CHAPTER 12

Intraprotein Proton Transfer – Concepts and Realities from the Bacterial Photosynthetic Reaction Center

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1 Introduction

For almost 40 years - since the discovery by Chance and coworkers\textsuperscript{1,2} of cytochrome photooxidation at very low temperatures in the photosynthetic bacterium, Chromatium - the bacterial photosynthetic reaction center (RC) has been the premier testing ground for our understanding\textsuperscript{3} of electron transfer in biological function, and even in aperiodic systems, generally.\textsuperscript{3} This position of influence significantly predates the publication of the X-ray structure of the RC from Rhodopseudomonas viridis\textsuperscript{4,5} but the latter event propelled the theoretical and conceptual developments in this area to new heights. The critical contribution of the X-ray structures, however, was not so much the spectacular details of the atomic structure but more simply the firm establishment of distances between cofactors and, in some cases, their relative orientations.

Because of the significant distances involved, the large majority of biological electron transfers are adequately accounted for by non-adiabatic theories as developed by Marcus, Levitch and Dogonadze, Hopfield, and Jortner, among others.\textsuperscript{6-10} The underlying physics has been described with ever increasing sophistication, which has built steady confidence in our understanding of the fundamental events. However, these refinements have had only a small impact on the quantitation necessary to understand the biological processes and, especially, the evolution and design of extant systems. Indeed, there has been a long and occasionally contentious - but for the outsider usually amusing - discourse between proponents of the atomic level and broad-brush views of biological electron transfer.\textsuperscript{11-21} The result is that, within reason, it is a rather simple matter to
understand why biological electron transfer is a robust activity, and how different behaviors can arise in natural and mutant variants.

In principle, the wavefunction that describes ET does correspond to the detailed structure of the medium and in that sense, at least, the network of bonds between a donor, D, and acceptor, A, provides the basis of the connectivity between them – the electronic coupling in the Hamiltonian matrix (see below). Furthermore, it is possible to demonstrate the influence of the bonding pathways between synthetically engineered redox sites, including, for example, better coupling along β-strands than across α-helices. However, there is little or no indication that Nature utilizes these features as a design principle in any natural functions. The overwhelming evolutionary approach is to hang cofactors on the protein scaffold by any suitable liganding, at separations that ensure ET rates that are non-limiting, with the intervening protein acting purely as a homogeneous medium characterized by the β-factor in the term $\exp[-\beta(r-r_0)]$, which approximates the electronic coupling in the non-adiabatic limit. The constraint of what is non-limiting is determined by the turnover time of whatever overall process the ET is functioning within. With few exceptions, the separation between donor and acceptor cofactors in a functional sequence is less than 14 Å, which provides ET times of 100 μs, or faster, for reactions that are thermodynamically neutral or even somewhat unfavorable. Since there is no specific pathway involved, the ET step is insensitive to a wide range of mutations – the biological meaning of robustness. Of course, mutations that influence the energetics of the reactants or products will affect the equilibrium constant as expected, and very likely the kinetics, but in a manner unrelated to the electronic coupling.

In contrast, proton transfer is potentially very sensitive to the structural and energetic details and dynamics of the environment, and it is a significant challenge to discern whereby it achieves the robustness that often characterizes successful, naturally evolved systems.

## 2 Proton Transfer vs. Electron Transfer

The obvious and critical distinction between electron and proton transfer is the almost 2000-fold difference in the mass of the particle. The tiny mass of the electron allows transfer by quantum mechanical tunneling to proceed with modest driving forces at a biologically meaningful rate over distances of up to 15 Å, and non-physiologically beyond 25 Å. At these and even much shorter distances, the donor-acceptor interaction is very weak and the electron transfer is clearly in the non-adiabatic regime.

Tunneling probabilities (rates) are proportional to (mass)$^{1/2}$ and, within the same functional time constraint of 0.1 ms, proton tunneling is limited to no more than 1 Å. (Figure 1). The necessary close approach of the heavy atom systems will result in sufficient perturbation of the wave functions that adiabatic processes will usually dominate. Indeed, even if the tunneling rate at some average non-bonding distance is adequate to support function, it is evident that it will be greatly modulated by thermal fluctuations of the distance between neighboring atoms - a fluctuation of 0.1 Å, around 1 Å, changes the tunneling rate by 1-2 orders of magnitude. Thus, the elementary (pairwise) transfer is usually controlled by the dynamics of the system, regardless of the nature of the heavy atom framework.
In bioenergetic systems, which are designed to catalyze proton coupled electron transfer (PCET) and net proton translocation, proton transfer is invariably between “normal” acids and bases, as defined by Eigen, e.g., most oxygen- and nitrogen-containing functional groups. These exhibit intrinsically fast rates of PT, partly due to their favorable electronic structure, but also, and perhaps more importantly, because the donor and acceptor associate by hydrogen bond formation and the tunneling distance of \( \approx 1 \, \text{Å} \) corresponds roughly to the separation of alternate proton positions in the hydrogen bond (Figure 1). This facilitates the formation of the acceptor bond as the donor bond is breaking and leads generally to low activation barriers and fast pairwise transfer. The question is, therefore, what and where is the rate limiting step?

3 The Grotthuss Mechanism and Hydrogen-bonded Chains

It has long been recognized – remarkably, for 200 years - that protons have the potential for a unique mode of transport in water and, by extension, in other highly connected hydrogen bonding systems. The Grotthuss mechanism involves a simple shift of

\(^{a}\) “Normal” acids and bases are defined operationally as being those that exhibit near diffusion controlled rates when thermodynamically favorable, with the reverse rate smaller by a factor \(10^{16}\).\(^{b}\) It seems especially remarkable, today, that this proposal was made prior to the normally cited date for the atomic theory of matter – clearly the pressure to publish was different in those days! - and before the empirical formula of water was correctly known. Grotthuss actually presented his idea as a mechanism for transfer in electrolysis according to the description: \( \text{OH} \cdots \text{OH} \cdots \text{OH} \rightarrow \text{HO} \cdots \text{HO} \cdots \text{HO} \). (John Dalton’s atomic theory was first presented in public lectures at the Royal Institution in 1803, but was published only in 1808, as Vol.1 of *A New System of Chemical Philosophy*).
hydrogen bonds to effectively relocate a net protonic charge from one position to another without significantly moving the mass of the proton. In water, this process contributes at least four fifths of the measured transfer number of hydrogen ions, and the ionic mobility of H’ is about 7 times that of Na’. Current views of the Grotthuss mechanism have the rate limiting event as the breaking of one or two critical hydrogen bonds outside the primary solvation sphere of the proton charge, allowing reorganization of the first shell from a hydronium, H₃O⁺ (or Eigen ion, H₂O⁺ = H₃O⁺(H₂O)₃), to a Zundel ion, H₂O₂⁺, as a transition intermediate (Figure 2). The proton then redistributes along its own coordinate between the two O atoms and further solvation adjustments can trap it at the new oxygen, in the form of a new H₂O⁺. The electrostatic energies are the same in the initial and final states, of course, and are also of little consequence in the transition intermediate – the energies of the Eigen and Zundel ions are not greatly different. The activation energy for the anomalous proton mobility that characterizes the Grotthuss mechanism (approx. 2.5 kcal/mol) arises from the breakage of a “typical” water-water hydrogen bond. Recent models suggest that the rate limiting step may involve the coordinated rearrangement of hydrogen bonds as far away as the third solvation shell.

