry a net charge, a model based on direct electrostatic interactions between the enzyme and the substrate cannot account for the proton-galactose co-transport. Accordingly, the substrate transport is probably mediated by timed conformational changes of the protein, which are coupled with replacement of protein-substrate interactions by solvent-substrate stabilization.

In the present study, we monitored the accessibility of the substrate-binding domain to the bulk by labeling it with a covalently bound pH indicator (fluorescein maleimide) and monitored the rate of its reaction with protons released in the bulk. The site for labeling was the native cysteine residue (C148), which stabilizes the substrate in the conducting cleft and blocking of it by maleimide derivative inhibits the flux through the enzyme [169, 170, 186, 187]. The lac permease has a substrate-binding site that can accommodate a hydrophilic moiety as large as a disaccharide, while maintaining a tight seal conformation that does not allow a proton to leak through the protein. Thus, by probing the proton accessibility into the protein, we gain a quantitative parameter for characterizing the inner cavities, which are comparable in depth with the penetration of the native substrate. The usage of an indicator with a molecular weight comparable to that of the substrate (427Da and 342Da respectively), which is attached to the same residue (C148) that was implicated in the binding of the native substrate, offers the opportunity to probe the inner space of the enzyme under conditions that mimic the state of the enzyme when loaded with the substrate. Indeed,
the fluorescein and the lactose are not identical in hydrophobicity, charge and geometry; however, this dye offers the best opportunity for first time analysis of the dynamics of proton transfer between the amino acid’s side chains with a reporter group located on the substrate binding site. Thus, even though the binding could have modulated the probed space, the measurements provide novel information about the interaction between moieties within the substrate-binding site.

The selection of a pH indicator as a reporter group offers the possibility to probe the environment of the dye by free diffusing proton, which is a particle that drives the substrate flux through the enzyme and is the best gauge for probing an environment. The solvated proton is the most studied ion in solution and its equilibration and kinetics are well recognized [38, 40, 188-190]. The rate constant of protonation of a residue by a free diffusing proton is given by the Debye-Smoluchowski equation. For a well-exposed site, the rate constant is $1-2 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ [16]. A slower rate constant implies that the site is located in a hydrophobic environment or that intensive, positive potential repels the proton. The very same parameters, hydrophobic environment, or nearby electric charge also affect the pK value of a residue. Thus, the chemical-physical properties of the site can be deduced by combining kinetic and thermodynamic information.

The analysis reveals that the lac permease-fluorescein adduct exists in two conformation states, which are controlled by the pre-pulse pH. In the low pH range (pH $\leq$ 7.1), the accessibility of the proton to the substrate-binding site is of a diffusion-controlled reaction, while the interaction of the dye with the nearby carboxylate of E126 is poor. In the high pH regime (pH $\geq$ 7.4), the proton accessibility is reduced while the carboxylate of E126 and the dye exchange protons at a very high rate, suggesting that the two residues are no more than one water molecule apart. The correlation between these conformations and the catalytic mechanism will be discussed.

The subject described in this chapter was published in Biochimica et Biophysica Acta, 2001, 1514, 33-50.
MATERIALS AND METHODS

Preparation of lac permease

The enzyme used in the present study was a generous gift of H. Ronald Kaback and J. Le-Coutre (Howard Hughes Medical Institute, UCLA, U.S.A.). The preparation was a 6 His-tagged enzyme, on the C terminus, Cys-less background, enzyme that was back mutated (S148C) to introduce a single cysteine at the native C148 site, thus reconstructing the native substrate binding site with no other maleimide binding domain on the whole enzyme. The labeling was carried out at pH =7.5 at 0°C in the dark with a final yield of 90-95% labeled protein. Before usage the protein was eluted from a P10 column by 0.018% lauryl maltoside (LM), 100 NaCl, to remove the phosphate buffer in which the enzyme was suspended.

Kinetic measurements

The kinetic measurements were carried out in solution containing pyranine (15 to 35 µM) and lac permease labeled by fluorescein maleimide with 97% efficiency. The enzyme was suspended in 100 mM NaCl, 0.5 mM LM to a final concentration of 5-10 µM (with respect to the bound fluorescein) and its precise concentration was determined by its absorbance at 500 nm.

Kinetic analysis

For the purpose of analysis, all proton transfer reactions taking place within the perturbed space were defined and linked by equilibrium and rate equations. The implementation of the differential rate equations for the lac permease-fluorescein adduct necessitates definition of the various proton-binding sites involved in the reaction. Naturally, accounting for each of the proton binding residues is a precise presentation of the system, but the penalty would be an overwhelming increment in the complexity of the system. For this reason, the number of independent proton binding sites that were included in the numeric reconstruction was reduced as described below.

1) The two dyes, pyranine (φOH) and fluorescein (Flu), were explicitly presented by their formal concentrations.

2) All proton binding sites on the periplasmic side of the protein were grouped into two subpopulations: the carboxylates (COO⁻av)pri and the histidines (Hisav)pri. The
number of elements \( (n_i) \) in each subpopulation was an adjustable parameter with the limitation \( n_i \leq n_{\text{max}} \), where \( n_{\text{max}} \) is the number of residues on the surface as estimated by Kaback’s structural model [164].

3) All proton binding sites on the cytoplasmic side of the protein were grouped into two subpopulations; the carboxylates \( (\text{COO}^-_{\text{av}_{\text{cyt}}} \) and the histidines \( (\text{His}_{\text{av}_{\text{cyt}}} \). The number of elements in each subpopulation was an adjustable parameter as described above.

4) The intra-protein proton binding sites were equated with the reactive residues identified by Kaback and coworkers [164] as essential for the enzymic activity; histidine \( \text{H}322 \) was treated explicitly while the four carboxylates were split into two subpopulations. The effects of the E126A mutation on the apparent pK of the bound fluorescein suggested some coupling between the two proton binding sites. Accordingly, one of the intra-protein carboxylates was treated as an explicit group \( (\text{COO}^-_{\text{E126}}) \), while the other carboxylates were grouped in one subpopulation \( (\text{COO}^-_{\text{in}}) \) that was assigned by average parameters. Because of their high pK, the lysine and arginine moieties were assumed to be constantly protonated and made no contribution to the buffer capacity of the protein.

All proton-binding sites were assigned with rate constants for their reactions with free protons, free diffusing-pyranine anions and for proton exchange reactions with all other proton-binding sites. The distinction between the sites located on the periplasmic vs. the cytoplasmic side was attained by setting \( (k_{j:i}=0) \) for all proton exchange between groups located on opposite faces of the enzyme.

The set of parameters that reconstructs the experimental system made of pyranine and the fluorescein-lac permease adduct, over the whole pH range, is given in Tables 3a.1, 3a.2 and 3a.3. The quality of the reconstructed dynamics is demonstrated by the deviation plots (insets to Figure 3a.5 below). Along the full length of the observation time, neither the pyranine nor the fluorescein reconstruction exhibits a systematic deviation from the experimental data.

The reconstruction of the two signals, as in Figure 3a.3, is a summation of all parallel reactions that link the transient protonation of the moieties in the bulk, on the protein’s surface, the two dye molecules and the protonable residues located inside the cavity. The mode of analysis and its results will be described in the text while the rate constants are summarized in Tables 3a.1 and 3a.2.
Table 3a.1 lists the reactions of free protons with the various proton-binding sites. These parameters are the minimal number of reactants needed to reconstruct the whole set of observations. In accordance with the structure proposed by Kaback and coworkers [164, 184, 186, 191] and Brooker and coworkers [185, 192, 193], the number of exposed residues was estimated and set as the upper limit for the number of residues that react with free protons in a diffusion controlled reaction. The cytoplasmic surface of the enzyme was efficiently represented by two populations of proton binding sites: one consisted of 6 carboxylates and the other of 3 histidine residues. It is of interest to point out that although the enzyme was expressed with a His-tag of six residues, its contribution to the proton binding dynamics of the protein was rather small. Each subpopulation was assigned an average pK value and rate constants of proton exchange with other types of proton binding sites. The periplasmic surface was also satisfactorily represented by two subpopulations, one consisting of 5 carboxylates, the other of two histidine residues. Within the substrate-transporting cavity, the analysis was consistent with one carboxylate (E126) and one histidine residue (H322) that were each characterized by specific rate constants, and three more carboxylates that were characterized by average values. A more defined system of non-identical sub-populations would not have lead to any gain in the accuracy of fitting of the experimental signals.

RESULTS

Physical properties of the covalently bound fluorescein

The binding of fluorescein maleimide to C148 of lac permease shifts the absorption maximum of the anionic state of the dye from 490±1 to 500±2 nm, indicating that the local environment modulates the molecular orbitals of the dye (Figure 3a.1, Panel A). In the first singlet state, the electronic orbitals are more prone to delocalization by the intra-protein electrostatic field than in the ground state. Thus, the red shift of the absorption band of the bound fluorescein implies that the cavity provides a polarizing force that narrows the energy gap between the ground state and the excited one. Considering the proposed structure of the substrate binding site [169, 170, 194], it is plausible that the polarization is caused by the positive charge of R144 and the negative charge of E126 that flank the substrate from both its sides.
**Figure 3a.1**: Absorption spectra of the C148-fluorescein maleimide adduct of lac permease and its pH titration curve. Panel A depicts the absorption spectra of the alkaline state of free fluorescein (4.7 µM, pH 8.0) (Line A), and the fluorescein-lac permease adduct (line B). For the sake of clarity, line B was shifted upwards with respect to line A. The maxima of the two spectra are at 493 and 500 nm respectively. Panel B depicts the pH titration curves while monitoring the fluorescence (excitation at 500 nm, emission at 515 nm) of the fluorescein C148-lac permease adduct (○) and of the adduct with a protein where E126 was mutated to alanine (E126A; ▲). Please note that the systematic shift of these data points to lower pH values. The titrations were carried out in 0.5 µM lac permease in 100 mM KCl, 5 mM Tris-Mes buffer.

The pK of the bound dye was measured by monitoring the excitation spectrum of the bound dye while the pH of the solution varied gradually from 5.5 to 9.5. The results, presented in Figure 3a.1, Panel B, follow a smooth titration curve with an apparent pK value of pK$_{\text{app}} = 7.0 \pm 0.08$. This value is significantly higher than that of the free dye (pK = 6.3 ± 0.05 at I=50 mM). The slope of the titration curve is larger than 1 ($\alpha=1.35$; $R=0.98$), indicating that, during the titration, the protein as a polyelectrolyte responded to the pH variation by changing the pK of its surface groups. As the pH was raised, the surface became more negative with an appropriate downshift of the apparent pK. The pK shift of the bound dye is attributed to an extra stabilization of the protonated state of the dye by two forces. One is the hydrophobicity and low dielectric constant of the binding cavity, which favors the
uncharged state of the dye. The second force comes from the nearby negative charges that enhance the proton attraction of the domain. Measurement of the pK of the fluorescein bound to the glutamate 126-less mutant (E126A) supports this assumption. As seen in Figure 3a.1 Panel B, the elimination of the charge caused a small but systematic shift of the titration curve to change the dye to just slightly lower values, indicating that the immediate vicinity of the dye is less negatively charged.

The pK of the fluorescein incorporated into 3:1 PE:PG liposomes that was found to be pK=6.5. This value is significantly lower than in the micellar system, indicating that the fluorescein moiety is even closer to the positive charge in the liposomal system than in the micellar one.

**Protonation kinetics of free and bound fluorescein**

The dynamics of the acid-base perturbation, imposed during a proton pulse experiment, are demonstrated in Figure 3a.2. The protons, discharged from the excited pyranine molecule, are released within the response time of the measuring system, and an equal quantity of free protons and pyranine anion is formed. The protons react with the pyranine anion (Curve 1) and with the fluorescein (Curve 2) in a diffusion-controlled reaction. During the first phase of the reaction, lasting ~5 µs, both dyes react with the free protons and reduce their concentration to an almost pre-pulse level [32, 37]. At a latter phase (t>5 µs), the relaxation proceeds by a collisional proton transfer, where the rate-limiting step is the encounter between a protonated fluorescein molecule and a pyranine anion. This mechanism is evident from the mirror symmetry of the relaxing curves; all protons not present on the pyranine (Curve 1) reside on the protonated fluorescein molecules (Curve 2).

Repeating the same measurements with a protein bound fluorescein generates a different kind of relaxation curve (Figure 3a.2, Curves 3 and 4). The protein, with its many proton-binding sites, competes with the pyranine for the free protons and the rapid phase of pyranine re-protonation is missing. What is more, as the protons are bound to the protein, the main mechanism of relaxation is a collisional proton transfer from the protonated site on the protein with a free diffusing pyranine anion. This modulation of the pyranine transient is the kinetic signature of the protein buffers’ capacity [25, 26, 32]. The pyranine signals measured with the native lac permease or with the fluorescein adduct were identical within the limit set by the electronic noise, indicating that the fluorescein residue makes a minor contribution to the total buffer
capacity of the protein and that its binding does not modulate the structure of the protein in such a way that affects its proton binding capacity.

**Figure 3a.2:** Transient absorbencies associated with acid-base perturbation of pyranine and fluorescein in solution. The experiments were carried out with pyranine (29µM) and either free fluorescein (9µM) (Curves 1 and 2) or fluorescein-labeled lac permease (9µM with respect to the bound fluorescein) (Curves 3 and 4). The measurements were carried out in 100 mM KCl at pH=7.70. The upper two curves were measured at 458 nm and recorded the re-protonation of the pyranine anion. The bottom curves were measured at 496 nm and monitored the reversible bleaching of the fluorescein chromophore due to its protonation.

The dynamics of fluorescein, bound to lac permease, are characterized by fast rise and slow relaxation times. The fast protonation indicates that the protein facilitates the access of proton to the dye, while the slow relaxation suggests that the presence of a local proton reservoir replenishes the dye with protons. Accordingly, the reconstruction of the dye’s dynamics should account for these processes.

**Effect of pH on the protonation dynamics**

The velocity of chemical reaction is a function of the reactants’ initial concentrations. Accordingly, variation of the pre-pulse [ionized]/[protonated] ratio of the reactants, through modulation of the pH, should affect the velocity of the proton transfer reactions that follow the pH jump. For this reason, the kinetic measurements were repeated at varying initial pH values and the dependence of the reaction on the pH was investigated. However, the procedure is limited to a certain pH range. At
pH>pK\textsubscript{OH} the concentration of the φOH species decreases and a smaller perturbation is expected. Similarly, at pH<pK\textsubscript{(flu)}, the dye will mainly be in its protonated state and the incremental protonation, in response to the proton pulse, will diminish.

Figure 3a.3 depicts a set of kinetic measurements carried out at varying initial pH values. Panel A depicts the pyranine relaxation signals as they vary with the initial pH. As the pH increased from 6.0 to 8.03, the initial φOH population decreased, reducing the amount of protons released by the laser pulse (Panel A). The pH also affected the shape of the curve, and at high pH values a slower phase appears, representing an increment in the protein’s buffer capacity.

**Figure 3a.3:** The effect of the pre-pulse pH on the protonation dynamics of labeled lac permease. The experiments were carried out in 100 mM KCl, 29 \(\mu\)M pyranine and 9 \(\mu\)M fluorescein-lac permease. The transients shown in Panel A were recorded at 458 nm and the pre-pulse pH of the solution, where Curves 1, 2, 3 and 4 were measured, was 6.0, 6.6, 7.45 and 8.03 respectively. Panels B and C depict the transient protonation of the bound fluorescein measured at pH 6.0 and 6.3 (Curves 1 and 2 in Panel B). Panel C depicts the fluorescein protonation curves as measured at pH 7.45 and 8.03 (Curves 1 and 2, respectively). Each pair of curves is presented with its simulated dynamics, which is the continuous line superpositioned over the experimental one. The set of rate constants that reconstructs the observed dynamics is given in Tables 3a.1 and 3a.2. Please note the different scale of the ordinate corresponding to the fluorescein signal.

The fluorescein signals retain their general shape over the whole pH range, even below 7.2, where its relaxation is faster than at the higher pH range (compare Panels
B and C). The initial pH also affected the amount of fluorescein protonated during the perturbation, as the pH increased, the maximal yield of protonated fluorescein, normalized with respect to the increment of $\phi O^-$, increased from 3.2% at pH=6.0 to 9% at pH=8.03. This enhancement is surprising considering the enhanced proton binding capacity of the protein and a ~ 40-fold increment in the ground state $\phi O^-$ population. To account for the enhanced reactivity of the fluorescein at higher pH values, it was suspected that the mechanism of the bound fluorescein protonation varies with the pH, thus necessitating a precise kinetic analysis of the signals.

**Table 3a.1:** The kinetic and thermodynamic parameters of the reactions of free diffusing protons with proton binding sites on lac permease. All rate constants are given in $M^{-1}s^{-1}$ units. Bold face reactions vary in their parameters when the pre-pulse pH shifts between the high and low pH regimes. The value $n$ represents the number of residues per protein.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$n$</th>
<th>pH&lt;7.1 Rate constant</th>
<th>pK</th>
<th>pH&gt;7.4 Rate constant</th>
<th>pK</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYTOPLASMIC SURFACE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 H$^+$ + COO$_{av}$</td>
<td>6</td>
<td>$3 \times 10^9$</td>
<td>4.0</td>
<td>$7 \times 10^9$</td>
<td>4.2</td>
</tr>
<tr>
<td>2 H$^+$ + His$_{av}$</td>
<td>3</td>
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<td>6.3</td>
<td>$1 \times 10^9$</td>
<td>6.3</td>
</tr>
<tr>
<td>3 His$_{av}$ + $\phi O^-$</td>
<td>3</td>
<td>$1.5 \times 10^8$</td>
<td></td>
<td>$1.5 \times 10^8$</td>
<td></td>
</tr>
<tr>
<td>PERIPLASMIC SURFACE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 H$^+$ + COO$_{av}$</td>
<td>5</td>
<td>$1 \times 10^9$</td>
<td>4.8</td>
<td>$1 \times 10^9$</td>
<td>4.8</td>
</tr>
<tr>
<td>5 H$^+$ + His$_{av}$</td>
<td>2</td>
<td>$1 \times 10^9$</td>
<td>7.5</td>
<td>$1 \times 10^9$</td>
<td>7.1</td>
</tr>
<tr>
<td>6 His$_{av}$ + $\phi O^-$</td>
<td>2</td>
<td>$1.5 \times 10^8$</td>
<td></td>
<td>$1.5 \times 10^8$</td>
<td></td>
</tr>
<tr>
<td>PROTONATION OF INTRACAVITY RESIDUES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 H$^+$ + Fluorescein</td>
<td>1</td>
<td>$10.10^9$</td>
<td>7.0</td>
<td>$2.5 \times 10^9$</td>
<td>7.0</td>
</tr>
<tr>
<td>8 H$^+$ + COO$_{E126}$</td>
<td>1</td>
<td>$1 \times 10^9$</td>
<td>5.7</td>
<td>$2.5 \times 10^9$</td>
<td>5.75</td>
</tr>
<tr>
<td>9 H$^+$ + His$_{H322}$</td>
<td>1</td>
<td>$1 \times 10^9$</td>
<td>7.3</td>
<td>$1 \times 10^9$</td>
<td>7.3</td>
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<tr>
<td>10 H$^+$ + COO$_{av}$</td>
<td>3</td>
<td>$1 \times 10^9$</td>
<td>5.0</td>
<td>$1 \times 10^9$</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Finally, the dynamics of reversible protonation of the lac permease were measured with the protein incorporated in 3:1 PF/PG liposomes. The kinetic features of the liposomal system were very similar to those of the micellar one, as can be evaluated by comparing the signals measured with the liposomal system (Figure 3a.4) with those recorded with the micellar ones (Figure 3a.3).
Figure 3a.4: The effect of the pre-pulse pH on the protonation dynamics of labeled lac permease incorporated in 3:1 PE/PG liposomes. The experiments were carried out in 100 mM KCl, 43 µM pyranine and 20.5 µM fluorescein-lac permease. The transients shown in Panel A were recorded at 458 nm and the pre-pulse pH of the solution, where Curves 1, 2, 3 and 4 were measured, was 6.25, 6.67, 7.35 and 7.8 respectively. Panels B and C depict the transient protonation of the bound fluorescein measured at pH 6.25 and 6.67 (Curves 1 and 2 in Panel B). Panel C depicts the fluorescein protonation curves as measured at pH 7.35 and 7.8 (Curves 1 and 2, respectively). Please note the different scale of the ordinate corresponding to the fluorescein signal.

The fraction of proton taken up by the liposomal enzyme is small (Panel A), indicating a slow rate of protonation of the enzyme's surface groups. The protonation signals of the fluorescein (Panels B and C) exhibit a pH variance similar to that of the micellar system. In the high pH regime, the signals were relatively large, and declined in magnitude due to the depletion of the $\phi$OH population at pH~ pK ($\Phi$OH). In the low pH range, the signal of the fluorescein (pK=6.5) was smaller partly due to the low concentration of unprotonated indicator, but the signals measured at pH=6.25 and 6.7 were almost identical in size, indicating that the protein's system assists in the delivery of the proton to the dye. Yet, due to the uncertainty about the polarity of the incorporated protein, a detailed kinetic analysis was avoided.
Table 3a.2: The virtual second order reaction of proton exchange between the proton binding sites of lac permease. Bold face reactions vary in their parameters when the pre-pulse pH shifts between the high and low pH regimes.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>PH&lt;7.1</th>
<th>PH&gt;7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>REACTION ON THE CYTOPLASMIC SURFACE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 COOH&lt;sub&gt;av&lt;/sub&gt;→His&lt;sub&gt;av&lt;/sub&gt;</td>
<td>10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>2×10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>REACTION ON THE PERIPLASMIC SURFACE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 COOH&lt;sub&gt;av&lt;/sub&gt;→His&lt;sub&gt;av&lt;/sub&gt;</td>
<td>10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>CYTOPLASMIC → CAVITY</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 COOH→COO&lt;sub&gt;E126&lt;/sub&gt;</td>
<td>10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>8×10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 His&lt;sub&gt;a&lt;/sub&gt;H&lt;sup&gt;-&lt;/sup&gt;→His&lt;sub&gt;H322&lt;/sub&gt;</td>
<td>10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Negligible</td>
</tr>
<tr>
<td><strong>PERIPLASMIC → CAVITY</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 COOH&lt;sub&gt;av&lt;/sub&gt;→Fluorescein</td>
<td>7.5×10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Negligible</td>
</tr>
<tr>
<td>6 COOH&lt;sub&gt;av&lt;/sub&gt;→His&lt;sub&gt;H322&lt;/sub&gt;</td>
<td>1×10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>7 His&lt;sub&gt;a&lt;/sub&gt;H&lt;sup&gt;-&lt;/sup&gt;→His&lt;sub&gt;H322&lt;/sub&gt;</td>
<td>0.1×10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1×10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>INTRACAVITY PROTON TRANSFER</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 COOH&lt;sub&gt;E126&lt;/sub&gt;→Fluorescein</td>
<td>k&lt;1×10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>2.5×10&lt;sup&gt;12&lt;/sup&gt;</td>
</tr>
<tr>
<td>9 Fluorescein H→His&lt;sub&gt;H322&lt;/sub&gt;</td>
<td>5×10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>2.5×10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Reconstruction of the pyranine signals

The relaxation of pyranine anion is regulated by the total buffer capacity of the protein, to which the fluorescein makes a negligible contribution. As a result, the relaxation of the pyranine signal is affected by the concentration of the protein’s proton binding sites and their pK values. An attempt to simulate the pyranine signals, assuming that all histidine residues of the his-tag reacted with free proton in a diffusion controlled reaction, failed to fit the observations. The number of histidine residues on the cytoplasmic surface that participate in the microsecond proton uptake reactions had to be adjusted to three. By the same reasoning, the number of histidine residues on the periplasmic face was set as two. It seems that a combination of steric hindrance and interaction between the surface groups reduces the number of residues on the enzyme’s surface which are capable of reacting with the free proton within the 2 µs time frame, where the free proton concentration exceeds the pre-pulse level [25, 32, 33, 35, 36].
The reconstruction of the experimental pyranine signal, over the pH range, was essentially attained by the same parameters (see Table 3a.1) except for two: the rate of protonation of the average carboxylate population of the cytoplasmic face of the enzyme, and the pK of the average histidine residues on the periplasmic side. Both parameters were found to vary between the two pH regimes in a range beyond the limit of confidence in the analysis.

**Reconstruction of the fluorescein signals**

The reconstruction of a single pair of tracings can be obtained by more than one combination of the rate constants. Yet, as the number of independent observations increases, the range of variance of the rate constants converges. The limits of variance of each parameter are given in Tables 3a.1, 3a.2, and 3a.3. In a case where one parameter exceeds its boundary, the whole set of measured signals cannot be reconstructed even if all the other parameters are allowed to vary.

The protonation of the fluorescein is a summation of two processes: reactions of the dye with free diffusing species (protons and pyranine anion) and proton exchange between protonable residues located within ~20Å from the dye [32, 37, 40]. The shape and amplitude of the fluorescein signal is mostly controlled by the parameters presented in Figure 3a.5. Each panel in the figure depicts the reconstructed dynamics (measured at pH=7.45), where one parameter varies within the indicated range. The rate constant that mostly affects the amplitude and initial velocity of the reaction is the protonation of the fluorescein by free diffusing protons. In Panel A, the well-fitted curve was calculated with k=2·10^9 M⁻¹s⁻¹. This value is significantly smaller than a rate constant of a diffusion-controlled reaction. Attempts to reconstruct the signal with larger rate constants caused gross deviation from the experimental curve. The slow protonation of the bound dye indicates limited accessibility of protons to the substrate-binding cleft of the enzyme.

Panel B demonstrates how the intra-protein proton exchange with E126 affects the fluorescein signal. The variation of the rate constant from 2·10^{12} (characteristic to proton exchange between sites that are less than 10 Å apart) to 1·10^{10} affects both the amplitude and its evolution with time. The quality of the measured signal is good enough to establish that the rate constant of the reaction is k~10^{12}. The rapid accumulation of the protonated dye is a consequence of a rapid shuttle mechanism, where the carboxylate of residue E126 delivers proton to the dye.
Figure 3a.5: The effects of the rate constants on the observed dynamics. The curves in each panel were generated by the parameters given in Tables 3a.1 and 3a.2 except for one parameter which varied within the indicated range, as given below in ascending order. For comparative purposes a fluorescein signal (measured at pH=7.45, 100 mM KCl, 19 µM pyranine, 9 µM covalently bound fluorescein) is presented by the experimental points. Panel A depicts the effect of the rate of reaction between the dye and free diffusing protons (Line 1, k=2·10⁹ M⁻¹s⁻¹; line 2, k=6·10⁹ M⁻¹s⁻¹; line 3, k=1·10¹⁰ M⁻¹s⁻¹). Panel B demonstrates the effect of the 'virtual second order' rate constant of proton transfer between the E126 carboxylate and the bound fluorescein. (Line 1, 1·10¹⁰; line 2, 1·10¹¹; line 3, 2·10¹²). Panel C depicts reconstructed dynamics where the proton exchange rate between histidine 322 and the fluorescein was modulated (line 1, 2·10⁹; line 2 6·10⁹; line 3, 1·10¹⁰). Panel D corresponds with the dynamics calculated for three pK values of histidine 322 (Line 1, pK=7.; line 2, pK=7.3; line 3, pK=7.6).

The effect of proton exchange between the fluorescein and the intra-cavity histidine (H322) is shown in Panel C. In contrast to the proton exchange between the carboxylate and the fluorescein, the proton transfer reaction between the dye and the nearby histidine is much slower, and the best fit was calculated with k=2·10⁹. Thus, at pH ≥7.4, the mechanism of proton exchange with the histidine is less efficient than with E126. The shape of the fluorescein relaxation curve is very sensitive to the pK value assigned to histidine residue H322 (Panel D). The best estimation, based on analysis of 27 independent measurements, is pK =7.3±0.08.
Effect of pH on the lac permease conformation

To obtain a large number of experimental tracings, with varying protein/pyranine ratios, the experiments were carried out within the pH range $6.0 \leq \text{pH} \leq 8.0$, where good quality data could be obtained. Altogether 56 pairs of signals were recorded. The analysis of the signals was carried out, starting with those gathered at the high pH range, and proceeding systematically to the lower pH values. In the pH range of 8.1 to 7.4, we analyzed 27 pairs of signals that were all solved by one set of parameters.

![Figure 3a.6: Reconstruction of the measured dynamics by the rate constants of the high and low pH regimes. Panel A depicts the dynamics measured at pH = 6.0 with its reconstructed dynamics. Lines 3 and 4 were calculated with the parameters adequate for the high pH regime. Lines 1 and 2 were reconstructed by parameters that were fitted for the low pH regime. The quality of the fitting is recorded in the inset. Panel B depicts the kinetics measured at pH = 8.03. Lines 3 and 4 were calculated with the parameters suitable for the low pH regime. Lines 1 and 2 were calculated with the parameters of the high pH regime and the quality of the fit is recorded in the inset. In the two insets, for the sake of clarity, the plots for the fluorescein and pyranine were shifted along the Y-axis. Please note the different ordinate scale used for the fluorescein signals.](image)

When the same solution was tested on signals measured at pH $\leq 7.1$ (Figure 3a.6 Panel A, Curves 1 and 2), they totally failed to resemble the shape of the curve. Panel A
presents the experimental signal measured at pH=6.0 together with two reconstructed dynamics. The curves 3 and 4 (for pyranine and fluorescein, respectively) were generated by parameters of the high pH regime, and grossly deviate from the experimental data. It is obvious that these parameters are inadequate to reconstruct the transients in the low pH regime. A new set of parameters had to be searched for. The results of 29 kinetic measurements, obtained below pH=7.1, were subjected to kinetic analysis and a new set of rate constants was obtained. These parameters were appropriate to reproduce the results of the low pH regime (Figure 3a.6 Panel A, lines 1 and 2 and the deviation plot in the inset). The new set of parameters was found to be totally inadequate for the reconstruction of the signals gathered at high pH range (see Panel B, lines 1 and 2). Thus we conclude that the proton pulse measurements revealed two states of the enzyme, each having its own kinetic parameters for reaction with free protons.

The transition between the two conformations was very sharp. Up to pH=7.1, all measurements could be fitted by the parameters of the low pH regime, while above pH=7.4 all observations were simulated by the other set of parameters. In the intermediate range, the two conformations coexisted and the signals could not be fitted by either set. The two sets of solutions are given in Tables 3a.1 and 3a.2, and the difference between them is sufficiently large to associate each solution with a different conformation of the enzyme.

Quantitative evaluation of the rate constants

A. Interaction of free protons with the protein’s surface

The following tables summarize the pK values and rate constants associated with the reversible protonation of the fluorescein maleimide attached to C148 of the lac permease, and reflect the combined features of the covalently bound dye and residues of the substrate-binding site. Table 3a.1 lists the rate constants of the diffusion-controlled reactions between the various residues with free diffusing species (H+ and φO−). The reactive groups are defined in the first column. The two chromophores and the intra-protein residues E126 and H322 are marked as such, while the others are labeled with generic names (such as Hisave etc.) The properties of the imidazol residue of H322 were assigned to the high pK intra-cavity residue that regulated the decay dynamics of the fluorescein (see Figure 3a.5, Panels C and D). The kinetic features of the E126 carboxylate were corroborated upon analysis of the cysteine less/C148/E126A double mutant (see Figure 3a.7 below). The number of residues in each subpopulation is given in the second column. It should be pointed out that the number of residues was equal, or smaller, than the estimation based on the
structure proposed by Kaback [164]. Taking the full content of histidine residues, including the His-tag groups, yielded a proton binding capacity too high to fit the measured signals. The reconstruction of the signals was attained with only three, fast reacting histidine and six carboxylate residues.

The other four columns in Table 3a.1 refer to the rate constants of protonation and the pK of each subpopulation when the protein is either above pH=7.4 or below pH=7.1 (the high and low pH regimes, respectively).

Of all reactions with the free proton, only that of the fluorescein, in the low pH regime, is fast enough to comply with the diffusion controlled reaction. All other reactions are smaller than $1 \cdot 10^{10} \text{ M}^{-1}\text{s}^{-1}$, a value measured for exposed residues on other proteins [25, 26, 32, 33, 35-37] or for phosphatidyl serine in a lipid membrane [7]. Apparently, the surface carboxylates of lac permease are well shielded from reaction with bulk protons either by adjacent positive residues or partial insertion in a non-polar environment. The slow reaction of the surface groups with free protons cannot be attributed to the presence of the supporting lauryl maltoside micelles as the same phenomenon has been found with lac permease in lipid vesicles (Figure 3a.4). Furthermore, the surface groups of cytochrome oxidase [25, 32], stabilized by the same concentrations of lauryl maltoside, reacted with free protons at rate constants compatible with the Debye-Smoluchowski equation ($k>10^{10} \text{ M}^{-1}\text{s}^{-1}$).

The rate constant values shown in bold print in Table 3a.1 merit special attention, as they differ markedly in the high and the low pH regimes. The most prominent value is the protonation of the bound fluorescein. In the low pH range, this rate constant is compatible with that of the diffusion controlled reaction, indicating that the cleft where the dye is bound is fully exposed to the bulk. (Kinetic analysis cannot determine whether the opening is in the direction of the extracellular space or on the periplasmic side of the protein). In the high pH range, the reaction is four times slower, suggesting that the cavity closed over the bound dye, thus barring the free entry of protons into the cleft. In contrast to the fluorescein, the protonation of E126 and the cytoplasmic surface carboxylates is faster in the high pH regime. Thus, the effect of the pH on the accessibility of the proton binding sites exhibits site selectivity and is not a general property of the protein.

On the periplasmic side of the protein, the rate constants are pH-insensitive but the pK of the histidine residues varies between the two conformations of lac permease. As evident from this data, the transition of the protein from one
conformation to the other appears to be selective, and the various proton-binding sites respond independently to the transition.

Finally, we wish to emphasize that although the pK values of the carboxylates determined with high accuracy even though their values are lower than the pH range where the measurements were carried out. This confidence is gained by the mode by which the pK is derived. Unlike equilibrium titration, where the pK is measured by relating the protonated fraction with the pH, in the kinetic analysis of the pK, it is determined by the ratio of the rate constants for the reversible protonation of the residue. As both rate constants are independent adjustable parameters, the pK can be derived even when the system is far from its state of equilibrium.

B. Proton transfer between fixed sites

The virtual rate constants measured for the proton transfer reactions in the fluorescein-labeled lac permease are listed in Table 3a.2 and those values that exhibit a major variation to the pH regime, are printed in bold. The most dramatic diminution of a virtual rate constant was noted for the proton transfer from the intra-cavity carboxylate, identified as E126, and the bound fluorescein (reaction 8, Table 3a.2). As demonstrated by the reconstruction (Figure 3a.5, Panel B), the rate of this reaction controls the velocity of the protonation of the bound fluorescein. The mechanism supporting this fast reaction is proton transfer from the nearby carboxylate (E126) that shuttles the protons from the bulk to the dye. The reconstructions presented in Figure 3a.4, Panel B indicate that the rate constant of the reaction, in the high pH regime, is \(10^{12}\). In the low pH range, the same rate constant of proton is slowed down by a factor of 100,000, indicating that the connectivity between the two sites is lost.

The other intra-protein proton transfer reaction is the proton exchange between the fluorescein and the high pK residue located in the cleft (reaction 9). The virtual rate constant of the reaction is \(~1000\) fold slower than the one with the E126 and less sensitive to the conformation change. Thus, the transition between the two conformations does not affect all intra-cavity proton transfer to the same extent.

The other virtual rate constant of proton transfer, in which H322 is involved, also varies between the two pH regimes, indicating that the conformation change is not just limited to helix IV, that carries E126, but is widespread over various domains of the protein.
Positive identification of E126 by its kinetic features

The kinetic analysis indicated that a single carboxylate residue is located at very close proximity to the bound indicator and facilitates its protonation. Of the four carboxylates located in the transmembranal section of the protein, E126 has been implicated as the residue that mostly interacts with the transported galactosides [172, 195]. For this reason, the kinetic properties of the {cysteine less\C148\E126A} mutant were investigated. The reprotonation of the pyranine, measured with the E126A mutant, was indistinguishable from that measured above, in accordance with the minor contribution of E126 to the total proton binding capacity. On the other hand, the kinetic analysis of the protonation dynamics of the fluorescein (Figure 3a.7) failed to reveal the transition between the two pH regimes.