Computational studies on proton migration in water have provided a fairly good picture of the operation of the mechanism that underlies the idea of PT through a hydrogen bonded chain or network. Given the flatness of the potential energy surface in bulk water, it might not be surprising to encounter such a mechanism there, but even in water the result is not a long distance concerted transfer over multiple oxygen centers. The excess mobility of the proton arises simply from the increased step size of the random walk, i.e., diffusion, as the proton charge is moved across the diameter of a single water molecule (~2.5 Å) in about 1 ps, the Debye (rotational) relaxation time of water.

Caution should be exercised in transferring this picture to the inside of a protein where donor-acceptor pairs are unlikely to be so well matched in pKₐ. At the same time, however, the reduced dimensionality in structured systems increases the opportunity for hydrogen bonded chains to provide proton wires with high conductivity and specific directionality. Consequently, a great deal of speculation and a modest amount of computation has been expended in exploring the importance of such structures in biological processes. It was pointed out early on, by Nagle and coworkers, that steady state conduction by a hydrogen bonded chain must involve two types of activity – a hopping mode as the proton charge moves from one end of the pre-oriented hydrogen bond chain to the other, and a turning mode, in which the hydrogen bond network reorients to restore the original configuration.

Various levels of microscopic calculation and dynamic simulation have shown this in action, but the results can be misleading. While transfer of the protonic charge across hydrogen bonds undoubtedly occurs, and is responsible for the excess diffusion of protons in water, in a low dielectric environment like a protein interior, additional factors intrude, including an electrostatic (Born) energy barrier to entry, as well as site-differences, which can easily dominate the structural aspects of the conduction (i.e., the Grotthuss mechanism) once inside. Of course, the protein structure could be designed to minimize the Born energy factor, as seen in the specialized channel proteins for K⁺ and Cl⁻ conduction, but such a system has not yet been described for protons. Certainly, the precise balance of these factors is still unclear and PT, like ET in earlier times, is currently enjoying its own contentious debates. This is well illustrated by the literature
The Grotthuss mechanism in water, showing Eigen and Zundel ions. From top to bottom: The hydronium ion (b) is almost planar and is solvated by 3 water molecules forming an Eigen ion, H₃O⁺. Each solvating water is hydrogen bonded to approx. 3 additional neighbors – this is shown only for one solvating water (c). A hydrogen bond in the second solvation shell (c–d) is broken and the remaining ion rearranges to yield a Zundel ion, H₅O₂⁺. The excess proton fluctuates along the "proton coordinate", between the two oxygen atoms and is trapped at either one as a new hydrogen bond (here, from a to b) reforms an Eigen ion – in this case on oxygen c. (Figure adapted and redrawn from Agmon.²³)

on gramicidin A, an ionophorous antibiotic that may come as close as we know to a proton conducting, hydrogen bonded chain with a low Born energy factor.

Proton transport through the gramicidin A (gA) channel has been analyzed and simulated in a number of studies, using a variety of computational approaches with conflicting apparent outcomes. Pomès and Roux, for example, found the main activation barrier to H⁺ movement within the gA channel to be the turning motion of the channel water molecules, while the H⁺ hopping process along the ordered water chain was
essentially activationless.\footnote{47} This surprising result implied a negligible electrostatic penalty for moving the protonic charge within and through the channel, and the authors suggested that this was due to very favorable dipole interactions with the water chains on either side of the charge site. In apparent contrast, Warshel and coworkers found a significant electrostatic penalty to the presence of the charge and believe this to be almost exclusively the source of the kinetic barrier,\footnote{48-50 i.e., they consider the motions necessary to ensure the hydrogen bond connectivity and reorientation are sufficiently small as to be energetically insignificant. This, of course, is the very motion that comprises the “turn” action of a hydrogen bonded chain.} i.e., they consider the motions necessary to ensure the hydrogen bond connectivity and reorientation are sufficiently small as to be energetically insignificant. This, of course, is the very motion that comprises the “turn” action of a hydrogen bonded chain.

In fact, these two results are not comparable. Pomès and Roux calculated the motion of the charge once inside the gA channel, and did not consider the entry or exit of the proton (the cap of water molecules at each end of the water file were “non-dissociable”). On the other hand, Warshel and coworkers calculated the proton energies relative to the bulk phase, and found a total barrier height of 5-7 kcal/mole. This is in quite good agreement with the experimental activation energy for H\(^+\) conduction in gA, which is quite small. However, the estimated internal barriers were also small and, although not indicative of an activationless hopping motion, are probably not so far from those of Pomès and coworkers, given the errors and differences in both approaches.

Interestingly, Schumaker and colleagues, using a macroscopic formalism that incorporates the microscopic results of Pomès and coworkers,\footnote{51} have estimated the steady state proton conduction behavior of gA, including the entrance and exit events.\footnote{52} They find that the activation energy for the turn steps is lower than originally estimated, and that it is the proton’s exit that is rate limiting. This intriguing result suggests that the channel, possibly including the induced polarization of the water file, “holds on” to the proton as it tries to leave. NMR studies have provided structural information on the conduction of alkali metal cations through the gA channel, which may also be relevant to understanding proton conduction.\footnote{53-55} Entry to the channel is accompanied by the
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sequential removal of waters of hydration from the cation in three distinct steps as the ion moves from one site to the next. Ultimately, the ion is associated with only 2 waters – one ahead and one behind in the single file of the channel. In this same entrance (and exit) region of the channel, the ion has a significant electrostatic interaction with the 4 tryptophans (approx 1 k_BT per indole) that comprise a well-defined collar in the membrane headgroup region of the channel. Conceivingly, this provides the interaction that limits proton exit from γA.

A similar discourse has developed on the topic of aquaporin, a membrane protein designed to conduct water but not protons. Here, the water chain that spans the channel reverses the polarity of the hydrogen bonded water dipoles at the mid-point of the membrane span, and this structural feature was suggested to impede proton transport by interrupting the conduction pathway. However, subsequent calculations and molecular dynamics simulations have shown that a significant contribution to the barrier for proton conduction comes from the electrostatics and related desolvation penalty for a protonic charge to penetrate into the channel interior. The relative magnitudes of these contributions are, of course, under intense debate! In fact, the result for aquaporin is hardly surprising, but it does not contribute to the discussion about how proteins do conduct protons.

The conclusion from these disparate works might be that the kinetic barriers to proton transfer are small and conduction rates can be controlled by electrostatic penalties to populating internal sites, or be facilitated by a flat profile. From an experimental point of view, we need to know how to probe the mechanism by, for example, changing the proton donors and acceptors or otherwise changing the driving force for PT, and how to analyze the experimental results. These questions are not yet settled but are partly addressed in the following sections.

4 Free Energy Relationships – Marcus and Brønsted

Correlations between reaction rates and free energy changes (or equilibrium constants) are often observed in chemistry, and significance for these free energy relationships (FERs) has long been sought. In general, it is obscure except at a qualitative level, but the simplicity of ET has allowed genuinely theoretical descriptions to be developed. These are commonly referred to as Marcus theory, although many names are associated with current formulations of it. The essential ingredients of Marcus theory are that the reaction coordinate for ET is controlled by the nuclear reorganization of the environment (solvent polarization), and that the donor and acceptor are weakly interacting, so that the ET is non-adiabatic.