The analysis of the signals measured at pH=6.85 was based on the parameters of the low pH regime, while setting the rate constants of all proton transfer reactions in which E126 is a reactant to zero ($k_{E126j}=0$). The parameters that reconstruct the signals measured with the E126 mutant are given in Table 3a.3.
Table 3a.3: \textit{Comparison of the proton transfer kinetic parameters that vary with the pH upon mutating E126 to E126A}

<table>
<thead>
<tr>
<th>Reaction</th>
<th>E126</th>
<th>E126A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH&lt;7.1</td>
<td>pH&gt;7.4</td>
</tr>
<tr>
<td>\text{COO}_{E126}\text{H}^+\text{Flu}</td>
<td>k&lt;10^9</td>
<td>2.5 \times 10^{12}</td>
</tr>
<tr>
<td>\text{Flu}^++\text{H}^+</td>
<td>1.0 \times 10^{10}</td>
<td>0.25 \times 10^{10}</td>
</tr>
<tr>
<td>\text{FluH}^++\text{His}_{E122}</td>
<td>5 \times 10^9</td>
<td>2.5 \times 10^9</td>
</tr>
</tbody>
</table>

At the high pH range, the amplitude of the fluorescein signal measured with the E126A mutant was found to be smaller than at pH=6.85, in contrast to that measured with the E126 enzyme (see Figure 3a.3), where the amplitude increased in the high pH regime. The reduced protonation of the dye indicated that the proton shuttle pathway via E126, which sustains the high flux above pH=7.4, is indeed missing in the mutant. The reconstruction of the curve, shown in Figure 3a.6, Panel B, was attained by the rate constants used for the reconstruction of the low pH conformation of the enzyme, except that the accessibility of free protons to the bound fluorescein was reduced to \(\sim 50\%\), while in the parent protein the rate of the reaction was slowed, with the pH change, to 25\% of the rate in the low pH regime. The rate constant of proton exchange between the bound fluorescein and H322 was found to vary with the pH, having the same values as in the E126/C148-fluorescein. Thus, the E126A mutant retains the pH dependent regime but the absence of the proton shuttle mechanism alters the protonation dynamics of the dye in the substrate-binding cavity. We conclude that the E126 carboxylate is crucial to the protein-substrate interaction but does not trigger the conformation change.

\textbf{DISCUSSION}

The lac permease utilizes the electrochemical gradient of protons, built across the bacterial inner membrane, for uphill transport of galactosides. The reaction with the two substrates (lactose and proton) is coupled and reversible; thus the enzyme can build up a proton gradient at the expense of unequal lactose concentrations. To carry out these reactions, the enzyme should exist in at least four major configurations. Two of them represent the different protonation states of the enzyme, while the other two differ in their affinity to the substrate. The catalytic cycle alternates between states
where the stabilization of the substrate is attained by strong interaction with ligand groups of the protein, and those where water penetrates to the binding cavity and replaces the protein’s ligands by hydrogen bonds with the water molecules. The transition between these two states is controlled by the state of protonation of the enzyme [172, 186, 192, 195]. According to a recent study where the effect of pH on the substrate binding was measured, it seems that the affinity of the site to the substrate decreases with the pH. Upon replacement of H322 by an unprotonable residue, the binding was significantly weakened and became pH-independent [182]. While Kaback and coworkers [182] quantitated the accessibility of a substrate analogue to the site by measuring its capacity to protect C148 from reacting with N-ethyl-maleimide, in the present study the site is irreversibly labeled by fluorescein maleimide and the accessibility of proton to the site was determined by real-time kinetic measurements. Accordingly, the measured parameters are not identical but may converge to the same conclusions.

The present studies were carried out with lac permease stabilized in the micellar system: thus both sides of the enzyme were exposed to the same pH. Yet, as the enzyme maintains a leak-proof proton seal, any event that is driven by selective protonation of a site, which is not accessible from both sides of the protein, will affect the observations just like a membrane-embedded enzyme. On the basis of kinetic analysis, we concluded that below pH = 7.1 the substrate binding cleft is fully exposed to bulk protons, residue E126 is partially inaccessible to the bulk and, as evaluated by the rate constant of proton exchange between the carboxylate and the chromophore, the connectivity between the bound dye and E126 is rather weak. Based on these characteristics, the low pH state of the enzyme is compatible with a configuration where the cleft is well solvated and the substrate is about to be released to the bulk. The results of Kaback [182] suggest that, in the low pH regime, the accessibility of the substrate analogue to the site is high and 0.1 mM of TDG suffices to protect the cysteine from reacting with the maleimide.

The second state of the enzyme, favored at pH ≥ 7.4, is characterized by a closed cleft and high proximity of the E126 carboxylate (on Helix IV) to the dye. In the high pH state, the two residues exchange a proton among them with a virtual rate constant of $10^{12}$. This state of the enzyme corresponds with the tight complex where the intracavity polar groups fold on the substrate and hold it tight. In this
configuration [182], the interaction between H322 and a substrate analogue is weakened, thus resembling our observation that the rate constant of proton exchange between the dye and H322 is halved. It is of interest to point out that the two pH regimes, measured in the present case by the proton accessibility to a dye molecule attached to C148, were also noted by the Kaback’s group [182] (pK=8.1). Yet, as the mode of detection varied between the two experimental systems, the pH range and transition are not identical. Thus the two modes of observation corroborate each other.

During the catalytic cycle, some of the helixes interact with the substrate in an alternating mode [196-199]. The involvement of helix IV in the process may be deduced by the alternating accessibility of E126 to the bulk. In the high pH configuration, the fluorescein is secluded from the bulk, but E126 is better exposed to reaction with bulk protons. This observation might suggest that the major proton pathway to the bound fluorescein is through the side antipodal to E126, i.e. the periplasmic one. In the low-pH state, the fluorescein (bound to the substrate anchoring C148) is the better-exposed moiety, while the E126 reaction with free protons is four times slower than in the high pH state. This inverse relationship may reflect a variation in the solvation of micro-domains inside the substrate-conducting pathway and corroborates the scissor mechanism of Kaback [164, 184]. In a previous publication [178] we reported that an intra-protein space of lac permease indeed exhibits a variation in solvation during conformation changes of the protein.

According to current molecular models, the galactose in its bound state appears to be sandwiched between helix V and helix IV, where residues C148, M145 (both on Helix V, [169, 170]) and E126 (on helix IV) and R144 (on helix V) [172, 198] seal it from all sides (see Figure 3.3 above and [164]). In that state, the glucosyl and galactosyl residues and the oxygen atom that links them are roughly on the same plane. The fluorescein molecule with the planar xanthene chromophore has a molecular weight comparable to that of the galactose (342Da vs. 427Da, respectively). When the fluorescein is anchored by a C-S covalent bond with the C148 sulfur atom in the substrate-conducting cavity, it may assume a position similar to that of the substrate. This location is in accord with the spectral red shift of the dye (Figure 3a.1), as caused by the polarizing field generated by the positive (R144) and negative (E126) residues in the cavity. Naturally, due to the rigidity and planarity of the xanthene structure, plus the bulkiness of the benzoic acid-maleimide residue of the
fluorescein-maleimide, the fitting of the dye inside the cavity is not identical to that of the substrate and some distortion of the protein’s structure is expected. However, kinetic evidence for the involvement of E126 in the protonation of the fluorescein supports the proposed location of the dye.

The experiments we carried out indicate that the fluorescein-lac permease complex retains the structural flexibility of the native enzyme. The protein can alternate reversibly between two conformations. One dominating conformation in the low pH regime has an open-cleft structure and protons react with the chromophore in a diffusion-controlled reaction. As the pH is raised, the other conformation appears. The transition between the two states is within the 0.5 pH unit. Such a steep shift between the conformers suggests a cooperative interaction, similar to that proposed by Kaback [195] and Brooker and coworkers [199] between residues that trigger the transition.

Figure 3a.8: pH titration of active lactose transport by E. coli wild type (○), K358H or D237H (▲; no difference is observed between cells expressing K358H or D237H permease), D237H/K358H (○), D237C/K358C (●), or D237H/K358A (△) permease. Measurements were carried out as described in ref. [200]. Initial rates of [1-14C] lactose transport were measured during the initial linear portion of the time course at each pH value given. The figure was taken from ref. [196].
According to the current models of the catalytic cycle [182], the transition between the two states is tightly associated with the substrate pumping mechanism. The active transport by lac permease, as shown by Kaback and coworkers [196, 200], has a sharp maximum at pH = 7.0-7.5 (see Figure 3a.8). This pH range is similar to the region where the enzyme can easily transform between the two conformers. It is of interest to point out that only the active transport, and not the partial reactions, exhibits maximum activity at ~7.5. The equilibrium-exchange reaction is almost pH independent, and the efflux of lactose exhibits a continuous decrease in the activity between pH=4 to pH=9.5 [196, 200]. The downhill lactose accumulation by whole cells was reported to be practically pH-independent [192]. Apparently, the transition between the two conformers is exclusive for the active transport mechanism.

The identity of the residue(s) controlling the transition has not been confirmed. The mutations described by He et. al., [200] suggest that an ion pair, between residues 237 and 358, is essential for activity, but in all these cases the pH optimum was conserved. Thus, neither D237 nor K358 is involved in the reported transition. The carboxylate E325 (with pK~10) is essential for catalysis and should be in its protonated state before the substrate can react with the enzyme [164, 195]. There are two reasons to negate E325 as the residue involved in the conformation transition; the first one is its high pK, while the second is that the E325D mutant, just like the WT, exhibits maximal transport at pH~7.5 [196]. It is of interest to point out that our analysis revealed the presence of one intracavity proton-binding site with a pK of 7.3. It is possible that this residue, tentatively identified as H322, might have a fundamental mechanistic function.

We propose that the catalytic cycle of lac permease combines a shift of the protein between two un-isotropic configurations (periplasmic vs. cytoplasmic orientation of the open cleft) together with high and low affinity for the substrate. In one conformation, the substrate is readily accessed by water and its interaction with the protein is weak. In the other state, the substrate is stabilized by interaction with the side chains of the residues in the active site. During the substrate release step, the lactose interactions with the protein are replaced by its solvation. Our observations with the bound fluorescein could record these two states. The replacement of protein-dye interaction by water-dye interactions was detected by the protonation rate of the bound fluorescein. In the high pH regime, the fluorescein is at almost contact distance from the E126 carboxylate moiety, while the accessibility of free protons to the dye is
slower than a diffusion-controlled reaction. In the other configuration, the carboxylate-chromophore interaction was canceled and the protonation of the dye is diffusion-controlled. Thus, the transition from the high to low pH regime exhibits some of the characteristics associated with the substrate-expelling step of the catalytic cycle.

Finally, the same basic features, where the enzyme exhibits two conformation states that differ in the accessibility of the bound fluorescein to bulk protons with a sharp transition at the same pH range, were recorded with lipoprotein vesicles of lac permease.
Chapter 3b: Time resolved study of the inner space of lactose permease

INTRODUCTION

The substrate transported by lac permease is an uncharged hydrophilic molecule that is driven by vectorial binding and dissociation of a proton. Thus, there must be a mechanism that couples the reversible charging of the protein with a replacement of the hydration shell of the free substrate by protein-substrate interaction. In the present study, we looked for evidence for the modulation of the hydration of the protein during its interaction with its substrate. The detection of the hydration level is based on time-resolved fluorescence measurements of an excited pyranine molecule. In its excited state, the redistribution of the $\pi$ orbital electrons lowers the pK of the hydroxy moiety from pK$_0$ =8.2 to pK* =1.4 and the proton dissociates with a time constant of 100 ps. This rate of dissociation is very sensitive to the capacity of the immediate vicinity to solvate the proton within a timeframe comparable to the stretching frequency of the OH bond [6]. Thus, monitoring the fluorescence of the pyranine molecule can reflect a local variation of the activity of the water within the most immediate vicinity of the dye. The pyranine molecule has three negative charges suitable for interaction with positive residues, and an extended $\pi$ orbital surface that can participate in hydrophobic interactions with the protein. The combination of these two forces enables it to adsorb both to neutral phospholipid membranes [201, 202], and to charged domains on a protein [17]. In the present study we noticed that, at low ionic strength, the pyranine forms a 1:1 tight complex with the protein ($\Delta G \sim -7.5$ Kcal/mol), which is stabilized by electrostatic interactions.

The observed parameter in this study is the well-documented property of pyranine to gain thermodynamic stability in its excited state by dissociating to an ion pair ($\phi O^*+H^+$) [189, 203]. The dynamics of dissociation [20, 205] were detected by picosecond fluorescence measurements and analyzed by the geminate recombination model of Agmon [205, 206]. The analysis is based on numerical reconstruction of the translational diffusion of a proton within the electrostatic field of the excited anion, according to the Debye-Smoluchowski time-dependent diffusion equation. The analysis incorporates the contribution of the shape of the diffusion space, the effective
dielectric constant and the diffusion coefficient of the proton into a set of transition probabilities along a diffusion trajectory. The propagation of the proton, in time and space, according to the transition probabilities, reconstructs the observed fluorescence decay dynamics [3, 4]. By this method, we investigated the physical properties of a microscopic space at the heme binding site of apomyoglobin [19,20], the anion-specific channel of PhoE of E. Coli [18] and the inter-membranal space in multilamellar phospholipid vesicles [203].

The analysis of the fluorescence decay dynamics of the pyranine, when bound to lac permease, revealed that it is located in a rather deep cleft, where the water molecules strongly interact with the protein. The binding site appears to be coupled with the substrate-binding site but is not identical with it; the substrate or its analog modulates the fluorescence properties of the dye without displacing it from its binding site. We regard this observation as evidence that the insertion of the substrate alters the structure of the protein in a way that affects the physical properties of its solvation water.

Chapter 3b was published in Biophysical J. 2001, 80, 1498-1506 [179].

MATERIALS AND METHODS

Purified lac permease was a generous gift from R. Kaback and J. Le Coutre (Howard Hughes Medical Institute, UCLA, U.S.A.).

Pyranine (8 Hydroxy-pyrene 1,2,6 trisulfonate (Na⁺ salt), laser grade) was purchased from SIGMA. (St. Louis, Missouri, USA).

**Binding of pyranine to lac permease.** The protein was dissolved in 0.018% n-dodecyl-β-D-maltoside (LM) to a final concentration of 5.7μM and the pyranine was added to the desired concentration. To maintain a constant pH and low ionic strength, the solution was buffered by a low concentration (50 μM) of Hepes buffer (pH 6.8) in the absence of other salt.

Steady state fluorimetry was measured with a Shimadzu RF540 spectrofluorometer. Time-resolved fluorescence was measured with 200 μl solution of the dye-protein complex. Time-resolved fluorescence was measured using the time-correlated single-photon counting (TCSPC) technique. We used a cw mode-locked Nd:YAG-pumped dye laser (Coherent Nd:YAG Antares and a 702 dye laser) providing a high repetition rate (>1MHz) of short pulses (2 ps at full width half maximum, FWHM) in the spectral range 285 - 315 nm.

The fluorescence of the protonated excited pyranine, φOH⁺, was monitored by a Time Correlated Single Photon Counting system (for more experimental details see [3]).
MATHEMATICAL MODEL OF ANALYSIS

The analysis of the fluorescence decay of φOH* emission was carried out by the program of Agmon that reconstructs the experimental signal through numerical integration of the differential form of the Debye-Smoluchowski equation for diffusion controlled reaction [3-5, 205-207]. The reconstruction of the proton propagation, after the excitation, incorporates two parallel processes:

1) The reversible dissociation at the surface of the reaction sphere ($r_0$). The process is quantified by two rate constants: the dissociation ($\kappa_f$) and recombination of the proton with the excited pyranine anion ($\kappa_r$).

2) The dispersion of the proton in the reaction space. This process is operative in the space defined by $r>r_0$ and is modulated by three parameters:
   i. The intensity of the electrostatic potential that attracts the proton to the pyranine anion, which is quantitated by the Coulomb cage radius ($R_D$; $E= R_D/\kappa_1$ (in kT units)).
   ii. The entropic term that favors the dispersion of the proton. This term reflects the geometry of the dispersion space.
   iii. The diffusion coefficient of the proton $D_{H^+}$ that regulates how fast the proton will probe the reaction space.

The analysis of the observed signal combines all these terms into a set of transition probabilities that define the probability that a proton in a concentric shell ($i$) will progress to the next one ($j$) as given by Eq. 3b.1:

$$TP_{ij} = \frac{D_{H^+}}{\Delta r^2} * f^{(n)}(r_i/r_j) * \exp[-R_D/2*(1/r_i-1/r_j)]$$

The first term in Eq. 3b.1 is the diffusion coefficient ($D_{H^+}$) of the proton and the width of the concentric shells that make the reaction space ($\Delta r=1\text{"A}$). This term sets the basic frequency of the transition between the shells and its value is $\sim 3-9 \times 10^{11}\text{s}^{-1}$, depending on the diffusion coefficient. The second term ($f^{(n)}(r_i/r_j)$) represents the preference of the proton to diffuse from shell ($i$) to shell ($j$) that differ in the number of equi-potential sites where $n$ is the dimensionality of the diffusion space. For $n=3$ the value of $f^{(3)}(r_i/r_j)$ is equal to $r_i/r_j$, while for a one-dimensional space, $f^{(1)}(r_i/r_j) \equiv 1$. 
The last term in Eq. 3b.1 denotes the gradient of the electrostatic pair potential. The Debye radius, $R_D$, (see Eq. 3b.2) is the distance at which the dielectric constant of the diffusion medium reduces the electrostatic potential to the level of thermal energy and its magnitude varies with the effective dielectric constant of the environment.

$$R_D = \frac{Z_1 Z_2 e_0^2}{\varepsilon_{\text{eff}} k_B T}$$

The program combines the chemical reaction at the surface of the excited molecule, $r_0$, defined as the rate of proton ejection ($\kappa_f$ in s$^{-1}$) and recombination ($\kappa_r$ in Å s$^{-1}$), with the diffusion process described by the transition probabilities given in Eq. 3b.1. It propagates the perturbation in time and space, calculating the probability density of the proton by the Chebyshev expansion method.

In the present case, the diffusion space is approximated as a well with a radius of $r_w$ ($r_w \geq r_0$) and a depth of $r_d$ [18, 19]. We consider a proton, which reaches the end of the channel, to be irreversibly lost to the bulk.

The program in a simple PC version has been published [206]. In the present study, we have used a modular unix variation that was adapted to account for the special case of a cavity in a protein [18, 20].

**RESULTS AND DISCUSSION**

**Binding of pyranine to the lac permease**

The pyranine molecule can be excited at two wavelengths: at 450 nm, only the ionized form $\phi O^-$ is excited and the measured emission is at 515 nm. Excitation at 400 nm excites the $\phi OH$ state and the measured emission appears as a main band at 515 nm with a shallow shoulder at ~440 nm. The reason for the enhanced emission of $\phi O^*$, even when $\phi OH$ is the excited species, is the rapid dissociation of $\phi OH^*$ at a rate that exceeds the decay to the ground state [204]. The fluorescence lifetimes of the two forms ($\phi OH^*$, $\phi O^*$) are almost the same: 5.0 and 4.8 ns, respectively. Accordingly, the enhanced emission of $\phi O^*$, even when the excited species is $\phi OH$, implies that in water most of the excited dye molecules discharged their proton before decaying to the ground state.
Figure 3b.1: Fluorescence emission spectra of pyranine–lac permease complex. The emission spectra were measured with excitation at $\lambda = 350$ nm with a solution containing 5 $\mu$M lac permease, 0.018% LM, 100$\mu$M Hepes buffer, pH=6.5. Traces a and b were recorded in the presence of 80 nM pyranine or 80 nM pyranine plus 20 mM sucrose. Trace c was recorded after addition of 20 mM TDG. Trace d was measured after addition of NaCl to a final concentration of 50 mM and is identical with the spectrum recorded with 80 nM pyranine in LM.

The emission spectrum of pyranine in the presence of lac permease or in LM is shown in Figure 3b.1. The spectra, as measured either in pure water or in the presence of the detergent, are identical in shape and the intensity of the emission of the $\phi$OH* is $5\% \pm 0.05\%$ with respect to that of the $\phi$O* (data not shown). This ratio implies that there is no adsorption of the dye to the detergent micelles. In the presence of the enzyme, the emission at ~440 nm was significantly intensified up to 27% of that measured at 515 nm (Figure 3b.1 Panel A, curve a). The enhanced emission of the $\phi$OH* implies that the dye is in a new environment, that slows the dissociation with respect to the fluorescence lifetime of the dye. Addition of sucrose had no effect on the emission spectrum (curve b), but addition of 20 mM TDG lowered the ratio to 19% (curve c). Considering the high affinity of TDG to the enzyme ($k_D=20\mu$M), the marginal reduction of the ratio implies that TDG did not displace the dye from its binding site, but rather modified the physical-chemical properties of the dye’s binding site. Thus, the site we are probing by the pyranine molecule is not that at which the substrate is bound, but is close enough to detect the conformational changes caused by its binding. The selective deformation of the dye’s emission by a substrate analogue, and not by a molecule of comparable mass and solvation properties, implies that the binding site is within the protein itself and is not some traces of co-purified lipids that
were carried into the micelle. At high ionic strength (100mM NaCl) the dye dissociates and the intensity of the emission at 435 nm (curve d) is identical to that measured for the free dye.

The binding of pyranine to the lac permease was measured by fluorescence titration, monitoring the ratio of the two emission bands as a function of the dye/protein ratio and analysis of the results according to Gutman and coworkers [19, 208, 209]. For each sample, the fraction of the bound ligand was calculated and the data were calculated according to a Scatchard plot. The intercepts yielded an association constant of the dye $K=2.5\pm0.7 \times 10^5 \text{ M}^{-1}$ and a dye/protein stoichiometry of 1:1. This ratio is also indicative that the dye is bound to the enzyme and not to phospholipids that were carried over with the protein.

The pK of the pyranine in the complex was calculated from the ratio of the excitation bands of the two ground state species (400 nm for $\phi\text{OH}$ and 460 nm for $\phi\text{O}^-$). The titration was carried out with 5.7 $\mu$M lac permease in the presence of 300 nM pyranine, conditions where ~42% of the pyranine was free. The apparent pK of the bound dye, where half of it was in the protonated state, was 7.4±0.05. This value is significantly lower than pK = 8.4 that is measured in water at vanishing ionic strength [3]. However, as we could not exclude the possibility that the titration curve was distorted by the emission of some free dye, the actual pK of the bound pyranine could be even lower. The low pK value of the bound pyranine and the reversal of binding at high ionic strength indicate that the binding is attained through electrostatic interaction of the dye with one or more positive charges in the protein. The steady state fluorescence measurements clearly indicate that the environment in which the pyranine is bound differs from bulk water. Yet, it provides no information about its nature and which mechanism leads to the diminished emission of $\phi\text{OH}$ in the presence of the substrate. For this reason, the time resolved fluorescence was investigated.

**Time correlated single photon counting**

Kinetic measurements of the fluorescence decay of excited pyranine bound to the lac permease were carried out, and the results are shown in Figure 3b.2, Panel A. The fastest relaxing curve (curve a) was measured for the dye (300 nM) in the presence of 0.018% LM. The relaxation commences with a fast decay corresponding to the dissociation of the excited molecule, followed by a slow, shallow ‘tail’ where
the recombination between the proton and the excited anion regenerates some of the \( \phi \text{OH}^* \). This kinetics is identical to those measured in pure water (not shown).

**Figure 3b.2:** (A) Fluorescence decay curves of pyranine complexed with lac permease. The measurements were carried out in 0.018% detergent, 5.7 \( \mu \)M enzyme, and 14.3 mM lactose (as indicated) at pH=6.8. Excitation wavelength 300 nm; emission 435nm. Total observation time 20 ns. The ordinate is the fluorescence intensity normalized with respect to the maximal measured value. Trace a is a control curve and corresponds with pyranine dissolved in lauryl maltoside and in the absence of enzyme; trace b was measured for the pyranine lac permease complex stabilized in lauryl maltoside; trace c was measured for the sample defined in b after addition of 14.3 mM lactose. The inset expands the transient during the first 3 ns. (B) Titration of lac permease by increasing concentrations of pyranine. Fluorescence decay curves measured as in (A) with increasing pyranine concentrations. The top curve (a) was measured with 300 nm pyranine. The bottom curve (d) was measured in the absence of lac permease and corresponds with the signal of the free dye. The other two curves from top to bottom were measured with 600 and 1500 nM pyranine, respectively. The inset expands the dynamics during the first 3 ns.
In the presence of 5.7 μM lac permease (curve b), the dye is partially bound to the protein and its relaxation dynamics have new features. The most initial rate of relaxation (first 100 ps) is significantly slower (see inset for the expansion of the first 3 ns), and the tail is more prominent. Addition of substrate (14.3 mM lactose, curve c) accelerated the decay but not to the level measured in the absence of the protein. Thus, either some of the pyranine was released from the protein, or the substrate (lactose) modulated the physical-chemical features of the dye’s environment. This effect was specific to the protein dye complex, as comparable concentrations of lactose, which were added to the dye in water or in LM solution, had no effect on the relaxation dynamics (not shown).

Figure 3b.2, Panel B depicts the fluorescence decay curves as measured in the presence of increasing dye concentrations. For comparative purposes, all signals were normalized to maximal amplitude. At low dye concentration (300 nm, curve a) the fraction of the bound dye is maximal and the measured decay is indeed the slowest of the whole series of measurements. At increasing pyranine concentrations, the fraction of the free dye increases and the measured dynamics gradually assume the features of the free dye. At pyranine concentrations of 2.0 μM and above, the signals are indistinguishable from those measured for pyranine in a LM solution (curve d).

Kinetic analysis

The analysis of the measured signals was carried out by the algorithm of Agmon [3, 18-20, 204, 206] and the results are shown in Figure 3b.3, where the analysis was carried out for the control signal as measured in 0.018% LM. The mainframe depicts the observed dynamics and its reconstructed curve on a linear scale. The inset depicts the same data on a logarithmic scale, emphasizing the accuracy of the fit at the longer time scale, where the signal had decayed to 1% of its initial size. The deviation of the reconstructed curve from the experimental one is presented in the bottom part of the mainframe, where for the sake of clarity the base line was shifted to –0.1. The kinetic parameters characterizing the reactions at the solvation shell of the dye (κf and κr), the dielectric constant of the reaction space and diffusion coefficient of the proton were identical with those determined for the reaction in pure water (Table 3b.1, column 2).
Figure 3b.3: Reconstruction of the fluorescence decay curve of pyranine in 0.018% LM. The signal is the one presented in Figure 3b.2, Panel A, curve a. The ordinate is the fluorescence intensity normalized with respect to the maximal measured value. The reconstructed curve is superimposed over the experimental one. The curves are presented on a linear scale (main frame) and logarithmic one (inset). The bottom section of the main frame shows the difference between the reconstructed signal and the experimental one. For the sake of clarity it was shifted to \( y = 0.1 \). The parameters reconstructing the experimental curve are listed in Table 3b.1.

Table 3b.1: The characteristic parameters of the pyranine-binding environment in lac permease.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pyranine in lauryl-maltoside</th>
<th>Pyranine in lac permease</th>
<th>Pyranine in lac permease + lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \kappa_f )</td>
<td>( 7.2 \times 10^9 \text{s}^{-1} )</td>
<td>( 0.65 \times 10^9 \text{s}^{-1} )</td>
<td>( 0.8 \times 10^9 \text{s}^{-1} )</td>
</tr>
<tr>
<td>( \kappa_r )</td>
<td>( 7.0 \times 10^9 \text{Å s}^{-1} )</td>
<td>( 0.75 \times 10^9 \text{Å s}^{-1} )</td>
<td>( 0.8 \times 10^9 \text{Å s}^{-1} )</td>
</tr>
<tr>
<td>( a_{\text{water}} )</td>
<td>1.00</td>
<td>0.71</td>
<td>0.73</td>
</tr>
<tr>
<td>( D_{H^+} )</td>
<td>( 9.3 \times 10^{-9} \text{cm}^2/\text{s}^* )</td>
<td>( 3. \times 10^{-9} \text{cm}^2/\text{s}^1 )</td>
<td>( 3. \times 10^{-9} \text{cm}^2/\text{s}^1 )</td>
</tr>
<tr>
<td>( R_0 )</td>
<td>28.3 Å(^*)</td>
<td>50. Å</td>
<td>42.5 Å</td>
</tr>
</tbody>
</table>

* The diffusion coefficient of proton in bulk water.
*\(^*\) The Coulomb cage radius of pyranine in water is the theoretical value calculated for a charge of \( Z = -4 \) at 25°C.

The first step in the analysis of the dye-protein complex was to eliminate the contribution of the free dye. Using the measured dissociation constant and the reactant concentrations, the fraction of the free dye equilibrated with 5.7 \( \mu \)M protein at 300nM and 600nM pyranine was calculated to be \( \alpha_{\text{free}} = 42\% \) and 46\% respectively. To
correct for the contribution of the free dye to the measured signal, the normalized fluorescence decay curve measured with the dye–lac permease complex was adjusted by subtracting the appropriate fraction $\alpha_{\text{free}}$ of the free dye signal from the measured signal. The adjusted signals were subjected to kinetic analysis, and experimental curves together with the reconstructed dynamics are shown in Figure 3b.4

**Figure 3b.4:** Kinetic analysis of the fluorescence decay curves of pyranine adsorbed to lac permease. (A) and (B) correspond with the transients of the signals measured with 300 nM and 600 nM Pyranine, respectively, after subtraction of the contribution of the free dye. The ordinate is the fluorescence intensity normalized with respect to the maximal measured value. The main frame depicts the reconstruction on a linear scale while the insets present the dynamics on a logarithmic scale to emphasize the quality of the fit on a longer time scale. The bottom section of the main panel shows that the fit has no systematic deviation from the experimental data. The fractions of bound dye for A and B are 58% and 54%, respectively. The measurements were carried out with 5.7 µM lac permease in 0.018% LM at pH 6.8.

The adjusted signals as measured with 300 and 600 nM pyranine (Panels A and B, respectively) are practically identical and were simulated with the same set of parameters. The adjusted signal of the experiment carried out with 1.5 µM dye was
similar to the previous two signals, but was too noisy for precise kinetic analysis. The parameters determined for the complexed dye are given in Table 3b.1, column 3.

The effect of lactose on the pyranine decay dynamics

Lactose in the range of 10-100 mM has no effect on the fluorescence decay of pyranine, either when measured in water or in LM solution. This is in accordance with our previous observation that sugars affect the decay dynamics of pyranine only at concentrations exceeding 1 M [22]. Addition of a substrate, either TDG or galactose, affected the steady state and the fluorescence decay emission of the bound pyranine (see Figures 3b.1 and 3b.2). The modulation of the emission can be attributed either to displacement of some of the dye by the substrate, or to the altered environment of the bound dye caused by the binding of the substrate to another site on the protein.

![Figure 3b.5](image)

**Figure 3b.5:** Fluorescence decay dynamics of pyranine adsorbed to lac permease in the presence of saturating concentration of lactose. The ordinate is the fluorescence intensity normalized with respect to the maximal measured value. (A) Depiction of the distortion of the shape of the kinetics when the fraction of free dye was increased to 45%. Please note the appearance of an artificial rise time followed by a delay in the appearance of the decay. (B) Kinetic analysis of the signal measured with 14.3 mM lactose corrected for 41% free dye. The kinetic parameters are listed in Table 3b.1. The inset depicts the same reconstruction on a logarithmic scale and the absence of systematic deviation is presented in the bottom section of the main panel.

To find out whether the lactose displaced the pyranine, we tried to subtract increasing fractions of the free dye signal from that measured in the presence of lactose. In the absence of lactose, the ratio (bound/ free of the pyranine), calculated on the basis of the dissociation constant, was 0.58/0.42. When lactose displaces some of
the pyranine from the site, a lower ratio is expected. Accordingly, we subtracted an increasing fraction of the free-dye signal from the signal measured with 14.3 mM lactose. This procedure caused an unacceptable distortion of the signal. The pyranine fluorescence rise time is the sum of the laser pulse width plus the instrumental response time, and is $\tau = 45\text{ps}$ with the present setup. Yet, as seen in Panel A, increasing the free dye fraction by as little as 4% distorted the shape of the curve by introducing an artificial rise time that is much longer than the instrumental one. A careful adjustment of the $\alpha_{\text{OH free}}$ value for the signal measured in the presence of lactose was carried out, in a search for a subtracted signal with no artificial rise time. The search yielded the curve shown in Panel B, where the ratio of bound/free equals (0.59/0.41) which is essentially identical to that determined in the absence of the substrate.

We conclude that the lactose did not displace the dye from the protein, but rather altered the immediate environment of the pyranine. This observation is in accord with the proposed conformational changes caused by binding of substrate [193, 199, 209]. The reconstruction of the fluorescence decay measured in the presence of lactose is presented in Figure 3b.5 Panel A, and necessitated alteration of some of the parameters. These values are given in Table 3b.1, column 4.

**Evaluation of the accuracy of the simulating parameters**

Before the magnitude of the parameters that characterize the reaction space is evaluated, it is necessary to establish their accuracy. Five parameters are needed to reconstruct the measured dynamics: $\kappa_f$, $\kappa_r$, $R_D$, $D_{H^+}$, and $r_d$.

The parameters $\kappa_f$, $\kappa_r$ are the respective rate constants at which the excited dye ejects or recombines with a proton at the surface of its reaction sphere ($r_0=6\ \text{Å}$) [3]. $R_D$ is the effective Debye radius of the pyranine anion and reflects the charge of the dye and the dielectric constant of the environment (see Eq 3b.2 above). $D_{H^+}$ is the diffusion coefficient of the proton (in water $D_{H^+}=9.3\ \text{cm}^2\text{s}^{-1}$). Finally, the dimensions of the cleft are given by its cross-section (considered to be comparable with $r_0$) and its depth ($r_d$). Once the discharged proton diffuses out of the cleft, it is irreversibly dispersed in the bulk of the solution [19].

The reconstruction of the measured signal is a propagation of the proton, in space and time, along the whole length of the trajectory according to the transition probabilities. The propagation is carried out at successive time intervals and the probability density of the proton in calculated for each step along the pathway. The probability density inside the space enclosed within $r \leq r_0$ is equated with the calculated
fraction of the un-dissociated excited-pyranine molecule \( \{ \phi \text{OH}^* \}_t \). The product of \( \{ \phi \text{OH}^* \}_t \) value times the radiative (plus non-radiative) decay of the excited molecule (\( \tau = 4.8 \) ns), constitutes the predicted fluorescence decay as measured at 435 nm.

\[
(I_{435})_t = [\{ \phi \text{OH}^* \}_t \times \exp(-t/\tau)]
\]

The fitting of the experimental signals was attained through an iterative search over the parameters’ space, looking for a set that reproduced the experimental observations. While a search in a five-dimensional space seems extremely laborious, it was facilitated by the fact that each parameter affects the dynamics in a very particular mode and within a limited time interval.

**Figure 3b.6:** Evaluation of the accuracy of the simulating parameters. In each panel, one of the parameters was varied and its effect on the dynamics was investigated. The range of variance is indicated below. The middle curve was calculated by the best fit parameter as listed in Table 3b.1. The ordinate is the fluorescence intensity normalized with respect to the maximal measured value. Panel A: The effect of \( \kappa_f \) on the reconstructed dynamics. Traces 1, 2 and 3 were calculated with \( \kappa_f = 4, 6.5 \) and \( 9 \times 10^8 \) s\(^{-1}\), respectively. Please note that the time scale of the panel is 6 ns. Panel B: The effect of the diffusion coefficient on the reconstruction dynamics. Traces 1, 2 and 3 were calculated with \( D_{H^+} = 1, 3 \) and \( 9 \times 10^{-5} \) cm\(^2\) s\(^{-1}\), respectively. Panel C: The effect of the electrostatic force, given by the Debye radius. Traces 1, 2 and 3 were calculated with \( R_D = 80, 50 \) and \( 28.3 \) Å respectively. Panel D: The effect of the length of the binding cavity on the dynamics. Traces 1,2 and 3 were calculated with \( r_c = 25, 17 \) and \( 9 \) Å, respectively.
Figure 3b.6 depicts the effect of these parameters on the reaction kinetics, using a logarithmic Y scale. This presentation expands the later phase of the dynamics and allows evaluation of the contribution of each parameter during the full length of the observation time. In each panel, the middle curve corresponds with the best fit values as listed in Table 3b.1, while the other two were calculated with higher and lower values as detailed in the legend. For each panel, only one parameter was varied, while all others are as listed in Table 3b.1. In Panel A, the rate constant of the proton ejection from the pyranine molecule ($\kappa_f$) to the surrounding solvent was varied within the ~ 50% of the best-fit value. It can be seen that deviation of the curves from the experimental data is noticed right from $t=0$ (please note that the time scale of Panel A is only 6 ns). Actually, $\kappa_f$ is the only term that controls the dynamics right from its most initial point. In the present case, any $\kappa_f$ value that exceeds the range $0.65\pm0.1 \times 10^9$ s$^{-1}$ generates a systematic deviation that cannot be adjusted by modulation of all other parameters. The rate of recombination at the surface of the reaction sphere is given by $\kappa_r$. As discussed before [40], this parameter is somewhat ambiguous and is not suitable for unique mechanistic interpretation. For this reason its value will not be discussed.