From the Golden Rule of perturbation theory, the probability of transition between initial and final states is:

\[ p(t) \propto \frac{2\pi|V_{ij}|^2}{\hbar} \rho_f \]  

\( \text{(1)} \)

This is commonly referred to as Fermi’s Golden Rule and it is certainly true that he coined the term for his lectures at the University of Chicago, in 1947-1951, indicating his great admiration for the power of this simple expression. However, the equation, itself – and the whole construct of perturbation theory that it represents – is due to Dirac, 20 years earlier.
$V_{if}$ represents the electronic coupling or matrix element that mixes the initial and final states – or, in molecular terms, the strength of interaction between the electron donor and acceptor. $\rho_f$ is the density of states that are accessible to the transition from $i \rightarrow f$ while maintaining conservation of energy. It is commonly called the Franck-Condon factor, or thermally weighted density of states, and is the ratio of states at the transition state relative to the reactant ground state. For a thermal reaction it provides the activation energy term.

For a non-adiabatic transition, the Franck-Condon factor defines the intersection of the diabatic curves for initial and final states, and, when these are represented as harmonic oscillators, it can be easily derived and calculated. This is the familiar, classical form of Marcus theory.

$$k = \frac{2\pi}{h} |V(r)|^2 \frac{1}{\sqrt{4\pi\lambda k_b T}} \exp\left(-\frac{(\lambda + \Delta G^0)^2}{4\lambda k_b T}\right) = k_0 \exp\left(-\frac{\Delta G^*}{k_b T}\right)$$

(2)

where the activation free energy, $\Delta G^* = (\lambda + \Delta G^0)^2/4\lambda = \lambda/4(1 + \Delta G^0/\lambda)^2$, is given as the intersection point of two parabolas representing the diabatic curves of the reactant and product states (Figure 4). The novel concept, here, is the reorganization energy, $\lambda$, which is the energy gap between the reactant state at its vibrational equilibrium position and a point on the reactant curve that is vertically above the product state equilibrium. It is the equivalent, in energy units, of the difference between the equilibrium positions of reactant and product along the vibrational coordinate. The reorganization energy describes the responsiveness of the vibrational system (including solvent) to the electronic changes in the reaction. Together with the free energy of the reaction, $\Delta G^0$, it quantifies the energy necessary to generate an intermediate nuclear configuration that has the same total energy before and after electron transfer.

The reorganization energy is determined both by the magnitude of the distortion and the stiffness of the bond vibrations that respond to the electronic transition. This gives rise to a crude categorization of contributions to $\lambda$ - an inner component, $\lambda_i$, which reflects the molecular response of the donor and acceptor cofactors, and an outer component, $\lambda_o$, which arises from the solvent polarization response to the change in charge distribution. In many cases, the structures of biological cofactors, such as large conjugated macrocycles, minimize $\lambda_i$ and the major contribution is from the solvent-environment, which is primarily the protein matrix rather than the true aqueous solvent, as biological redox centers are at least partially shielded from the latter. More sophisticated versions that include quantization of the nuclear vibrations, allow the distinction between $\lambda_i$ and $\lambda_o$ to be made explicit.

From the Marcus equation, one can relate the activation free energy (and hence the temperature dependence) of a reaction to the standard free energy change, $\Delta G^0$, and to the reorganization energy of the “solvent coordinate”, which includes all vibrations that are coupled to the electronic transition.

Marcus theory was originally formulated for bimolecular reactions in solution, and two additional terms were included to account for the necessity of forming an encounter complex between the reactants. These are $w_r$ and $w_p$, the work involved in bring the reactants together and in separating the products, respectively. Incorporating these, the classical expression becomes:
Marcus theory diagram for electron transfer, showing weak electronic coupling $V_{\text{if}}$. A single reactant surface (black) and four possible product surfaces are represented as parabolas. The reorganization energy, $\lambda$, is given by the position on the reactant curve that lies above the equilibrium coordinate of the product curves. The product surfaces are shown for several net driving forces ($\Delta G^0$ values). For $\Delta G^0 = 0$ (black), the intersection between reactant and product curves is at $\lambda/4$, indicating the activation free energy for the thermodynamically neutral reaction ($\Delta G^*_0$, the “intrinsic” barrier). As the free energy decreases from unfavorable (pink) to favorable, the activation free energy decreases and the reaction accelerates. $\Delta G^*$ is zero and the rate is maximum when $\Delta G^0 = -\lambda$ (red). At very large (negative) driving forces ($\Delta G^0 < -\lambda$) (blue), Marcus theory predicts an “inverted region” where the activation free energy increases again and the rate of reaction slows. At each intersection point the reactant (initial) and product (final) states interact weakly to yield two curves separated by $2V_{\text{if}}$ (shown for the black curves), where $V_{\text{if}}$ is the coupling or interaction energy. For non-adiabatic electron transfer, the splitting is very small, yielding what is called an “avoided crossing”. In solution, the formation of the reaction complex (to which Marcus theory may be applied) and dissociation of the products involve work terms, $w_r$ and $w_p$, which raise the reactant and product curves – often by similar amounts.

$$\Delta G^* = w_r + \frac{\lambda}{4} \left(1 + \frac{\Delta G^0_{\text{obs}} - w_r + w_p}{\lambda}\right)^2$$  \hspace{1cm} (3)$$

where $\Delta G^0_{\text{obs}} = \Delta G^0 + w_r - w_p$, and $\Delta G^0$ is the free energy of reaction within the encounter complex. In cases where $\Delta G^0$ is large, the influence of $w_r$ and $w_p$ is small and they are commonly neglected. In biological ET, the cofactors are often preorganized and the work terms are appropriately absent.

The concept and investigation of free energy relationships (FERs) predate Marcus theory by many decades. The earliest example is the Bronsted relationship, which relates the rates of proton transfer reactions (and hence the activation free energy) to the
differences in pKₐ's of the donor and acceptor (the standard free energy of the reaction, ∆G⁰). A limited range of ∆G⁰ yields linear FERs, but more extensive data sets invariably show curvature suggestive of a quadratic dependence. The success of Marcus theory as a mechanistically meaningful, theory-based description of a quadratic FER in ET, led to adoption of it as a basis for PT and for more complex reactions, including atom and small group transfers and enzyme catalysis and it has been widely used and explored. However, while undoubtedly useful, it is fundamentally inappropriate and its success relies on correlation rather than any real mechanistic underpinnings.

A very general (“interpretation free”) analysis of the relationship between activation free energy and standard free energy of reaction is to consider a FER as a Maclaurin series expansion:

\[ \Delta G^* = \Delta G^0 + \frac{d\Delta G^*}{d\Delta G^0}\Delta G^0 + \frac{d^2\Delta G^*}{d(\Delta G^0)^2}\Delta G^0 + \ldots \]

\[ = \Delta G^0 + \alpha \Delta G^0 + \alpha' (\Delta G^0)^2 \]

\[ \Delta G_0^* \] is the “intrinsic” activation free energy encountered for a reaction with no driving force (\(\Delta G^0 = 0\)). \(\alpha = d\Delta G^*/d\Delta G^0\) is the Brönsted coefficient, and reflects the change in activation free energy as the driving force changes, and \(\alpha_0\) is the value of \(\alpha\) when \(\Delta G^0 = 0\). When recast in the form of the original Marcus theory, we find:

\[ \Delta G^* = \frac{\lambda}{4} \left(1 + \frac{\Delta G^0}{\lambda}\right)^2 = \Delta G_0^* \left(1 + \frac{\Delta G^0}{4\Delta G_0^*}\right)^2 \]

i.e., \(\Delta G_0^* = \lambda/4, \alpha_0 = 1/2, \alpha' = d\alpha/d\Delta G^0 = 1/(8.\Delta G_0^*) = 1/(2\lambda)\).

In the language of physical organic chemistry, the activation free energy, \(\Delta G^*\), is considered to comprise two distinct contributions — a “kinetic” component or intrinsic activation free energy, \(\Delta G_0^*\), and a “thermodynamic” component, \(\Delta G^0\) (Figure 5). \(\Delta G_0^*\) being equal to the free energy required to distort the reactant or product states to the transition state configuration for a reaction with \(\Delta G^0 = 0\), is identical in concept to the reorganization energy, since \(\lambda = 4\Delta G_0^*\) under these circumstances. Like \(\lambda\), \(\Delta G_0^*\) reflects the response of the environment and any molecular vibrations coupled to the PT reaction.

Although possibly no more than phenomenological, mechanistic significance has long been sought for free energy relationships. The interpretations are mostly of a qualitative nature, the most notable being the Hammond postulate, from which it follows that the slope (\(\alpha\)) of the FER (\(\Delta G^*\) vs. \(\Delta G^0\)) reflects the position of the transition intermediate along the reaction coordinate. This in turn is interpreted as indicating whether the structure of the transition intermediate is more like the reactants (\(\alpha < 0.5\)) or the products (\(\alpha > 0.5\)). The change in slope (\(\alpha\)) along the quadratic curve suggests that the transition state of exergonic reactions resembles reactants, while that of endergonic reactions resembles products.

By comparison with the ET formulation, it is clear that the intrinsic activation free energy (\(\Delta G_0^* = \lambda/4\)) is determined by the point of intersection of the diabatic curves. For non-adiabatic ET this is a reasonable approximation as the electronic coupling is small
Marcus formalism and adiabatic modification for facile proton transfer. Diabatic curves (parabolas) are shown for reactants (black) and for products for neutral (blue) and endergonic (pink) reactions. The intersection of the black and blue diabats gives the intrinsic barrier of the reaction, $\Delta G_0^*$ or $\lambda/4$. The actual activation free energy, $\Delta G^*$, on the corresponding adiabatic surface (green) is much smaller and is modified by the coupling energy, $V_{ij}$, which is substantial. For the endergonic reaction, $\Delta G^*$ on the adiabatic surface (magenta) is also much smaller than the equivalent diabatic intersection, although not by the same amount - $V_{ij}$ is not constant but varies along the reaction coordinate. Note, also, that the peak ($\Delta G^*$) is not at the same position on the two adiabatic surfaces - the transition state is closer to the product for the endergonic reaction (Hammond’s postulate).

Figure 5

due to the substantial distance between electron donor and acceptor. For PT, however, the close approach necessary for bond breaking and formation (even if the proton tunnels), means that substantial electronic perturbations occur between proton donor and acceptor. Thus, the coupling is large and often on the same order as $\Delta G_0^*$, itself, so the true activation free energy is much less than that indicated by the Marcus equation (Figure 5). If this adiabatic character is not accounted for, the analysis can only yield a small activation energy by virtue of an improbably small reorganization energy, and it therefore grossly misrepresents the microscopic events.

Nevertheless, a beguiling correlation remains because the adiabatic transition state energy varies in parallel with the diabatic intersection. The absolute magnitudes of $\Delta G^0$ and $\Delta G^*$ may not be related by any simple FER, but a linear relationship between $\delta \Delta G^*$ and $\Delta G^*$ still holds so long as the coupling energy is insensitive to $\Delta G^0$. Thus, any change in the diabatic intersection is reflected in $\Delta G^*$ even after correction by $V_{ij}$.

A characteristic feature of Marcus theory for ET, and for the Marcus (quadratic) formalism, generally, is the prediction of an "inverted region" at very large driving forces, i.e., when $\Delta G^0$ becomes more negative than $-\lambda$, the rate slows down. The rate peaks at $\Delta G^0 = -\lambda$ and the width of the log (rate) vs. $\Delta G^0$ parabola is proportional to $\sqrt{\lambda k_B T}$. This prediction for ET was finally confirmed after 20 years. For proton transfer, it was long debated whether an inverted region was possible, and certainly attempts to observe it were commonly confounded by diffusional limitations. However, inverted region behavior has been reported for PT between the radical cation of
dimethylaniline and a series of substituted benzophenone radical anions in aprotic solvents.\textsuperscript{77,78} A more convincing demonstration has come from studies using an electron photoinjection method at an electrode interface.\textsuperscript{79}

5 Proton Transfer in Biology

PT is of major importance in two distinct areas of biochemistry – acid-base catalysis in enzyme activity, and proton coupled electron transfer in bioenergetics. In the former, the PT events are generally highly localized and the critical purpose is to transfer a proton between adjacent groups, for example an active site amino acid and a substrate. However, although water is generally excluded from active sites (except as a reactant), there are many examples where a chain of water molecules maintains a specific connection to the bulk phase.\textsuperscript{80} This may present a polarizable element that can respond to charge shifts associated with the catalytic events, including local PT. Nevertheless, given the generally pairwise nature of PT in active site chemistry, descriptions of general acid-base catalysis are applicable and some simple relationships might be expected. In contrast, proton transfer in bioenergetics is usually over long distances, the primary purpose being to translocate protons into and across the membrane, e.g., of the mitochondrion, chloroplast or bacterial cell.

An intermediate example, from active site proton transfer, is the reaction of carbonic anhydrase (CA), where a proton is taken up (or released) as part of the stoichiometric turnover:

$$\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$$  \hspace{1cm} (Scheme 1)

Somewhat surprisingly, the transport of H\(^+\) in and out of the active site is the rate limiting step \textsuperscript{81,82}. PT occurs over a distance of 8-10 Å and is associated with the regeneration of the active site Zn\(^{2+}\)-OH\(^-\) complex:

$$\text{Zn}^{2+}\cdot\text{OH}_2^- + \text{B} \leftrightarrow \text{Zn}^{2+}\cdot\text{OH}^- + \text{BH}^+$$  \hspace{1cm} (Scheme 2)

The communication between the zinc-bound water and the aqueous phase is mediated by a short chain of 2-3 water molecules and an amino acid side chain, represented by B/BH\(^+\), which is in contact with the bulk phase. In carbonic anhydrase II, the fastest of the many mammalian isozymes, B/BH\(^+\) is a histidine (His\textsuperscript{64} in Figure 6).