The other panels in Figure 3b.6 depict the effect of parameters characterizing the dispersion space. Panel B demonstrates the effect of the diffusion coefficient on the dynamics. The diffusion is expected to have a minor effect on the early phase of the reaction, while the probability density of the proton is still clustered near the pyranine anion. Indeed, we find that during the first 1-2 ns, the diffusion coefficient has no effect on the $\phi\text{OH}^*$ decay curve. With the progression of time, as the proton probability density spreads over an increasing fraction of the diffusion space, the effect of $D_{H^+}$ on the reconstructed curves increases. A small diffusion coefficient implies that the proton will remain near the pyranine anion with a subsequent increase in recombination probability and a larger fraction of the $\phi\text{OH}^*$ population would decay by the relatively slower radiative pathway. Fast diffusion would allow the proton to escape out of the cleft and the fast dissociation of $\phi\text{OH}^*$ would dominate fluorescence dynamics. The lower curve on Panel B was calculated with the normal value of the diffusion coefficient of proton in bulk water ($9.23 \times 10^{-5}$ cm$^2$s$^{-1}$), while the upper one corresponds with only 10% of this value. Reconstruction carried out with $D_{H^+} = 3 \pm 1 \times 10^{-5}$ cm$^2$s$^{-1}$ exhibited no systematic deviations from the measured transient.

Panel C depicts the effect of the intensity of the electrostatic forces expressed by the Debye radius (see Eq. 3b.2). Strong electrostatic attraction between the reactants (large $R_D$) implies that the proton will remain in the vicinity of the pyranine
anion for an extended period and recombination that regenerates φOH* will be a frequent event. In such a scenario, the decay of φOH* emission will be slow (lower curve). A weak electrostatic interaction will reverse the situation and the φOH* population will vanish rapidly. The shape of the curves, calculated with varying intensity of the electrostatic potentials, exhibits ‘spreading’ from a common time point earlier than in Panel B. The best-fit curve has a value of ~50 ±10 Å, indicating that the electrostatic attraction of the bound dye extends beyond the dimension of the cavity where it is located. Such an extension of the electrostatic field suggests that a low dielectric constant matrix surrounds the space where the dye is bound.

In panel D, we investigated to what extent the geometry of the reaction space affects the reconstructed dynamics. The calculations were carried out for a cylindrical cavity having a radius comparable to that of the pyranine anion and a depth of 9Å (upper curve) or 25 Å (bottom curve). In a deep cavity, the proton will remain under the influence of the intensified electrostatic attraction so that the probability density of protons in the cleft is prolonged with subsequent slower relaxation of the φOH* population. According to the shape of the curve we estimate the depth of the cleft to be between 9 to 17 Å. The shape of the curves calculated with varying cavity depths of cavity exhibit a special feature not observed in the other Panels; the time point at which the curves ‘spread’ varies with the depth of the cavity. Thus, each of the parameters leaves a distinct mark on the shape of the relaxation curve, which assists in the fitting of the parameters to the measured curve.

During the reconstruction of proton dissociation in other proteins such as apomyoglobin [19] or the PhoE channel [18], an additional parameter had to be incorporated in the reconstruction: the reaction of the discharged proton with the carboxylates lining the cavity. In the reconstruction of lac permease, we found no evidence for that reaction. Moreover, inclusion of an intra-cavity proton-carboxylate reaction distorted the dynamics to a point where no combination of the other parameters could make them fit the experiments.

Quantitative evaluation of the parameters

The adjustable parameters reproducing the measured dynamics are listed in Table 3b.1. The first parameter, κf, is the rate constant of proton dissociation from the excited pyranine molecule and controls the most initial phase of the decay. This
parameter can be easily measured with an accuracy of ± 5%. The modulation of this rate by the environment can be used for quantitating the activity of the water at the first solvation shell of the excited molecule (Table 3b.1, row 2) [6]. The rates measured for the lac permease-bound pyranine are much slower than in bulk water, indicating that the water molecules in the cavity strongly interact with the protein. The ordering of the solvent molecules by the protein retards their capacity to rotate and solvate the discharged proton at a rate that is competitive with the proton’s tendency to recombine with the excited anion. The activity of the water, as estimated by the rate of dissociation is rather low (a$_\text{water}$=0.71), and comparable with the activity measured for the heme-binding site of the apomyoglobin, where the pyranine almost fills the whole intraprotein cavity [19]. Addition of saturating concentrations of lactose increased $\kappa_f$ by 25%. This effect is not to be confused with a general feature of concentrated solution of sugars (over 1 M) where enhancement of the protein’s solvation shell was reported [210, 211]. For this reason we consider the effect to be a specific one, resulting from the interaction of the substrate with its native binding site. Although the acceleration of $\kappa_f$ is experimentally significant, because of the logarithmic correlation between rates of dissociation and the a$_\text{water}$ [6, 16, 208], the calculated increment of water activity is rather small. Both lactose and the TDG used in the steady state fluorescence measurements failed to eject the pyranine from the binding cleft, suggesting that the dye is not located within the space where the substrate is bound. Yet, the two domains are not isolated and binding of the substrate affects the environment of the dye. The lac permease is sufficiently flexible and dynamic in nature [185, 192] so that the scissors motion of the helices, induced by the substrate [184], propagates the perturbation to other sites of the protein.

The diffusion coefficient (D$_\text{H}^+$) of the proton in the cavity is ~30% of its diffusivity in bulk water. Considering that the diffusion of proton in water proceeds by the Grotthuss mechanism, which is sensitive to the rate of random rotation of the water molecule [213], the reduced diffusion coefficient is in accord with the lower activity of the water in the site. We wish to point out that while the estimation of a$_\text{water}$ on the basis of $\kappa_f$ mainly reflects the water molecules next to the dye, the measured diffusion coefficient suggests that most, or all, of the water in the cleft is effectively immobilized by the protein.
The electrostatic potential in the binding site was derived according to Eq. 3b.2 using the value of $R_D$ as needed to reconstruct the measured signals. In comparison with the standard value for pyranine anion in water, $R_D = 28.3 \, \text{Å}$, (25°C), the values determined for the pyranine binding site and in the presence of lactose were 50 Å and 42Å, respectively. Both values correspond with an environment that amplifies the electrostatic potential within the cleft, and expands the region of influence of a charge beyond the limits measured in water. Reflection of similar properties to the substrate-binding site suggests that enhancement of the charge-dipole attractions tighten the interactions between the substrate and the protein. Addition of lactose reduced the pyranine’s apparent $R_D$ value beyond the limits of experimental error. Thus, the conformational change imposed by the binding of lactose modulated the electrostatic potential within the pyranine-binding site in a way that corresponds with better screening of the anion’s charge. After combining this effect with the acceleration of the dissociation reaction, we suggest that the binding of the substrate enhanced the solvation of the pyranine-binding cleft.

CONCLUDING REMARKS

In this chapter, I have presented evidence for the binding of pyranine to lac permease at a site that differs from the substrate-binding site, yet the two sites can interact with each other through the flexible matrix of the protein. In the bound state, the pyranine exhibits physical and chemical properties that differ from those of the dye in bulk water. The analysis of the fluorescence decay signals indicates that the pyranine-binding site is a cleft (some 9 to 17 Å deep), where at least a single positive charge is present. Water molecules, which strongly interact with the protein’s side chains, fill the intracavity space. As a result, the diffusion coefficient of the proton, the chemical potential of the water and the effective dielectric constant generate a local environment that differs from the bulk. In such a space, the enhancement of the electrostatic potential affects polar molecules through charge-dipole or dipole-dipole interactions, and the reduction of the activity of the water suppresses the dissociation of acidic residues. These properties, if attributed to the substrate-conducting pathway, may explain some of the forces operating on the substrate in the cavity. The reduced activity of the water strips the substrate of some of its solvating water molecules and replaces them by direct interaction with the protein. In parallel, the lower dielectric
constant enhances the binding of the proton to the protein, thus keeping a tight seal that prevents protons from leaking through the enzyme.

The lac permease is a flexible protein that assumes more than one conformation. The transition between the states is associated with the catalytic cycle. Both protonation of the protein and the binding of substrate shift the dominant conformation of the enzyme. The present study demonstrated that lac permease could react with pyranine by a combination of electrostatic and hydrophobic interactions, to form a complex where the dye is inserted into a rather deep cleft in the protein. Furthermore, the dye’s binding site is not in the substrate-conducting cavity, as the addition of lactose (but not sucrose) modifies the site without expelling the dye. We suggest that the inter-helix, scissor like motion imposed by the substrate binding [184] causes the pyranine site to be better solvated.
Chapter 4: Fast kinetics measurements of reversible protonation of transhydrogenase

INTRODUCTION

Transhydrogenase is a conformationally coupled proton pump linking a proton gradient to the redox reaction between NAD(H) and NADP(H), catalyzing the reversible reaction:

\[ \text{H}^+_{\text{out}} + \text{NADP}^+ + \text{NADH} \leftrightarrow \text{NADPH} + \text{NAD}^+ + \text{H}^+_{\text{in}} \]

Reduction of NADP\(^+\) by NADH is linked to an inward translocation of protons from the periplasm (in bacteria) or cytosol (in mammalian cells) into the bacterial cytosol or mitochondrial matrix, respectively [214-222].

The transhydrogenase of \textit{E. coli} is composed of two subunits, \(\alpha\) (510 residues) and \(\beta\) (462 residues), organized as an \(\alpha_2\beta_2\) tetramer (Figure 4.1). The \(\alpha\) and \(\beta\) subunits of the \textit{E. coli} enzyme probably bind mainly through their membrane regions [223].

![Figure 4.1: A sketch of the proton-translocating transhydrogenase from E. coli. Domain Ia and Ib refer to the subdomains of dI where domain Ib contains the NAD(H)-binding site. The figure was taken from ref. [224].](image)

Three domains are recognized in all known transhydrogenase sequences. Domain I (dI) and domain III (dIII) are extramembranal structures carrying the NAD(H) and NADP(H) binding sites, respectively. Three-dimensional structures for domains I and III have been proposed recently [216, 225, 226]. Domain II (dII) is inserted in the cell (or mitochondrial) membrane. A sketch of the intact \textit{E. coli} transhydrogenase is shown in Figure 4.2.
In *E. coli* domain II is composed of the C-terminal 100 residues of the α subunit, organized as four transmembranal α helices, and the 260 residues of the β subunit N-terminal, probably organized as nine transmembranal α helices [227] (Figure 4.3). Recently, J. Rydstrom and his coworkers [223] suggest that the two hydrophilic dI and dIII in *E. coli* transhydrogenase, even when covalently attached to their respective membrane parts, are not permanently in contact but move away from each other often enough to leave free access for dI from *R. rubrum* transhydrogenase (rrl) to bind to dIII. These two hydrophilic domains do not bind efficiently to each other when artificially separated from their membrane parts [215].

The catalytic cycle consists of the reversible binding of the two nucleotides, each on a different domain, and a hydride transfer between them. The two substrates have the same redox potential and their affinity to the protein is comparable [228]. The directionality of the catalysis is ensued by the passage of a proton through dII that modulates the structure of the other domains, leading to preferential release of the NADPH from domain III [220, 229-231]. This proton driven reaction should not be mixed with the general pH dependence of the catalysis, which was also noticed in the absence of dII [222]. The rate constant of the hydride transfer is \( \sim 500 \text{ s}^{-1} \), while the
turnover rate of the enzyme is $\sim 30 \text{s}^{-1}$. Thus, in order to ensure the vectoriality of the hydride transfer, the proton must be retained in its bound state for a time frame that is at least comparable with the hydride transfer ($\sim 2\text{ms}$). Otherwise the hydride transfer will reverse the reaction and no $\Delta \mu H^+$ driven bias between the NAD(H) and NADP(H) populations will take place.

**Figure 4.3:** upper panel: The membrane topology of E. coli transhydrogenase. Encircled residues are the His91, N222, D213 and R265.

Lower panel: Predicted helix packing of transhydrogenase domain II viewed from the cytoplasmic side. Residues conserved in at least 85% of the approx. 25 known transhydrogenase sequences are depicted, polar residues in blue, non-polar residues in green and basic residues in black. No conserved acidic residues are found in the transmembranal helices. The figure was taken from ref. [224].
Binding of NADP⁺ and NADPH causes a marked change in the conformation of the enzyme [232, 233]. It is likely that the difference in binding energies of NADPH and NADP⁺ is the principal driving force for the proton pumping [229, 234].

**Structure and function of domain II**

Domain II of transhydrogenase consists of 370-400 proximal residues, which form the membrane spanning part of the enzyme and are responsible at least partly for the proton translocation. The topology of the transmembranal domain has been systematically studied using cysteine-free *E. coli* transhydrogenase, which has essentially wild-type properties, in which single cysteines were introduced and their reactivities towards membrane impermeable thiol-specific maleimide reagents were investigated [227].

The packing of the 26 α helices in the active dII dimer has been only partly proven (Figure 4.3 lower panel). The very tight binding between the peptides in the *E coli* and the dI dimer [229] suggests that the four helices of each α helix of the α subunit are also closely located. An examination of conserved residues in the transmembrane helices reveals that helices 3, 9, 10, 13 and 14 possess more than 50% conserved residues (Figure 4.3, [224]), suggesting that they form the basic structure for a functional helix bundle containing the proton channel.

The loops on the periplasmic side were identified as short ones, containing mainly unconserved residues. This suggests a lack of peripheral structural elements, which could have had regulatory effects on the entry and exit orifices of the proton pathway [224]. The loops on the cytosolic side are generally longer and relatively well conserved: four of the eight loops contain at least 50% conserved residues. Surprisingly, most of the cytosolic loops are very accessible to large molecules such as fluorescein maleimide, indicating a rather flexible contact between the hydrophilic domain and dII [227].

The pathway by which protons are pumped across domain II is still uncertain. The recently determined crystal structures of cytochrome c oxidase and bacteriorhodopsin indicate that protons are translocated across biological membranes through a hydrogen-bonded network which involves the side-chains of charged and polar amino acids and water molecules [92, 119, 141, 235-237]. Such a system needs a mechanism by which a conformational change mediated by a redox change (as in
cytochrome c oxidase) or light absorption (as in bacteriorhodopsin), induces changes in the pK of one or more ionizeable amino acids. In the case of the transhydrogenase, it is coupled with variation of the ligand binding.

Nearly all conserved acidic, basic, and other polar residues in dII of *E. coli* transhydrogenase were mutated and not a single amino acid that is truly essential has been found, although some positions are severely affected by substitution [238-242]. Only the mutation of βH91N resulted in significant effects on proton translocation. This mutant retained normal activity for hydride transfer between NAD(H) and NADP(H) but lost the capacity for proton translocation [240]. The residue βH91 is located in helix 9 (Figure 4.3, upper panel) and may be the residue that alters its pK and its state of protonation in response to substrate binding. It is of interest that some mutants of this residue showed impaired activity and binding of the substrate NADP(H) [238].

Mutation of two other residues βD213 [243] and βN222 [242] in dII also affected binding of NADP(H): It is possible that they are part of the proton pathway. Another interesting residue, in this regard, in dII is βE124, that is located at the periplasmic end of helix 10, adjacent to helix 9. The mutant βE124Q showed an enhanced rate of proton translocation [242, 244]. Examination of the accessibility to (NEM) and (pCMB) of cysteine mutants of βE124, and residues on the same face of helix 9 as βH91 suggested the presence of a water-filled cavity at the cytosolic end of helix 9. It was shown that βN222 on helix 13, a potential residue in the proton pathway [242], is at the same depth in the membrane as βH91 and is also fully accessible to pCMB. This result suggests that βN222 also faces an aqueous cavity.

The sites we have selected for the study (T81 and S105) are located at the vestibule of the proton channel of dII [227] on the same helix (IX) as βH91. The two residues were substituted in a cysteine-less mutant to generate either T81C or S105C as the single cysteine on the entire protein. Each site was labeled by fluorescein maleimide and the dynamics of protonation of the dye, when subjected to a brief proton pulse, were measured in the time resolved domain.

The kinetic analysis of the transients indicates that the entry of protons to the vestibule is assisted by a few residues that facilitate the encounter, suggesting that the ∆µH⁺ bias is mediated by an ensemble of a few interacting residues that cooperate in the reaction.
MATERIALS AND METHODS

The purified enzyme used in the present study was a generous gift of J. Rydstrom (Department of Biochemistry and Biophysics, Göteborg University, Sweden).

Labeling of mutants

The purified proteins, suspended in 0.01% lauryl maltoside were incubated with a ten-fold molar excess of fluorescein maleimide. The extent of labeling was 75% to 80% for the two samples.

Kinetic measurements

The kinetic measurements were carried out in 100 mM NaCl solution containing pyranine (17 to 27 \( \mu M \)), as proton emitter, and fluorescein labeled transhydrogenase. The enzyme was stabilized in the reaction mixture by addition of 0.01% lauryl maltoside and its final concentration varied from 1 to 4 \( \mu M \).

RESULTS

Determination of pK

The pK of the bound fluorescein moiety was measured by monitoring the absorption of the fluorescein anion as a function of the pH. The titrations were carried out in a mixture composed of 100 mM KCl, 0.01% lauryl maltoside and 5 mM Tris-HCl. The pK of the fluorescein maleimide, attached to the periplasmic side of the enzyme, is 7.55 \( \pm 0.1 \) and on the cytoplasmic surface it is 7.40 \( \pm 0.1 \). These values are significantly higher than that of the free dye (pK=6.3 at 100 mM NaCl).

Kinetic measurements

The kinetic tracings, measured for the fluorescein maleimide adducts with the two preparations (S105C and T81C), are shown in Figures 4.4 and 4.5, respectively. In each panel, the upper signal corresponds with the dynamics of the pyranine anion and the bottom one is for the transient protonation of the fluorescein. Both transients are presented on the same time scale but the amplitude of the fluorescein signal was magnified five-fold.
The pyranine curve exhibits an unresolved rise in absorbance, corresponding to the few nanoseconds formation-time of the H⁺ and the ΦO⁻ ion pair [30]. The relaxation of the curve commences with a rapid phase, lasting ~3 µs, that merits special attention. During this time frame, all the free protons were consumed in a diffusion-controlled reaction by all proton-binding sites: the pyranine anion, the fluorescein and the various sites on the protein. The fraction of the free proton population taken up by the various proton-binding sites depends on the relative concentration of the reactants and the rate constant of their reaction with the protons. As shown in Figures 4.4 and 4.5, about 40% of the pyranine anion generated by the photo-dissociation was reprotonated within the first 2-3 µs. The large fraction of protons that reacted with the pyranine suggests that the transhydrogenase is not designed for rapid reaction with free protons.