In a series of detailed and elegant studies, Silverman and coworkers have used site directed mutations and natural isozyme variants to alter the pK\(_a\) of both Zn-OH\(_2\) and B/BH\(^+\), roughly over the range of ΔpK\(_a\) = 0 ± 3 units.\textsuperscript{83-86} The measured rates have generally fit well to a quadratic relationship for log k vs. ΔpK\(_a\) and have exhibited significant curvature. In the Marcus formalism, curvature over such a relatively narrow range of ΔG\(^0\) (ΔpK\(_a\)) indicates that the ΔpK\(_a\) values are in the range of ΔG\(_0^*\), or λ/4, and that λ is small. From a Marcus analysis, the implied value for ΔG\(_0^*\) is very small and the bulk of the activation energy is provided by the work term, w\(_r\). Silverman’s interpretation of this is simple and appealing – work is required to assemble or order the water chain into a functional arrangement. However, the waters are crystallographically resolvable and therefore cannot be highly disordered. More disturbing is the value of ΔG\(_0^*\) ≈ 1-2
kcal/mol, i.e., $\lambda \approx 5-8$ kcal/mol, whereas computational methods would suggest values at least 10-times larger for a charge transfer reaction of this nature.\textsuperscript{73,74,87-90}

From a conceptual point of view, in fact, it is curious that a process that clearly involves intermediate water molecules, with relatively extreme $pK_a$ values, appears to conform to a Marcus analysis that considers only the $pK_a$'s of the terminal donor and acceptor, which are much more closely matched. PT in such a situation is not a two-state system and there is no reason why a Marcus analysis would give meaningful parameters. Computational studies of CA have yielded a very different picture, which serves well to illustrate the fundamental problems of applying a simple FER to PT.\textsuperscript{48} These are two fold. First, the Marcus formalism yields an intersection point for the two diabatic curves. However, the correction to this, due to the coupling between the states, is very large and the resulting transition state on the adiabatic surface is much lower energy. Second, the involvement of more than two states, i.e., with intervening water molecules between the identified donor and acceptor, invalidates the Marcus formalism altogether, as far as the overall PT is concerned (Figure 7).

The impact of the coupling or mixing between reactant and product states is so large that the free energy of the transition state between each site is comparable to the free energy differences between the sites, which are almost entirely determined by electrostatics. Thus, in this view, the kinetic barriers in CA are dominated by the

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**Figure 6** The active site of carbonic anhydrase II. The reactive water/hydroxyl is bound to a zinc(II) ion (black), which is liganded by three histidines. The fourth histidine, His64, is at the entrance of the active site cleft and is observed in two distinct configurations – the “out” position is essentially in the bulk phase. Up to four additional water molecules are seen in different crystal structures, as shown, bridging the zinc-bound water and His64 in the “in” position. (Image courtesy of David N. Silverman: www.med.ufl.edu/pharm/facdata/silvermn/silvermn.html)
Diabatic curves and adiabatic surface for sequential, multistep proton transfer. Three states are shown for the reaction sequence at the top (charges on PT donors/acceptors not shown, for generality). The true reorganization energy for the first step, $\lambda_{AB}$, is shown, and that for the second step would be similar (but not identical as the horizontal displacement is different). The adiabatic surface (dotted) shows that the actual barrier, $\Delta G^*$, is barely related to any of the diabatic intersections. Note that the barrier height is not very different from the maximum free energy difference at the intermediate state (pink). A 2-state Marcus formalism based on $pK_a$ values for the initial donor (A) and final acceptor (C) would require a very different (and smaller) reorganisation energy, “$\lambda_{AC}$”, to yield the right magnitude for the activation free energy (dashed green parabola, shown intersecting the reactant curve at the same height as the intermediate state).

Repeating these calculations for various mutations that resulted in the quadratic dependence of log $k$ the $pK_a$ difference between the terminal donor and acceptor, i.e., B/BH$^+$ and Zn-OH/Zn-OH$_2$\textsuperscript{91} Schutz and Warshel find that the $pK_a$ values for the intervening water molecules in the chain also change, and the effect on the total activation energy (and hence log $k$) rather coincidentally aligns with the $\Delta pK_a$ between terminal donor and acceptor.\textsuperscript{48} The individual, pairwise PTs, however, are associated with large values of $\lambda \approx 80$ kcal/mol, which is considered much more realistic for this type of reaction. In contrast, the work terms are negligible, indicating that it takes very little effort to arrange the water molecules for adequate function.

This is an area of active discussion, but it is important to note that approaches that focus on $pK_a$ values and their role in determining kinetics as well as equilibria, automatically take in to account the relevant electrostatics – that is what determines local electrostatic energies associated with protonating internal sites, rather than structural considerations such as the dynamics of hydrogen bonded chains.\textsuperscript{48,49}
pKₐs. Furthermore, regardless of the correct balance of electrostatic vs. structural control of PT, long range PT is certain to be a multistep process. This is an inevitable corollary of the insignificant distances that protons can tunnel, so that long range PT must be achieved in several, essentially adiabatic steps, not by non-adiabatic two-state tunneling as in ET.

It is conceivable that concerted PT could provide a 2-state description for PT over some small number of bonds but, in general, one expects concerted processes only when the step-wise mechanism is very expensive.²⁹,³⁰ To some extent, this is related to the energetics of the hopping mode of HBC activity, but the degree of coherence required is substantially greater than that implied by most computational results (but see ⁹⁴). In general, computational studies have given little support for it in bioenergetics, although the methods may yet be inadequate to the task. Nevertheless, highly polarizable hydrogen-bonded protons have been proposed as almost endemic to densely packed, hydrated, weak acid-base matrices, like proteins, giving rise to very broad – almost continuum – infrared absorbance, ⁹⁵,⁹⁶ and these represent a potentially 2-state, long distance PT system. It is intriguing to note that a broad infra-red feature has been reported for the “proton release complex” of bacteriorhodopsin ⁹⁷ and the quinone region of reaction centers, ⁹⁸ and these have been suggested to be signatures of such “delocalized protons”.

6 “Normal” Acids and Bases

As described above, analyses based on free energy relationships are meaningful only when the process under study is correctly identified as a single PT step. In acid-base catalysis this is often the case, but proton translocations in bioenergetics are likely to extend over several functional groups or molecules, including water. From a phenomenological view, this is similar to the situations investigated by Eigen and coworkers, working with proton donor and acceptor pairs in solution.²⁷,⁹⁹ Here, the proton transfer reaction is preceded by the transient association of donor and acceptor in an encounter complex:

\[
\begin{align*}
\text{AH} + \text{B} & \quad \overset{k_e}{\rightleftharpoons} \quad \text{AH} \cdots \text{B} \quad \overset{k_p}{\rightleftharpoons} \quad \text{A} \cdots \text{HB} \quad \overset{k_d}{\rightleftharpoons} \quad \text{A} + \text{HB} \\
\text{Scheme 3}
\end{align*}
\]

The “⋅⋅” symbol strictly implies only the solvent cage-induced association of the encounter complex, but for oxygen and nitrogen species it will almost certainly involve hydrogen bonding, too. For weak association, the concentration of complex will be very small and a steady state analysis can be used, in which the total passage time is the sum of the component step times:

\[
\tau_f = \frac{1}{k_f} = \frac{1}{k_e} + \frac{1}{k_pK_e} + \frac{1}{k_dK_eK_p} \quad \Rightarrow \quad k_f = \frac{k_kk_pk_d}{k_pk_d + k_ck_d + k_ck_p}
\]

where \(K_e = k_e/k_c\) and \(K_p = k_p/k_c\). Often, but not always, \(k_e \approx k_d\) and \(k_e \approx k_d\). Counter examples include products with different charges from reactants, such as AH + B vs. A⁺ + BH⁻, or vice versa, and other sources of ‘sticky products’.
The free energy relationships discussed above apply only to the actual PT process in the encounter complex: $\text{AH} \cdots \text{B} \quad \leftrightarrow \quad \text{A} \cdots \text{HB}$. If this is rate limiting it will be directly observable by monitoring the reactants or products, i.e., for $k_p \ll k_e$ and $k_p \ll k_d$:

$$k_f = \frac{k_e k_p k_d}{k_p k_d + k_\alpha k_d + k_- k_p} \quad \Rightarrow \quad k_f = k_p K_e$$  \hfill (7)

$K_e$ is generally very weak, on the order of $1 \text{ M}^{-1}$, and is also likely to not vary much for a series of reactants providing the charge types are the same.

Alternatively, if PT is fast in the encounter complex, $k_p \gg k_\alpha$, $k_\alpha$ and $k_p \gg k_d$, and we obtain the common behavior of “normal” acids and bases:

$$k_f = \frac{k_e k_p k_d}{k_p k_d + k_\alpha k_d + k_- k_p} \quad \Rightarrow \quad k_f = \frac{k_e K}{K + k_\alpha / k_-}$$  \hfill (8)

where $K = \frac{k_e k_p k_d}{k_\alpha k_d}$ but also, from a thermodynamic cycle: $K = 10^{pK_e(B) - pK_e(A)} = 10^{\Delta pK_e}$

Hence: $k_f = \frac{k_e 10^{\Delta pK_e}}{k_\alpha / k_- + 10^{\Delta pK_e}} \approx \frac{k_e 10^{\Delta pK_e}}{1 + 10^{\Delta pK_e}}$, \hfill (9)

the latter because $k_\alpha \approx k_d$.

This predicts two distinct regimes of behavior of the rate with respect to changes in the relative strengths of the acid and base ($\Delta pK_e$). When the donor, AH, is a stronger acid than BH$^-$, $\Delta pK_e = pK_e(B) - pK_e(A) > 0$ and the rate constant is equal to the association rate constant, $k_e$. The reaction is described as “diffusion limited” and it is insensitive to the individual $pK_a$ values so long as $\Delta pK_e > 0$. However, if the donor becomes relatively weak enough that $\Delta pK_e < 0$, the reaction becomes endergonic and the observed rate then slows down by a factor of 10 for each unit decrease in $\Delta pK_e$. This is nominally a Brønsted relationship with $\alpha = 1$, but the rate limiting step is not PT and the slope relates two independent processes. The proton transfer step is in rapid equilibrium and the equilibrium constant appears simply as a multiplier of the actual rate limiting process, which is the formation of the encounter complex. The reorganization energy or the related concept of intrinsic activation energy, $\Delta G^e_{\circ}$, for the PT is irrelevant. (Note that, in this regime, the reaction is diffusional but it is not “diffusion limited” because the rapid proton transfer equilibrium disfavors the forward reaction and not all encounters are successful.)

Although not a PT limited process, the rate in this regime is revealing with regard to the proton transfer reactants. For example, if a series of donors is used with an unknown acceptor, the rate dependence on the acid $pK_a$ can provide the $pK_a$ of the acceptor. This method has been used to advantage in studies of proton uptake by bacterial reaction centers, as described below.

7 Proton-coupled Electron Transfer in the Acceptor
Quinone Function of Photosynthetic Reaction Centers

Photosynthetic reaction centers (RCs) from purple bacteria and Photosystem II take up protons as the result of quinol production: $\text{Q} + 2e^- + 2\text{H}^+ \longrightarrow \text{QH}_2$. Although RCs do not carry out transmembrane proton pumping, which is characteristic of cytochrome
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oxidase$^{105,106}$ and bacteriorhodopsin,$^{107}$ the uptake associated with quinone reduction constitutes the first half of a proton translocating redox loop$^{108,109}$ that is completed with the oxidation of quinol by the cytochrome bc$_1$ (b$_d$f) complex. Furthermore, H$^+$ transfer to the buried quine site is over similar long distances to those that are encountered in pumping mechanisms, and the lack of gating in the RC provides a useful simplification in the study of the essential features of proton conduction pathway(s) in proteins. Indirect coupling of electron transfers to proton uptake is also seen in the reponse to the light-induced perturbation of the charge distribution of the protein, and the rich spectroscopy of the RC provides a unique system for studying the dielectric responses of proteins.

The single turnover events of the RC from Rhodobacter (Rba.) sphaeroides are summarized in Figure 8A, with an electron transferred from the excited state of the primary donor (P*) to the quinones, Q$_A$ and Q$_B$, on the other side of the membrane. All cofactors are tightly bound except Q$_B$, which is in weak binding equilibrium when fully oxidized or reduced. However, when reduced to the one-electron, semiquinone state, Q$_B^-$, it is tightly bound. In the presence of a secondary donor (cytochrome c$_2$ in vivo), P$^+$ is rereduced and the RC can undergo another photoactivated turnover, which provides a second electron, also via Q$_A^-$, and Q$_B$ is fully reduced to the quinol form (hydroquinone, QH$_2$) with the uptake of protons from the solution. The quinol unbinds and leaves the RC and is replaced by an oxidized quinone from the membrane pool. This returns the acceptor quinones to their original state and allows RC turnover to proceed under multiple-flash activations. Under such conditions, binary oscillations can be seen in the formation and disappearance of semiquinone and in the uptake of protons from the medium (b<1 on the first flash, 2-b on the second) (for review, see$^{100,105}$). This sequence of events is summarized by the acceptor quinone cycle shown in Figure 9, where it is clear that protons are involved in both electron transfers, although covalent attachment to Q$_B$ only occurs on the second turnover with full reduction to quinol.

The two quinones constitute a functional “acceptor quinone complex”, organized around the central iron atom and its ligand field of four histidines and a glutamate (Figure 8B). Q$_A$ and Q$_B$ are both bound with the C1 carbonyl hydrogen bonded to a backbone NH, and the C4 carbonyl hydrogen bonded to the N$_3$H of one of the four histidines liganded to the iron atom. In the semiquinone anion form, Q$_B^-$ is also hydrogen bonded by the O$_H$ of Ser$^{1223}$. Both quinone binding pockets are predominantly non-polar, with the notable exception of Glu$^{1212}$ and Asp$^{1213}$ in the Q$_B$ site. These residues are involved in the terminal delivery of protons to the quinone headgroup, during the second turnover (see below).