After the fast reaction phase, lasting few µs, the pyranine anion exhibits a slower relaxation, with a time constant of τ~40µs. During this phase of the reaction, the protons are redistributed among all proton-binding sites present in the system, while the pyranine anion is gradually re-protonated. The main pathway for the pyranine re-protonation is collisional proton transfer with the surface groups of the protein [7, 16, 245].

The fluorescein signals, given at the bottom of Figures 4.4 and 4.5, are much smaller than the pyranine signals. In the present case, out of the ~2.6 µM protons released by the pulse, ~ 2 µM reacted with the multitude of carboxylates and histidine moieties of the transhydrogenase. Of these protons, only 5% were detected on the fluorescein no matter on which end of the proton channel it was bound. In view of this, the fluorescein signals were enlarged five-fold for the sake of clarity.

**The protonation dynamics of fluorescein-S105C adduct**

According to the latest model of the enzyme [224, 246], the periplasmic facing section of transhydrogenase consists of short inter-helix loops and the N-terminal section of the β subunit (see Figure 4.3, upper panel). The only long loop, connecting helices 9 and 10, is made of ~20 residues (S105-E125) and contains the only three histidine residues of the cytoplasmic surface. As the fluorescein is attached at the base of the loop (S105C), we expect that the protonation of the histidine residues will affect the protonation dynamics of the dye.
Figure 4.4. Time resolved dynamics of proton pulsing of fluorescein attached to the periplasmic side of transhydrogenase. The S103C mutant of the cysteine-less transhydrogenase was labeled by fluorescein maleimide. The adduct was diluted to a final concentration of 3.0 µM in 100 mM NaCl solution containing 0.01% lauryl maltoside, and pyranine (17µM) at pH=7.5. The reaction mixture was subjected to a train of 1000 laser pulses (355nm; 1.6 mJ/pulse at a repetition rate of 10 Hz). The absorbance transients, following each laser pulse, were monitored at 458 nm (top curves, measuring the incremental concentration of the pyranine anion) or at 496 nm (transient bleaching of the fluorescein due to its protonation). The signals were recorded as a vector of 15000 data points of 8 ns each. For the sake of clarity, the fluorescein signal had been magnified 5-fold. The continuous lines represent the reconstructed dynamics, generated by integration of the differential rate equations with the pK values and the rate constants listed in Table 4.1. The inset depicts the deviation of the measured fluorescein signal from the calculated dynamics.

The reconstruction of 17 pairs of signals, each representing the protonation dynamics of the pyranine anion and the bound fluorescein as gathered under the identical conditions, could be attained by a minimal requirement of the reactants that are listed in Table 4.1.

Six reactants were required to reconstruct the fluorescein signals; the fluorescein, two histidine residues, two carboxylates and another reactant that represents the proton binding capacity of the proton-conducting channel of dII.

One histidine marked as Histidine$_{(1)}$ has a higher pK value and reacts with the pyranine anion at a faster rate than the other. This histidine is also very close to the fluorescein, as gauged by the high value of the virtual second order reaction ($1.2\cdot10^{11}$). As can be seen in the proposed structure (Figure 4.3, upper panel), the periplasmic face of the enzyme has only 3 histidine residues; two of them (H108 and H109) are only two residues apart from the fluorescein-binding site. Considering the
size of the maleimide linkage, the oxyanion moiety of the fluorescein’s chromophore can be in close proximity with the histidine pair. At the pH of our measurements, the two-histidine residues are in their unprotonated state, and their rate constant of protonation should be comparable to that of a single histidine. Furthermore, the proximity between H108 and H109 implies that a proton approaching the pair will have a high probability to react with either of them, leading to a higher pK value. For this reason, we tend to assign the pair H108 and H109 as Histidine(1). The other medium pK group (pK=6) on the periplasmic surface can be either H121, located on the loop between helices 9 and 10, or can even be the N terminal amine group. While we cannot suggest which of them is the second histidine detected by kinetic analysis, the magnitude of the rate constant implies that Histidine(2) and the dye are very close to each other.

Table 4.1: The kinetic characteristics of the proton binding sites on the periplasmic surface of transhydrogenase. The table denotes the pK of the proton binding sites, their rate constants of reactions with free protons, and the pyranine anion and the rate constants of proton exchange with the bound fluorescein. All other proton exchange reactions between the surface groups were found to be too slow to affect the reconstruction. The values listed in the table reconstructed 17 pairs of experimental signals, measured in the pH range 7-8 and in the presence of 17 and 27 µM pyranine, with the same quality as depicted in Figure 4.4.

<table>
<thead>
<tr>
<th>Proton binding site</th>
<th>pK</th>
<th>H⁺</th>
<th>ΦO⁻</th>
<th>Fluorescein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Fluorescein</td>
<td>7.55</td>
<td>2.10¹⁰</td>
<td>6 10⁷</td>
<td>-</td>
</tr>
<tr>
<td>2 Histidine(1) n=1</td>
<td>6.75</td>
<td>1 10⁷</td>
<td>7 10⁷</td>
<td>1.2 10¹¹</td>
</tr>
<tr>
<td>3 Histidine(2) n=1</td>
<td>6.0</td>
<td>1 10⁷</td>
<td>2.5 10⁶</td>
<td>2 10¹¹</td>
</tr>
<tr>
<td>4 COO⁻ n=2</td>
<td>4.75</td>
<td>1.75 10¹⁰</td>
<td>≤1 10⁷</td>
<td>6 10¹¹</td>
</tr>
<tr>
<td>5 “Channel” n=1</td>
<td>8.0</td>
<td>-</td>
<td>-</td>
<td>1 10¹⁰</td>
</tr>
<tr>
<td>6 COO⁻ average n=18</td>
<td>4.5</td>
<td>1 10⁷</td>
<td>≤10⁷</td>
<td>-</td>
</tr>
<tr>
<td>7 Histidine average n=10</td>
<td>6.5</td>
<td>1 10⁷</td>
<td>3 10⁸</td>
<td>-</td>
</tr>
</tbody>
</table>

The carboxylate residues that affect the protonation of the fluorescein are well exposed and react with free protons in diffusion controlled reaction. The rate of proton transfer from the carboxylates to the fluorescein is fast (6 10¹¹), indicating that the dye and the carboxylates are close enough to exchange a proton among them through a common water molecule in their solvation shell. Inspection of the
carboxylate residues on the periplasmic surface suggests that the nearby carboxylates are D239 (on Helix 14), D53 (Helix 7), D124 (Helix 10) or 184 (Helix 11).

A third residue labeled as “Channel” also appears in the table, representing a high pK group located at a site into which free protons can hardly reach. This buried moiety is in high proton connectivity with the dye \((1 \times 10^{11})\). The contribution of the residue labeled “Channel” to the reconstruction of the periplasmic-bound fluorescein is marginal but the same group appears to be crucial for the reconstruction of the protonated dynamics of the fluorescein attached to the cytoplasmic side of the enzyme.

The last two rows in Table 4.1 characterize the total proton binding capacity of the protein. The enzyme contains 15 histidine residues (including the His-Tag used for purification), 76 carboxylates and 56 lysines and arginines, most of which are on dI and dII. The \(\text{COO}^-\) average is a population of 18 carboxylates that reacts with protons in a diffusion controlled reaction. The Histidine average accounts for all histidine residues at the enzyme’s surface, excluding those at the immediate vicinity of the fluorescein. The very same populations of \(\text{COO}^-\) average and Histidine average were used for the reconstruction of the dynamics measured with the T81C-fluorescein adduct.

The protonation dynamics of fluorescein attached to T81C

The immediate environment of the channel’s exit port was investigated by monitoring the dynamics of the fluorescein bound at residue T81C. The analysis was carried out on 15 pairs of signals, each corresponding with fluorescein and pyranine traces that were measured under identical initial conditions, and the parameters that can reconstruct all those traces are given in Table 4.2. The pyranine signals that were measured in tandem with the fluorescein were all reconstructed by the same parameters used for the reconstruction of the enzyme labeled at S105C.

In contrast to the fluorescein on the periplasmic side, the dye bound to T81C is poorly approached by free protons. The rate constant of its reaction with free diffusing protons \((k= 1 \times 10^9 \text{ M}^{-1}\text{s}^{-1})\) is only 5-10% of the reaction rate predicted by the Debye-Smoluchowski equation. Such a slow reaction is an indication that the access of proton to the dye is hindered by the two lobes of dI and dIII.

The reconstruction of the fluorescein transients measured for the cytoplasmic surface was more difficult than that of the periplasmic surface, as the number of proton binding sites located on dI and dIII is very large. Accordingly, the analysis
proceeded by gradually increasing the complexity of the system. At first, the reconstruction was attempted by modulating the kinetic properties of the fluorescein residue, but as the signal could not be properly reconstructed, more proton binding sites that exchange proton with the dye had to be introduced. Two histidine and two carboxylates were the minimal number of reactants required for the reconstruction of the fluorescein signals. The two carboxylates exhibit an extremely fast rate of proton exchange with the dye, suggesting proximity up to a sharing of a common water molecule in the solvation shells. The two carboxylates can be identified as E82 and E80, which flank the dye-binding site on both sides.

\[ \text{Figure 4.5: Time resolved dynamics of proton pulsing of fluorescein attached to the cytoplasmic face of transhydrogenase. The T81C mutant of the cysteine-less transhydrogenase was labeled by fluorescein maleimide. The adduct was diluted to a final concentration of } 3.0 \mu \text{M in 100 mM NaCl solution containing 0.01% lauryl maltoside, and pyranine (17\mu M) at pH=7.15. The measurements were carried out as in Figure 4.4. For the sake of clarity, the fluorescein signals had been magnified five-fold. The continuous lines represent the reconstructed dynamics, generated by integration of the differential rate equations with the pK values and the rate constants listed in Table 4.2. The quality of the fit is similar to that given in Figure 4.4. The inset depicts experimental signal curve and one reconstructed without the contribution of the “Channel” site to the dynamics (see text).} \]

While the two carboxylates could be assigned with the same kinetic parameters, the histidine residues were not identical. Histidine_{(1)} has a higher pK and seems to be well exposed to the bulk, as gauged by its reaction rate with the pyranine anion. The rate of proton exchange between Histidine_{(1)} and the dye \( (k=6 \times 10^8) \) suggests that the histidine residue is some 15-20 Å from the chromophore. Histidine_{(2)} has a lower pK and reacts
with the pyranine at a slower rate. Histidine\textsubscript{(2)} seems to be close to the fluorescein moiety, exchanging a proton with a rate constant of $3 \times 10^{10}$.

Table 4.2: The kinetic characteristics of the proton binding sites on the cytoplasmic surface of transhydrogenase. The table denotes the $pK$ of the proton binding sites, their rate constant of reaction with free protons and the pyranine anion and the rate constant of proton exchange with the bound fluorescein. All other proton exchange reactions between the surface groups were found to be too slow to affect the reconstruction. The values listed in the table reconstructed 15 pairs of experimental signals, measured in the pH range 7-8 and in the presence of 17 and 34 $\mu$M pyranine, with the same quality as depicted in Figure 4.4, Panel B.

<table>
<thead>
<tr>
<th>Proton binding site</th>
<th>$pK$</th>
<th>$H^+$</th>
<th>$\Phi$\textsubscript{O}</th>
<th>$\Phi$O\textsuperscript{-}</th>
<th>Fluorescein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Fluorescein</td>
<td>7.4</td>
<td>$1 \times 10^9$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 Histidine\textsubscript{(1)} n=1</td>
<td>7.6</td>
<td>$1 \times 10^9$</td>
<td>$1 \times 10^{10}$</td>
<td>$6 \times 10^7$</td>
<td>-</td>
</tr>
<tr>
<td>3 Histidine\textsubscript{(2)} n=1</td>
<td>6.5</td>
<td>$1 \times 10^9$</td>
<td>$1 \times 10^8$</td>
<td>$3 \times 10^{10}$</td>
<td>-</td>
</tr>
<tr>
<td>4 COO\textsuperscript{-} n=2</td>
<td>4.8</td>
<td>$6 \times 10^9$</td>
<td>$\leq 1 \times 10^7$</td>
<td>$8 \times 10^{11}$</td>
<td>-</td>
</tr>
<tr>
<td>5 “Channel” n=1</td>
<td>8.0</td>
<td>-</td>
<td>-</td>
<td>$1 \times 10^{10}$</td>
<td>-</td>
</tr>
<tr>
<td>6 COO\textsuperscript{-}average n=18</td>
<td>4.5</td>
<td>$1 \times 10^{10}$</td>
<td>$\leq 10^7$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7 Histidine\textsuperscript{average} n=10</td>
<td>6.5</td>
<td>$1 \times 10^9$</td>
<td>$3 \times 10^8$</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The last proton-binding site that was required for the reconstruction of the fluorescein signals is marked in the table as “Channel”, and has very peculiar properties. Its presence was explicitly required in order to reconstruct the fluorescein signal. In its absence (see inset to Figure 4.5), the calculated signal exhibits a persistent overshoot that could not be corrected without modulation of the total buffer capacity of the enzyme, which spoiled the matching with the pyranine signals measured either with the T81C-fluorescein or the S105C-fluorescein adducts. The peculiar kinetic feature of this proton-binding site was fast equilibration with the dye but not with bulk protons, suggesting it may be located in the proton-conducting channel of dII. At present we cannot assign the proton-binding site of the intra-channel space to a single residue and prefer to treat it as ensemble property residues lining the channel. To account for the fact that the channel can function as a proton acceptor with respect to its orifice, this acceptor function was also incorporated into the reactants characterizing the periplasmic-bound fluorescein, and its rate constants are given in Table 4.1. The addition of the “Channel” site improved the quality of the
fit of the S105C-fluorescein adduct. Considering that the structure of the channel is still unresolved, and more than one residue may be implicated in its proton binding capacity, a large number of mutations will be required for positive identification of the residue contributing to the “channel” proton binding site.

**DISCUSSION**

**The proton transfer reactions on the periplasmic surface**

The reconstruction of the observed signals through the differential rate equations is an *in silico* reproduction of the chemical reactions proceeding in the reaction space. This calculation can be used to visualize the sequence of events that are triggered by the proton pulse. The detailed reconstruction of the reactions that underlie the signals presented in Figure 4.4 are presented in Figure 4.6. The scenario is based on the parameters listed in Table 4.1 and retraces the proton transfer reactions of the periplasmic surface of the enzyme. The inset to Figure 4.6 depicts the dynamics of the components located near the fluorescein.

**Line a** (main panel) depicts the basic event of the perturbation, an instantaneous increment of the free proton concentration from $3.9 \times 10^{-8}$ M (pH=7.4) to $2.98 \times 10^{-6}$ M, followed by a rapid relaxation that is determined by the concentration of the reactants corresponding with the experimental conditions detailed in Figure 4.4 and the parameters listed in Table 4.1.

The increment of the free protons relaxes within less than 10 microseconds to their pre-pulse concentration, while in the same time frame the various proton-binding sites are protonated. The pyranine anion is the dominant proton acceptor (**line b**), yet its velocity of protonation is slower than the relaxation of the free protons, indicating that the proton binding sites of the protein compete for the protons. First population that reacts with the free protons is the exposed carboxylate of dI and dIII denoted as COO$^{-}$, and represented by **line c**. Because of the low pK of these carboxylates, they retain the protons for a short period, and within ~10 µs this population relaxes to the pre-pulse level. In parallel with the dissociation of protons from the COO$^{-}$ population, the Histidine$_{average}$ (**line d**) population reacts with the protons and retains its state of protonation for a long time, which is determined by the collisional proton transfer reaction with the pyranine anion. The last reactant presented in the main panel is the fluorescein (**line e**). Its protonation somewhat precedes the Histidine$_{average}$
population, reflecting its higher accessibility and the contribution of its accessory proton donors, as expanded in the inset to the figure.

**Figure 4.6:** Kinetic reconstruction of the proton transfer reactions associated with a proton pulse of transhydrogenase labeled by fluorescein at S105C. The transients were calculated with the rate constants and pK values listed in Table 4.1 and reproduce the events described in Figure 4.4. The inset is an expansion of the main panel, offering a better presentation of the smaller transients. **Line a** represents the free proton concentration; **line b** depicts the relaxation of the incremental pyranine anion concentration; **line c** depicts the state of protonation of the average carboxylate population of dI and dIII; **line d** depicts the reversible protonation of the average histidine residues on dI and dIII; **line e** (main panel and inset) depicts the protonation of the bound fluorescein; **line f** depicts the two carboxylates on the periplasmic surface next to the fluorescein binding site; **line g** is the protonation of Histidine$_{(1)}$ (pK=6.75); **line h** is the protonation of Histidine$_{(2)}$ (pK=6.0), and **line i** is the protonation of the “Channel” proton binding site.

The first acceptors within the immediate vicinity of the dye that is protonated are the two carboxylates (**line f**). They reach maximal amplitude within 3 µs, and relax to the pre-pulse level within 10 µs. Their deprotonation coincides with the protonation of the indicator (**line e**) and the nearby histidine residues (**lines g and h**). The last reactant to be considered is the proton-binding site of the channel (**line i**). Its fast reaction with the dye ensures rapid protonation, while due to its high pK it
functions as a dedicated proton reservoir, which can be discharged exclusively through the indicator. Thus, even though it is more basic than the dye, it replenishes the dye with proton whenever the dye looses it to the bulk. A similar scenario was generated to match the reversible protonation of the dye attached to the cytoplasmic surface of the enzyme, but as the features were rather similar to those in Figure 4.6, the calculations are not presented.

**Simulation of proton transfer through the dIII**

The catalytic turnover of transhydrogenase (k~30 s⁻¹) is significantly slower than the hydride transfer step (k~500 s⁻¹). In order to assure an efficient catalytic cycle, the proton should be bound to the protein for a time frame that is longer than the hydride transfer step, thus poising a constant selective bias for a preferential release of NADPH. The rate constants we had determined for the enzyme-proton interactions seem to implicate a sub-millisecond protonation of the protein, which is inconsistent with the expected long dwell time of proton in the regulatory site. To test whether the kinetic parameters that were determined by the proton pulse experiment can reconstruct the proton delay mechanism, the mathematical model was adjusted to match a scenario where the periplasmic side of the enzyme is subjected to a proton pulse (line a in Figure 4.7), but the protons are exclusively consumed by a large sink that reacts with the cytoplasmic surface of the enzyme (line b).