7.1 First Electron Transfer to Q$_B$ and Coupled Proton Uptake (Bohr Protons)

The first electron is shared between the two quinones, according to their relative redox midpoint potentials ($E_{m0}$). The negative charges of both anionic semiquinones induce pK$_a$ shifts in ionizable residues of the protein that are close enough to sense the electric field, resulting in net proton uptake.$^d$ A pK$_a$-shifted residue may or may not

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$^d$ Commonly referred to as Bohr protons, after the phenomenon in haemoglobin in which H$^+$ uptake is coupled to oxygen binding.$^{105,111}$
Figure 8  The reaction center (RC) complex from Rhodobacter sphaeroides, and quinone binding sites. A. The RC comprises three subunits, a heterodimer of similar, but non-identical L and M subunits, and subunit H that caps LM on the cytosolic side of the membrane. The LM dimer binds all the cofactors, while subunit H stabilizes the structure and is involved in $H^+$-ion uptake and transfer associated with electron transfer to the quinones. The L and M subunits and all associated cofactors are arranged around a quasi-2-fold rotational symmetry axis, normal to the plane of the membrane, and passing through the primary donor (P, a dimer of Bchl) and a ferrous ($Fe^{2+}$) iron midway between the two quinones. Electron transfer proceeds from the excited singlet state of the primary donor ($P^*$), via the monomer Bchl ($B_A$) to the Bph ($H_A$) bound to the L subunit. From $H_A$, the electron is transferred to the primary quinone, $Q_A$, which is bound
undergo change in its ionization state, depending on how close the pK_a is to the prevailing pH. This results in substoichiometric proton binding, e.g., H^+/Q_b^- < 1, that is distributed over several residues and pH-dependent.\textsuperscript{112,113} Net proton uptake and internal redistribution of H^+ between ionizable residues contribute substantially to the partial shielding and stabilization of the semiquinones. The difference in net H^+ uptake associated with Q_A^- and Q_B^- formation is the major determinant of the relative redox midpoint potentials (E_m) of semiquinone states, and is what sets the first electron transfer equilibrium in favor of Q_A^-Q_B^-, i.e., the “forward” direction, at physiological pH. At high pH the ET equilibrium decreases and favors Q_A^-Q_B^- at pH > 11.

The detailed origin of this behavior resides in the structure of the Q_B domain, which features an unusually high density of ionizable residues with a striking excess of acidic groups (Figure 10). The electrostatic interactions in this domain are therefore very complex and have been reviewed recently,\textsuperscript{100} and will be revisited here only to the extent necessary to discuss essential features of proton transfer.

Although site directed mutagenesis has suggested specific roles for some residues in governing the properties of the semiquinone states, Q_A^- or Q_B^-, computational studies have been an important source of insight and caution. In response to the appearance of a negative charge on Q_B^-, the most prominent acidic residues in the Q_B domain - Asp\textsuperscript{L210}, Glu\textsuperscript{L212}, Asp\textsuperscript{L213}, Asp\textsuperscript{M17} and Glu\textsuperscript{H173} - experience significant pK_a changes. Depending on the pH, H^+ ions taken up are mostly distributed among these residues, but also with many small contributions from more weakly coupled residues. The responses to Q_A^- and to Q_B^- largely arise from the same cast of characters in the Q_B domain.\textsuperscript{114,115} This reflects the dearth of ionizable residues around Q_A^-, which translates into a low effective dielectric that allows the electric field from Q_A^- to spread further.

Glu\textsuperscript{L212} appears to have an unusually high pK_a ≈ 8.5 in the ground state, and this increases by a further 1 or 1.5 pH units upon appearance of Q_A^- or Q_B^-, respectively.\textsuperscript{112,114} It is therefore expected to be fully protonated at neutral pH, but it becomes ionized at pH ≥ 8.5 and is then the major site of light induced proton uptake. The nominal rate

\begin{center}
\begin{tabular}{cccc}
3 ps & 1 ps & 0.2 ns & 10-100 µs \\
\end{tabular}
\end{center}

\textbf{B.} The acceptor quinone complex of the Rba. sphaeroides reaction center. Q_A (green) and Q_B (cyan) are bound around an iron-histidine ligand complex (two histidines, L230 and M266, are omitted). The view is from within the membrane plane, similar to part \textit{A}. The two quinone binding sites are similar and are related by the pseudo-2-fold rotational axis of the reaction center. Q_B is shown in its “proximal” position. Not all contact residues are shown, but both sites are predominantly non- or weakly polar, except for Glu\textsuperscript{L212} and Asp\textsuperscript{L213} in the Q_B site. Hydrophobic residues are shown in yellow (Ala\textsuperscript{M240}, Ala\textsuperscript{M249}, Ala\textsuperscript{M260}, Ile\textsuperscript{M266}, Ile\textsuperscript{M224}, Ile\textsuperscript{L229}). (Main chain atoms only are shown for M259 (asparagine) and M261 (threonine) – the side chains do not contact the quinone headgroup.) Each quinone is hydrogen bonded through its C4 carbonyl to a histidine (NH) and through the C1 carbonyl to a backbone amide (NH). In the semiquinone anion form, Q_B^- is also hydrogen bonded by Ser\textsuperscript{L223} (O\text{H}) (dotted green line). Coordinates from 1dv3.pdb.
constant of the first ET, \( k_{AB}^{(1)} \), is pH independent below pH 8.5, but slows down progressively at higher pH, and approaches a “well behaved” slope of \(-1\) for log \( k_{AB}^{(1)} \) vs pH \(116\text{-}118 \). This has generally been interpreted as due to the need to (re)protonate Glu\(^{L212}\), with raised pK\(_a\) \( \geq 10 \) in the presence of Q\(_B\). As described above, the full identity of the ionized species is uncertain, but mutating Glu\(^{L212}\) to Gln (mutant L212EQ) abolishes the pH dependence \(117,118 \) and decreases H\(^+\)/Q\(_A\) and H\(^+\)/Q\(_B\) to almost zero at pH \( \geq 8 \), \(119,120 \) and it is convenient and simple to refer to this residue as the responsible party.

Most calculations yield the above result for Glu\(^{L212}\), but FTIR experiments strongly suggest that Glu\(^{L212}\) is only partially protonated at lower pH, and picks up protons in the Q\(_B\) state over a wide pH range.\(^{115,121\text{-}126} \) Calculations also show that at neutral pH, Asp\(^{L213}\) and/or Asp\(^{L210}\) are deprotonated in the ground state, but Asp\(^{L213}\), at least, becomes fully protonated in the Q\(_B\) state \(127 \). This is thought to be coupled to a mechanistically important structural change in which Ser\(^{L223}\)(O\(_H\)) is H-bonded to Asp\(^{L213}\)(-CO\(_2\)) in the ground state (neutral Q\(_B\)), but rotates to hydrogen bond with the C\(_1\)–O\(_\gamma\) of Q\(_B\) in the Q\(_B\) state.\(^{100,115,127\text{-}129} \) In the process, Asp\(^{L213}\) is protonated.