The reconstructed kinetics curves of all the reactants that take part in transfer of the protons through the membranal part of the transhydrogenase are depicted in Figure 4.7. In the simulation of the proton passage mechanism, 1.7µM protons were released on the periplasmic side of the enzyme, lowering the pH from 7.4 to 5.76, and poising an initial ΔµH⁺ of 1.9 pH units. The released protons, represented by line a, were allowed to react only with the proton binding sites of the periplasmic surface at a velocity controlled by the availability and rate constants given in Table 4.1. The proton binding sites of the periplasmic surface consisted of the two carboxylates and the two histidine residues. The curve corresponding with the protonation of the carboxylates was very shallow and not presented in the figure. The two histidine residues of the periplasmic surface react with most of the protons and their dynamics are given by line c. The deprotonation of these surface histidine residues proceed through proton transfer to the “Channel” site.
Figure 4.7: Simulation of proton passage through transhydrogenase based on the rate constants and pK values given in Table 4.1 and 4.2. The scenario assumes a situation where the transhydrogenase enzyme is placed in a virtual proton impermeable boundary so that its periplasmic and cytoplasmic sides are in equilibrium with the two bulk systems. The perturbation is initiated by injection of 1.7µM of free protons into the aqueous phase in equilibrium with the periplasmic side. **Line a** represents their depletion through all proton binding reactions; the protons can react, exclusively, with the proton binding sites of the periplasmic surface and only after passage through the channel, they finally combine with a massive proton sink that maintains the cytoplasmic space at a constant pH. The protonation of the sink is given by **line b**. **Line c** corresponds with the transient protonation of the histidine residues on the periplasmic surface. **Line d** traces the protonation of the “Channel” proton binding site; **lines e and f** correspond with the protonation of Histidine(2) and Histidine(1) (respectively) of the cytoplasmic surface, which are in equilibrium with a massive proton sink that maintains the cytoplasmic space at pH=7.5.

To simplify the mechanism in the present model, the surface groups delivered their proton to the “Channel” site with the same rate constant determined for the proton exchange between the fluorescein and the “Channel” site. The accumulation of the protons at this site is shown by **line d**. The deprotonation of the channel site proceeds through the same rate constant determined for proton exchange between the “Channel” site and the fluorescein attached to T81C; the acceptors were the proton binding sites on the cytoplasmic surface (**lines e** (Histidine(2)) and **f** (Histidine(1)). Finally, the protons bound to Histidine(1) are released to a massive proton sink (**line b** in the main panel) made of a buffer with pK=7.5 that reacted with the histidine.
residues with the rate constants given for the collisional proton transfer between the surface histidines and the pyranine anion (Table 4.2). Histidine_{(2)} has a slower collisional rate constant and remains in its protonated state for a longer time than the better exposed Histidine_{(1)}.

The pattern of events initiated by very fast perturbation seems to be extended for more than a millisecond by the rate constants determined by probing the surface of transhydrogenase by free diffusing protons. In that time frame, it can invoke some secondary conformational changes. During the catalytic cycle of transhydrogenase, the protonation of domain II derives, by a still unresolved mechanism, a conformation change on dIII, leading to preferential release of NADPH as a leaving group with a time constant of $\sim 30 \text{ s}^{-1}$. The present study demonstrates the presence of a proton-binding site within Domain II that is exposed to both surfaces of the enzyme. A proton reacting with that site is retained in the bound state for a period comparable with the hydride transfer step, ensuring that the enzyme can be kept sufficiently long in the biased state to support an efficient hydride transfer from NADH to NADP.
CONCLUDING REMARKS

Proteins are not static, and their general properties cannot be described by frozen structures. A characterization of their internal dynamics and how they are associated with their function is a necessity for the understanding of biological activities. Important aspects of the internal dynamics of any macromolecule are the conformational changes that are imposed by environmental changes.

The first step in this work was to establish the limits of our methodology, the Laser Induced Proton Pulse, for studying conformational changes, using the well-studied soluble protein cytochrome c, as a model. The knowledge gained by this small soluble protein was later implemented in the study of large membranal proteins. The experiments conducted on the tuna cytochrome c revealed that upon reduction of the iron atom, the proton connectivity between two sites (Glu44 and His26) was increased four-fold, an observation that could be explained by enhanced structure fluctuation that brings these two groups closer than in the oxidized state. This conclusion is supported by the higher R-value of the oxygen atom of the carboxylate moiety of the Glu44 (reduced vs. oxidized) and the assignment of a substantial fraction (23%) of unordered structure in the 19-44 domain [1]. Thus, the kinetic analysis can sharpen the structural understanding of the protein by providing direct evidence that could not have been deduced by the slow-scanning methods.

The global analysis of the kinetic signals also provides information about the pK of the reacting moieties. Although the pK of the carboxylates is much lower than the experimental pH range, the pK values we had determined are of high accuracy. This feature is gained by the way in which the pK is derived. Unlike equilibrium titration, where the pK is measured by relating the protonated fraction of the population with the pH, in the kinetic analysis the pK is determined by the ratio of the rate constants for the reversible protonation (K_{diss}=k_d/k_a). As both rate constants are independent adjustable parameters and affect the observed dynamics, the pK can be easily derived while the system is far from its point of equilibrium. This is a great advantage for the study of protein, as the pK values of most polar residues fall in the pH range, where the proteins are unstable. As a result, an acid base titration of a protein may lead to its destabilization before the titration is over. By using the kinetic
method for determination of pK, this limitation is avoided. With this methodology, we could have deduced the changes in the pKs of the surface’s residues with the pH.

The second step in this work was to look at surface conformational changes associated with the function of the proton pumping protein, bacteriorhodopsin, whose structure and photocycle mechanism is one of the best-studied proton pumps. It was in our interest to find out whether the proton collecting function of its cytoplasmic surface is still operative in the M state after the configurational changes. For this purpose we have investigated, in the present study, two mutants; D96N and the triple mutant (D96G/F171C/F219L). The first one can be readily pumped into the late M state (Mₙ) by constant illumination [90, 92, 93, 99]. The triple mutant has a lower tendency to accumulate in the M state, but its structure resembles the M configuration. Our studies indicated that the late M configuration of the protein exhibits an efficient proton collecting system that differs in details, but functionality exceeded that of the BR state of the protein.

The question of how slow the conformational change propagates within a proton-conducting channel in a protein is still poorly understood. In order to confront this problem, we found the WT bacteriorhodopsin to be a suitable model. The bacteriorhodopsin has the advantage that its chromophore also function as a pH indicator, revealing the state of protonation of the Schiff base. A joint, on-going project of our laboratory with D. Oesterhelt (Max-Planck-Institute for Biochemistry, Martinsried, Germany) and N. Dencher (Technical University, Darmstadt, Germany), showed a large variety in the rate at which the protein regains its proton. The proton transfer from D96 to the deprotonation Schiff base generates a proton hole, and only when it reaches the D38-D36 domain, does a proton from the bulk fill it within hundred microseconds. It was observed that the reprotonation of the protein’s surface was characterized by an extremely slow, sub-second reaction. This slow protonation reaction is attributed to slow proton hole migration inside the protein towards the surface. Selective replacement of charged residues on the inter-helices loops modulates the rate of proton hole propagation, as well as the ionic strength of the solution. These observations suggest that proton hole propagation is strongly coupled with conformation changes, in which the interaction between the loops and the solvent plays a crucial role.

Even though the bacteriorhodopsin and transhydrogenase have an entirely different biological function, they both transfer a proton through a narrow channel
with the aid of an isolated high pK residue buried in the pathway. We have shown that a proton channel of transhydrogenase has a protonable residue with a pK of ~8.0. This is comparable with the pK of D96 of the M state of bacteriorhodopsin, where the enhanced solvation channel’s space reduces its pK from 11 to 7 [144].

The kinetic analysis of the transient protonation of the pH indicator located at the vestibule of the proton-conducting channel of transhydrogenase revealed that the passage of H⁺ inside the protein could be slow enough to match the time constant of hydride transfer by the enzyme.

Direct coupling between proton-induced protein conformational changes and the catalytic cycle was observed in our studies with lac permease. We found that the intensity of the dye-protein interaction changes dramatically in a narrow range of pH, 7.1-7.4, with one proton-binding site involved with the transition. The only histidine in the interior of the permease is His322, which we found to have pK=7.3, comparable with the “switch residue” of the transporter. It is proposed that the high-pH state of the lac permease-fluorescein adduct represents the high affinity state of the lac permease-substrate complex, while the low-pH state of the adduct represents the substrate releasing conformation of the enzyme. The replacement of the protein-lactose interactions by the hydration of the substrate is the driving force that expels the substrate of the site. The last aspect was better demonstrated by a different experimental approach, which monitors the diffusing of proton within a nanosecond time frame. An intra-protein cavity with high affinity for pyranine was detected in lac permease and the activity of the water in the space was measured. The binding of substrate to the enzyme drives a conformational change of the protein, which collapses around the sugar, replacing its solvation shell by protein-substrate interaction. As the protein is made of rigid helices, the constriction of the substrate-binding site loosens the grip of the other part of the protein and the water molecule regains more freedom in that regime.

In the case of transhydrogenase, the location of the bound indicator was either at the orifice of the cytoplasmic or on the extracellular side of the proton channel. The results of the kinetic analysis yield a detailed scenario of proton transfer reactions between sites that could be identified with known reactive elements of the protein. The coherence between the two lines of information indicates that the kinetic analysis can provide a functional mapping of the membrane surface of the enzyme.
The apparent time constant of proton release into the cytoplasmic side of the protein, based on the detailed molecular events, is compatible with the time constant of hydride transfer between dI and dII.

To sum up, we have shown in this work that the Laser Induced Proton Pulse can be one of the many experimental techniques for characterizing protein dynamics: Nuclear Magnetic Resonance spectroscopy (NMR) that can detect regions of highly variable structure at short (fewer than ~6Å) length scales [247]; absorption anisotropy at visible wavelength (the rotational motion within the F_1-ATPase [248]), light microscope by covalent attachment of a fluorescently labeled actin filament [249, 250], energy-resolved elastic incoherent neutron scattering, which has been used to characterized the temperature and hydration dependence of dynamics in bacteriorhodopsin [251] and diffuse features in X-ray crystallography experiments that have been used to determine the length scales at which a protein ceases to appear rigid and begins to look flexible [252]. Each one of these techniques is focused on specific aspect of the protein’s dynamics. The proton can be used as a probe for characterizing a surface protein’s structure and for investigation of the changing of the protein structure as its function was proven to be useful with a variety of proteins.

The proton, which is a small reactive particle with a high diffusion coefficient, is an excellent natural probe to measure how the structure varies during the catalytical cycle. Quantitation of the proton’s reactivity with surface residues enables the determination of their accessibility to the bulk and, when supplemented by pK measurements, also the local charge next to the site. The fast proton transfer reaction between nearby sites, proceeding in the nanosecond up to hundred of microseconds time frame, is sensitive to the relative location and orientation of the residues. Thus the structure information derived from the kinetic analysis of these types of experiments can be used as an easily confirmable prediction of molecular structure changes.
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APPENDIX A

Genetic algorithm approach to the determination of microsecond rate constants

INTRODUCTION

Biochemical reactions typically involve many elements that interact with each other. The laws governing the reaction between every two elements are known (first-order, second order, etc.). The velocity of these reactions, however, depends on the values of the rate constants, which in many cases are unknown. The time evolution of the system as a whole is the sum of all chemical reactions that take place in the solution, and can be modeled by a system of coupled nonlinear ordinary differential equations. However, the mathematical model can replicate the biochemical reaction only when the values of the rate constants are given with reasonable accuracy. An upper bound for the values of the rate constants is given by the Debye-Smoluchowski equation for diffusion-controlled reactions. In many cases, however, local conditions such as electrostatic potential or steric hindrance considerably reduce the reaction rate. Therefore, the a-priori uncertainty in the value of a rate constant can be several orders of magnitudes.

The determination of rate constants of a rate-limiting step is relatively easy, because in this case steady-state kinetic measurements suffice and the analysis is well formulated even for complex systems [2]. Steady-state kinetics is an inadequate methodology, however, when the reaction step under investigation is faster than the rate-limiting step. Determination of fast rate constants is carried out by fast perturbation of the system out of equilibrium and measurements of how it regains its stability. The perturbation can be either a step function, in which case the new equilibrium will differ from the initial one, or a δ-function perturbation in which case the final state will be identical to the initial one. The pioneering experiments of fast perturbation of systems in chemical equilibria were carried out by Eigen [3]. The analysis in [3], however, was based on linearization of an inherently nonlinear process. The proton-pulse method [4, 5] was the first method that modeled the relaxation of the perturbed system as a nonlinear process. In this method the system (e.g., a protein in solution) is subjected to a nanosecond laser pulse that increases the
free protons’ concentration. These protons react simultaneously with all the proton binding-sites, thus initiating a sequence of reactions that occurs on both fast (nanosecond to microsecond) and slow (millisecond) time scales. The dynamics of the perturbed system are measured experimentally, and are also modeled mathematically by a system of coupled nonlinear ordinary differential equations with linear and quadratic terms. The coefficients appearing in these equations are complex (but known) combinations of the rate constants and the equilibrium concentrations of the reactants. The rate constants are found by a search within the parameter space for a set of values that produces simulated dynamics that are close to (ideally, identical to) the measured one [6-9].

The early applications of the proton-pulse method were for systems with a small number of reactants, e.g., a solution made of a proton-emitter molecule and a pH indicator [10]. In those studies, matching the experimental data with the mathematical model was based on the skill of the experimentalist, who carried out a relatively short manual search in the parameter space. The systems that are studied nowadays, however, are much larger, e.g. large membranal proteins with more than a hundred proton-binding sites on their surfaces [6]. In such systems the search for the value of the unknown parameters is extremely laborious, and experience and a thorough understanding of the chemical nature of the reactants become crucial for a successful search. Therefore, until now, application of the proton pulse method to complex systems relied on the availability of highly specialized human skills and was prone to personal bias. The proton-pulse method was sometimes criticized for failing to convince ‘beyond reasonable doubt’ that the manual search indeed converged to the physical values of the unknown parameters, because of the general belief that ‘with four adjustable parameters an elephant can be made to dance’. In other words, if one finds a set of kinetic parameters that reconstructs the dynamics of experimental signals gathered under different experimental conditions, can it be taken as evidence that it is the only possible solution to the kinetic problem?

The reliance on a manual search was the main weakness of the proton-pulse method that prevented it from having more widespread use. Viappiani and coworkers [1] showed that the search process for the rate constants could be automated by using a genetic algorithm. In that study, however, the reaction mechanism was rather simple and the number of undetermined rate constants was two. As we shall see, the genetic algorithm methodology requires substantial modifications when applied to larger and
noisier systems. In this study, we develop such a methodology and apply it to a noisy system with nine undetermined rate constants. Because this is the first application of this genetic algorithm methodology, our emphasis is on demonstrating its robustness and on assessing its reliability. Hence, important issues, such as optimization of the algorithm for faster convergence, more sophisticated statistical analysis etc., are going to be considered in future studies.

EXPERIMENTAL SYSTEM

To demonstrate the applicability of our methodology we selected a system of moderate complexity that consists of three reactants: 1) a pyranine molecule (ΦOH) that ejects a proton when excited by a photon, 2) fluorescein (Flu), a pH indicator that changes its color when reacting with a proton, and 3) a buffer molecule (BH) that reacts with the proton without generating a measurable signal. In this study the buffer molecules were bicarbonate ions supplied by the atmospheric CO₂ with which the solution was equilibrated. The manifold interactions between the reactants is summarized in Scheme 1.

Scheme I: the reactants involved in the proton transfer reactions and the definition of the rate constants.

The measurements were carried out as described in ref. [11]. Briefly, a solution with pyranine and fluorescein was equilibrated with air at a given pH and subjected to a train of laser pulses (1-1.5mJ/pulse; 10Hz, 355nm, 3ns FWHM). The absorption transients at 458 and 496nm, where pyranine and fluorescein are respectively absorbing, were recorded at time resolutions of 30ns or 300ns per data
point and converted into concentration units using the extinction coefficients 24,000 and 50,000M⁻¹cm⁻² for pyranine and for fluorescein, respectively.

An additional complication encountered in previous studies was that the buffer capacity of the protein was not measurable [6, 8, 9, 11]. In order to include this feature in the present study, the initial concentration of the buffer in the solution, as well as its dynamic values after the perturbation, were not measured. Rather, the buffer concentration was derived from the analysis and compared with the known solubility of CO₂ in water.

**THE MATHEMATICAL MODEL**

Let us denote by X, Y, and Z deviations from steady-state concentrations (all in M units) of the three reactants:

\[ X(t) = [\Phi \text{OH}]_\text{eq} - [\Phi \text{OH}]_t, \quad Y(t) = [\text{FluH}]_t - [\text{FluH}]_\text{eq}, \quad Z(t) = [\text{BH}]_t - [\text{BH}]_\text{eq}. \]

The evolution of this system is described by the following system of differential equations (see ref. [5] for details):

\[
\frac{dX}{dt} = a_{11}X + a_{12}Y + a_{13}Z + b_{11}X^2 + b_{12}XY + b_{13}XZ \\
\frac{dY}{dt} = a_{21}X + a_{22}Y + a_{23}Z + c_{22}Y^2 + c_{12}XY + c_{23}YZ \\
\frac{dZ}{dt} = a_{31}X + a_{32}Y + a_{33}Z + d_{33}Z^2 + d_{12}XZ + d_{23}YZ
\]

The initial conditions are

\[ X(0) = X_0, \quad Y(0) = 0, \quad Z(0) = 0, \]

where \( X_0 \) depends on the laser pulse energy. The coefficients of the polynomials are combinations of the rate constants (\( k_i \)) and the equilibrium concentrations of the reactants as follows:

\[
a_{11} = -k_1([H^+]_\text{eq} + [\phi \text{O}^-]_\text{eq}) - k_2 - k_{10} [\text{BH}]_\text{eq} - k_9 [\text{B}]_\text{eq} - k_{12} [\text{FluH}]_\text{eq} - k_{11} [\text{Flu}^-]_\text{eq}, \\
a_{12} = (k_1 - k_{12}) [\phi \text{O}^-]_\text{eq} - k_{11} [\phi \text{OH}]_\text{eq}, \quad a_{13} = (k_3 - k_{10}) [\phi \text{O}^-]_\text{eq} - k_9 [\phi \text{OH}]_\text{eq}, \\
b_{11} = -k_1, \quad b_{12} = k_1 - k_{12} + k_{11}, \quad b_{13} = k_1 + k_9 - k_{10}, \quad a_{21} = (k_3 - k_{11}) [\text{Flu}^-]_\text{eq} - k_{12} [\text{FluH}]_\text{eq}, \\
a_{22} = -k_3 ([\text{Flu}^-]_\text{eq} + [H^+]_\text{eq}) - k_4 - k_8 [\text{BH}]_\text{eq} - k_7 [\text{B}]_\text{eq} - k_{11} [\phi \text{OH}]_\text{eq} - k_{12} [\phi \text{O}^-]_\text{eq}, \\
a_{23} = (k_6 - k_3) [\text{Flu}^-]_\text{eq} + k_7 [\text{FluH}]_\text{eq}, \quad c_{22} = k_3, \quad c_{12} = k_1 - k_3 - k_{12}, \quad c_{23} = k_7 - k_6 + k_3, \\
a_{31} = (k_5 - k_9) [\text{B}^-]_\text{eq} - k_{10} [\text{BH}]_\text{eq}, \quad a_{32} = (k_7 - k_6) [\text{B}^-]_\text{eq} + k_8 [\text{BH}]_\text{eq}, \\
a_{33} = -k_6 ([\text{B}^-]_\text{eq} + [H^+]_\text{eq}) - k_6 - k_6 [\phi \text{OH}]_\text{eq} - k_{10} [\phi \text{O}^-]_\text{eq} - k_7 [\text{FluH}]_\text{eq} - k_8 [\text{Flu}^-]_\text{eq}, \\
d_{13} = k_9 - k_5 - k_{10}, \quad d_{23} = k_8 - k_7 + k_5, \quad d_{33} = k_5.
\]
The goal of the present analysis is thus to determine the rate constants of all proton transfer reactions ($k_i$) in the system by comparing solutions of the mathematical model for $X$ and $Y$ with their corresponding experimental signals.

3.1 Range of unknown parameters

A search was conducted for the unknown parameters, each within a given range depending on its nature. The rate constants in the thermodynamic-favored direction were allowed to vary between an upper limit set by the Debye-Smoluchowski equation and a lower one by up to four orders of magnitude, i.e., $5 \times 10^{10} < k_1 < 1.5 \times 10^{11} \text{ M}^{-1}\text{s}^{-1}$, $10^9 < k_3$, $k_5 < 5 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$, $10^7 < k_8$, $k_{10}$, $k_{12} < 10^{11} \text{ M}^{-1}\text{s}^{-1}$. We calculated the rate constant in the reverse direction from the relation $k_i = k_{i+1}/10^{-pK_i+1}$, and allowed the dissociation constants ($pK$) to vary somewhat from their known values (with a wider range for $pK_{65}$ of the bicarbonate), i.e., $7.5 < pK_{21} < 8.5$, $6.0 < pK_{43} < 6.7$, and $5.5 < pK_{65} < 7.0$. Similarly, the rate constant pairs of collisional proton transfer between the reactants are related through $k_7 = k_8 10^{pK_{65}-pK_{43}}$, $k_9 = k_{10} 10^{pK_{65}-pK_{21}}$, and $k_{11} = k_{12} 10^{pK_{43}-pK_{21}}$.

The total pyranine [$\phi OH\text{]}_{\text{total}}$ and fluorescein [$\text{FluH}\text{]}_{\text{total}}$ concentrations in the solution are easily measurable. Using the values of [$\phi OH\text{]}_{\text{total}}$, the pH, and the $pK$ of the reactant, the equilibrium concentrations of pyranine in its protonated [$\phi OH\text{]}_{\text{eq}}$ and deprotonated [$\phi O^-\text{]}_{\text{eq}}$ states were calculated from the two relations $[\phi OH\text{]}_{\text{total}} = [\phi O^-\text{]}_{\text{eq}} + [\phi OH\text{]}_{\text{eq}}$ and $[\phi OH\text{]}_{\text{eq}} = [\phi O^-\text{]}_{\text{eq}}/(1+10^{\text{pH}-pK_{21}})$. Similar relations also hold for fluorescein and the buffer. The total buffer concentration ([B]$_{\text{total}}$), however, was considered as an adjustable parameter that can vary between 1 and 50 µM. The pH of the solution drifted slightly during the data acquisition time; therefore, its value was considered as an adjustable parameter whose value can vary up to ±0.05 from the measured value (see Table 1). The initial magnitude of the perturbation $X_0$ was measured directly from the initial increase in the absorbance at 458 nm. Since the recording of the first few data points after the laser flash is affected by the radio-frequency radiation released by the laser capacitors, the initial value of $X_0$ was allowed to vary within a narrow range of ±3% of its measured value.
THE FITTING PROBLEM

Altogether the number of adjustable parameters is twelve: The six rate constants $k_1, k_3, k_5, k_8, k_{10}, k_{12}$, the three pK values $pK_{21}, pK_{43}, pK_{65}$, the effective pH of the reaction solution, the initial magnitude of the perturbation $X_0$, and the total buffer concentration $[B]_{\text{total}}$. For any given set of values of these twelve unknown parameters, the ordinary differential equations (1) can be solved using standard numerical subroutines for stiff ODEs (such as Matlab's ODE23s). The level of agreement between the experimental signals and the numerical solutions can be expressed by a fitness function $F$ which is a weighted average of the squares of the differences between the calculated solutions and the measured signals, i.e.,

$$F(pK_{21}, pK_{43}, pK_{65}, k_1, k_3, k_5, k_8, k_{10}, k_{12}, X_0, \text{pH}, [B]_{\text{total}}) =$$

$$\sum_i e^{X_i/X_{\text{max}}} (X_i^{\text{calculated}} - X_i^{\text{signal}})^2 + \sum_i e^{Y_i/Y_{\text{max}}} (Y_i^{\text{calculated}} - Y_i^{\text{signal}})^2$$

where the $i$ in the summation stands for the value at time $t_i$. We use the weight functions $e^{X_i/X_{\text{max}}}$ and $e^{Y_i/Y_{\text{max}}}$ since they give more weight to regions where the signal-to-noise ratio is large.

The problem is thus reduced to finding the combination of (twelve) parameters that minimizes the fitness function $F$. Because of the high noise level (see Figure 1), it is not possible to reconstruct the noisy experimental data with the numerical solution of the mathematical model. A more reasonable approach is to ask whether the numerical curve lies within the noise level distance from the smoothed curve of the experimental signal. In order to add this modification to the definition of the fitness function we first pass the experimental signal $X^{\text{signal}}$ through a smoothing filter to get the smoothed curve $X^{\text{smooth}}$ and estimate the noise level from $N^X = \max_i |X_i^{\text{smooth}} - X_i^{\text{signal}}|$. The ‘admissible band’ is defined as the region between the curves $X^{\text{smooth}} \pm N^X$. We then redefine the difference $(X_i^{\text{calculated}} - X_i^{\text{signal}})$ in the fitness function to be the distance of $X_i^{\text{calculated}}$ from the band (and zero if $X_i^{\text{calculated}}$ is inside the band). A similar approach is applied to $Y$. 
GENETIC ALGORITHM

The optimization problem, which is at the heart of our new methodology, is to find the values of the unknown parameters that minimize the fitness function $F$. In general, one can use various optimization techniques to solve this problem. Our choice of the optimization algorithm is motivated by the following characteristics of the optimization problem:

1) The dimension of the parameter space is large. For example, even in the present case with just three equations, there are twelve unknown parameters. In larger systems, the number of parameters can easily exceed fifty [6, 11].

2) There is no \textit{a priori} information on the properties of the fitness function. For example, it is not clear whether there is a unique minimum or several local minima.

3) Because of the high noise level of the experimental signals, it is not clear whether the fitness function is smooth.

In cases of high-dimensional optimization problems with possible non-smooth fitness function and multiple local minima, a natural choice is a \textit{genetic algorithm}. Briefly, a genetic algorithm starts with a randomly chosen population of solutions. Then, using a \textit{natural selection} strategy the fittest individuals of each \textit{population} tend to ‘reproduce' and ‘survive' to the next \textit{generation}. Thus, in general, the population of each successive generation is fitter, but inferior individuals can sometimes survive.

Our choice of Matlab as the computational platform is motivated by its portability across different platforms and by the availability of the genetic algorithm toolbox \textit{gaot} [12]. To demonstrate the robustness of our methodology we use the default parameters of \textit{gaot}, rather than trying to optimize performance by playing with \textit{gaot}'s parameters (the selection and termination functions, etc).

RESULTS AND DISCUSSION

We analyzed measurements of the pyranine (X) and indicator (Y) signals from five different experiments that were carried out under varying initial pH values and in two time frames, 25µs and 250µs (see Table 1). The short time frame emphasizes the early events of the reaction where the fast reactions with free protons are dominant, whereas the longer time frame emphasizes the slower collisional proton-transfer reactions that dominate the system once the free protons are consumed. Each pair
(pyranine and indicator) is analyzed in tandem by using a single fitness function (see Section 4).

**Table 1.** Conditions under which the five analyzed experiments were measured. In all experiments $[\Phi OH]_{\text{total}}=20\mu M$ and $[\text{Flu}]_{\text{total}}=10\mu M$.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>PH</th>
<th>Observation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.46-6.52</td>
<td>25µs</td>
</tr>
<tr>
<td>B</td>
<td>6.45-6.51</td>
<td>250µs</td>
</tr>
<tr>
<td>C</td>
<td>7.68-7.76</td>
<td>25µs</td>
</tr>
<tr>
<td>D</td>
<td>7.15-7.21</td>
<td>25µs</td>
</tr>
<tr>
<td>E</td>
<td>7.10-7.20</td>
<td>250µs</td>
</tr>
</tbody>
</table>

**Convergence of genetic algorithm**

A typical evolution of the reconstructed signals during the natural selection process of the genetic algorithm is demonstrated in Figure 1, in which we show the fittest pair of curves after one, ten, a hundred and a thousand generations. The first generation begins with a population of a hundred sets of values, where each set consists of values of the twelve parameters that were randomly chosen from their admissible range as described in Section 3.1. Although after one generation the fitting is quite poor, it is of interest to note that the shapes of the curves are already similar to the experimental ones. This similarity indicates that the mathematical model captures the chemical kinetics of the system. In other words, the fitting process is not an artificial parameter-fitting procedure, since the general features of the dynamics are reproduced even with a random assignment of parameters. After ten generations the fitting of the fluorescein signal (right) is already satisfactory as it falls within the 'band' set by the noise of the measuring system. The reproduction of the pyranine signal (left), however, is still poor. Since the mathematical model should reproduce the two transients with the same level of confidence, we judge the fitting after ten generations to be unacceptable. After a hundred generations the reconstruction of the pyranine signal has improved, yet in the 2-5µs time frame the calculated curve exhibits an undershoot, whereas after 5µs there is a systematic overestimation of the amplitude. Finally, after a thousand generations, both curves are well fitted and
constitute an acceptable solution. It is of interest to note that the curves generated after a hundred and a thousand generations have almost the same fitness value (3.98 vs. 3.88) yet the human eye can easily detect the improvements. This shows that the current definition of the fitness function is not optimal and can be improved.

Figure 1: Evolution of reconstructed curves (red) after one, ten, hundred and a thousand generations. The experimental signals (blue) of the reversible protonation of fluorescein (X) and of the incremental pyranine concentration (Y) are from experiment C of Table 1.

Ideally, fitting the experimental signals with the mathematical model should give a unique set of values for the unknown parameters. This is not the case in practice, because the mathematical model does not take into account various factors
such as experimental noise and the drift in experimental conditions during the measurements (pH, material deterioration, etc). Therefore, the ‘acceptable’ reconstructions define a region for the value of each parameter and not a single value. Thus, there is no point in continuing the genetic algorithm simulation of Figure 1 beyond a thousand generations. A better strategy is to calculate these parameter regions by applying the genetic algorithm search for the same experimental signals several times, and repeating this procedure for various experimental signals obtained under different initial conditions.

In Figure 2 we show the evolution of the fitness function along the generation axis (on a logarithmic scale) for ten independent applications of the genetic algorithm each for the five pairs of experimental signals listed in Table 1.

![Figure 2](image.png)

**Figure 2.** The evolution of the fitness function during the genetic algorithm search. Each frame shows ten independent runs carried out for the pair of experimental signals corresponding to the initial conditions in Table 1.
The variance of the fitness function after the first generation is very large, as can be expected from a solution generated by a set of randomly assembled parameters. Yet, within a few generations, the value of the fitness function stabilizes at a level that hardly improves with the number of generations. In order to fully automate the search procedure we reject runs whose final fitness value exceeds the lowest value by more than 2%. When we applied the genetic algorithm search to the five pairs of signals, between 50% and 80% of the searches passed this test and were considered as converging runs.

The repetitious analysis of each pair of signals can serve as an objective criterion for rejecting experimental data that, for some reason, was fouled during the observation. In fact, we originally accumulated six pairs of experimental signals. However, because the genetic algorithm search for the sixth pair consistently stabilized on fitness function values larger than 70, we concluded that this was a fouled measurement. This objective criterion for detection of fouled experiments is another advantage of the genetic algorithm search, since in the past it was less clear whether the inability to get a good fit in the manual search was due to data or the researcher conducting the search.

The apparent consensus between parallel runs of the genetic algorithm, where the final value of the fitness function differs only at the second decimal digit, does not imply that in all cases the magnitudes of each parameter converged to a range as narrow as that of the fitness function. Indeed, the inherent complexity of the reconstructed curves and their dependence on many parameters leads to a situation where, within the narrow variance of the fitness function value, the various parameters can exercise wider fluctuations. Accordingly, the test for convergence is not only for the fitness function value but also for the individual parameter values. For this reason we monitored the variation of the values of the parameters with generation and noticed that in the converging runs the values of the adjustable parameters were practically constant (less than 5% of the final value) during the last 200-400 generations (data not shown).
Figure 3: Parameters calculated by ten runs of the genetic algorithm for each of the five pairs of experimental signals (see Table 1): A. (○), B. (×), C. (▽), D. (∗), E. (∽). The y-axis range in each frame is equal to the range of search. Thick horizontal lines are averaged values over the ten runs.

In Figure 3 we compiled the parameter values calculated by ten runs for each of the five pairs of signals. Each parameter has values in a region whose size reflects the stiffness of the fitness function with respect to that parameter. For some sets of experimental results these regions are narrower than for others. Yet in each case the spread seems to be centered around a mean value that can be considered as the most
suitable value of the parameter for that experiment (represented by the horizontal lines in Figure 3). These averaged values are clustered in a narrower region within the admissible range that was defined for the genetic algorithm search.

The mean and variance of these values over the five experimental measurements are listed in Table 2 and constitute the final estimates for these parameters based on ten genetic algorithm searches for five pairs of signals. At the present level of analysis (only five pairs of signals) we consider it as the global minimum of the optimization problem, with a large variance for some parameters. A more intensive investigation with a larger number of experiments will probably reduce the variance in the parameter values.

**Table 2: Estimation of the values of the adjustable parameters.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Standard Deviation (of five averages)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK_{21}</td>
<td>7.9</td>
<td>0.12</td>
</tr>
<tr>
<td>PK_{43}</td>
<td>6.33</td>
<td>0.02</td>
</tr>
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<td>0.18</td>
</tr>
<tr>
<td>k_1</td>
<td>103 10^9</td>
<td>18 10^9</td>
</tr>
<tr>
<td>k_3</td>
<td>5.7 10^9</td>
<td>2.0 10^9</td>
</tr>
<tr>
<td>k_5</td>
<td>19 10^9</td>
<td>7.4 10^9</td>
</tr>
<tr>
<td>k_8</td>
<td>2.9 10^9</td>
<td>1.5 10^9</td>
</tr>
<tr>
<td>k_{10}</td>
<td>2.8 10^9</td>
<td>1.3 10^9</td>
</tr>
<tr>
<td>k_{12}</td>
<td>2.8 10^9</td>
<td>1.7 10^9</td>
</tr>
</tbody>
</table>

The results in Table 2 show that some parameter values are spread within a narrow limit while others are more dispersed. The pK values of the three reactants exhibit a small variance and are practically identical with the values determined, at vanishing electrolyte concentrations, by standard equilibrium methods (8.0±0.1, 6.5±0.1, and 6.37 for pK_{21}, pK_{43}, and pK_{65}, respectively). The values of the rate constant of protonation of the two dyes (k_1 and k_3) are also in a narrow range, reflecting the fact that the concentrations of the two dyes were measured and used by the fitness function.
An uncertainty common to all measurements where a protein is present is that even when the protein concentration can be accurately measured, it is not known exactly how many of its side groups will function as proton-binding sites in the microsecond time domain [7-8]. In the present study, this uncertainty was incorporated by allowing the concentration of bicarbonate (both initial and dynamic) to be unknown. The concentration values of the bicarbonate calculated by the genetic algorithm were comparable with the solubility of CO₂ in water at the measured pH values, but their standard deviation was ~50%. Accordingly, the values of the rate constants associated with proton exchange with the bicarbonate (k₅, k₆, k₁₀) exhibit a wider range. Determination of rate constants of diffusion-controlled reaction through analysis of only five observations to within 50% can still be considered as more than acceptable accuracy.

CONCLUDING REMARKS

In this study we showed that the search for a large number of values of the kinetic constants could be fully automated using a genetic algorithm methodology. This approach allows us to be more confidently assertive that the calculated values indeed correspond to the global minimum, and to identify fouled experiments. Preliminary results show that this methodology works well in more complex systems such as proton-transfer reactions on the surface of proteins.

We recall that Viappiani et al. [1] fitted all the experiments in tandem, by using a single fitness function for all experiments which is the sum of the fitness functions of all the experiments. Our results show that this approach might not work for larger and/or noisier system, since there is no single set of values of the rate constants that can fit all experiments. Indeed, in Figure 2 we see that for some parameters there is even no overlap between the regions obtained for different experiments (see e.g., the values of k₃ in experiments B and C, and of k₁₂ in experiments B and E). An additional advantage of fitting each experiment individually is the identification of fouled experiments. The ability to fit all experiments in [1] with a single set of rate constants may be due to the fact that in that study each experiment was fitted with four adjustable parameters; two rate constants common to all experiments and two concentration values which were allowed to vary between experiments. Therefore, roughly speaking, since 50% of the adjustable parameters were experiment-dependent, this provides enough flexibility to fit all
experiments with a single set of values of the two rate-constants. In our experimental system there are nine rate-constants and only three parameters, which are experiments specific. Thus, only 25% of the adjustable parameters are experiment-dependent, making it harder to fit all experiments with a single set of values of the nine rate-constants.

The genetic algorithm methodology can be improved in several ways. For example, one can reduce the number of generations needed for convergence of the genetic algorithm by playing with gaot’s parameters and/or switching to a local maximization algorithm at a certain stage [1]. The genetic algorithm can also be trivially parallelized, resulting in faster simulations. Statistical analysis of the noise can improve the quality of the signal, as well as lead to a better definition of the fitness function.

REFERENCES