Note that the pH dependence of \( k_{AB}^{(1)} \) in the wild type does not imply the process is PT limited, and the pH independence in the L212EQ mutant does not mean that it is not. The mutant result, however, does make it clear that H\(^+\) uptake is not rate limiting for ET to Q\(_B\). Related to this point, H\(^+\) uptake in response to the immediate formation of P\(^+\)Q\(_A\) (with no ET to Q\(_B\)) was found to be rate-limited by some, presumed conformational, process.\(^{130} \)

It is noteworthy that, although the kinetics of electron and coupled proton transfer at the Q\(_B\) site have been subject to much study, the rate determining process for the first electron transfer is still uncertain, and is generally thought to be neither electron nor proton transfer per se.\(^{131} \) The kinetics are not simple exponential and can be considered either polyphasic\(^{131\text{-}134} \) or genuinely dispersive.\(^{135} \) The major rate components are insensitive to the size of the ET driving force (\( \Delta E_m = E_m(Q_A) - E_m(Q_B) \)), varied by using artificial quinones as Q\(_A\) but native Q-10 as Q\(_B\).\(^{131,136} \) The insensitivity indicates that ET is not rate limiting. However, when \( \Delta E_m \) is large, Gunner and coworkers have observed a fast phase that is responsive to the driving force.\(^{134,137} \) This is consistent with ET from Q\(_A\) directly to Q\(_B\) in an “unprepared” state, which subsequently relaxes, for example due to structural changes and/or proton uptake.\(^{25,100} \)

On-going studies by Paddock et al. have detected ENDOR signals in the Q\(_B\) spectrum that correspond to proton coupling to Ser\(^{L223}\)(O\(_H\)), and have correlated these with the lifetime of the P\(^+\)Q\(_B\) state, as an indicator of the stability of Q\(_B\). Their results certainly implicate this residue in providing much of the stabilization that “gates” the ET from Q\(_A\). When P\(^+\)Q\(_B\) is prepared at room temperature and frozen in the light, it is stable for at least a year at 100 K.\(^{138,139} \) When prepared at low temperature, however, it decays in a few seconds – at least 10\(^7\) times faster.\(^{140,1} \) In a mutant with Ser\(^{L223}\) replaced by alanine, the charge separated state frozen in the light is much less stable. Tentatively, this is interpreted as indicating that the rotation of Ser\(^{L223}\)(O\(_H\)) to engage the Q\(_B\) carbonyl

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\(^6\) Mutations are designated by the subunit and residue number, followed by the wild type residue letter, and then the mutant residue letter. Thus, the single mutant Glu\(^{L212\text{-}\rightarrow}\)Gln is given as L212EQ.

\(^4\) Normal ET from Q\(_A\) to Q\(_B\) is blocked at low temperature in samples frozen in the dark. However, P\(^+\)Q\(_B\) can be prepared at low temperature in mutant RCs in which the B-side chain of cofactors (see Fig 8) has been made active and the normal A-side has been inactivated by a number of mutations, and in which Q\(_A\) is lacking.\(^{119} \) Yields of approx 15% P\(^+\)Q\(_B\) per flash can be obtained.
oxygen is required to stabilize the electron on $Q_B^-$ and is the rate limiting step in the 1st ET. However, this does not rule out proton transfer ($H^+$ redistribution) as a factor in this event, since the strongly stabilized state can only be generated in the wild type at room temperature.

### 7.2 Second Electron Transfer and Coupled Proton Transfer to $Q_B$ (Chemical Protons)

Full reduction of $Q_B$ on the second turnover is coupled with the delivery of two protons to the quinone head group, to form $QH_2$. As Figure 9 shows, the acceptor quinone cycle branches at this point, with two main mechanistic possibilities (a concerted process is a third possibility). In the upper branch, the second electron is transferred to yield the dianion, $Q_B^{2-}$, followed by uptake of the first proton to form $Q_BH^-$. In the lower branch, the first proton is taken up to give the protonated semiquinone, $Q_BH$, followed by transfer of the second electron to form $Q_BH^-$. In both scenarios, the second proton is taken up following formation of $Q_BH^-$. 

**Figure 9** The Acceptor Quinone Cycle. Following photoactivation, the first electron is shared between the two quinones. The negative charges of the anionic semiquinones induce proton uptake to the protein, contributing to the partial shielding and stabilization of the semiquinones. Followin a second photoactivation, full reduction of $Q_B$ is coupled with the delivery of two protons to the quinone head group, to form $QH_2$, which unbinds and is replaced by an oxidized quinone. Two possible routes are shown for the proton-coupled second electron transfer - the lower path (PT/ET) is the active one (see text).
Both paths begin with an unfavorable step followed by a highly favorable one. For the upper path (ET/PT), the midpoint potential of the Qb\textsuperscript{2+/−} couple is not well established, but I have estimated it to be about −200 mV (vs. NHE), although Zhu and Gunner estimated a somewhat lower value of about −360 mV\textsuperscript{114}. In either case, the electron transfer is significantly uphill from Q\textsubscript{A}\textsuperscript{−}, with \( E_m \approx -45 \text{ mV} \).\textsuperscript{142} It is also likely to be severely constrained by a large reorganization energy reflecting the substantial charge redistribution.\textsuperscript{100} However, the \( pK_a \) of Qb\textsuperscript{2+} (the second \( pK_a \) of the quinol) is expected to be high and protonation very favorable, thereby pulling the equilibrium through to QbH\textsuperscript{+}.

The lower route (PT/ET) in Figure 9 proceeds via the unfavorable protonation of Qb\textsuperscript{2−} unfavorable because the \( pK_a \) of the semiquinone is low. The reference values for this species are reasonably well established in the literature with a few studies on ubisemiquinone (Q-10), although not in water. Two \( pK_a \) values have been reported - 6.45 in methanol\textsuperscript{143} and 5.9 in 7 M isopropanol/1 M acetone.\textsuperscript{144} From the known solvent (dielectric) effect on the \( pK_a \) of durosemiquinone, one can estimate an aqueous value of \( pK_a = 4.9\pm0.1 \) for ubisemiquinone.

In RCs, we might expect the Qb semiquinone \( pK_a \) to be lower due to the local capacity to accommodate a single negative charge. In fact, experimentally, no indication of a \( pK_a \) has been detected - the Qb (and Q\textsubscript{A}) semiquinone is anionic at all accessible pH, even as low as pH 3.5 (R. R. Stein, Ph.D. thesis, University of Illinois, 1985). If the \( pK_a \) of Qb\textsubscript{H}/Qb\textsuperscript{−} were indeed lowered due to electrostatic stabilization of the anion, one should expect a quantitatively similar effect on the midpoint potential. The \( E_m \) of Qb\textsuperscript{2−}/Qb\textsuperscript{−} can be estimated at +30 mV in isolated RCs\textsuperscript{100,145} (it is about +100 mV in chromatophores\textsuperscript{146}), which is 170 mV higher than my estimate of the \( E_m \) for Q-10 in aqueous solution (\( E_m = -140 \text{ mV} \), C.A.W. unpublished – but see\textsuperscript{141}). This shift is consistent with a positive potential in the Qb binding site, but may be somewhat too large.

In fact, for the second electron transfer, the relevant \( pK_a \) for Qb\textsuperscript{2+} is in the state Q\textsubscript{A}\textsuperscript{−}Qb\textsuperscript{2+}, where the influence of the Q\textsubscript{A}\textsuperscript{−} anion is likely to raise the \( pK_a \) of Qb\textsuperscript{2+}. This is a reasonable expectation in view of the influence that Q\textsubscript{A}\textsuperscript{−} has on the \( pK_a \) values of amino acids in the Qb domain. It is also supported by results with rhodoquinone acting as Qb, which suggest the \( pK_a \) increases by about 0.7 units upon formation of Q\textsubscript{A}\textsuperscript{−}Qb\textsuperscript{2+}.

In the lower path of Figure 9, following protonation of Qb\textsuperscript{−}, the second electron transfer is very favorable, involving the relatively high potential redox couple, Qb\textsubscript{H}/Qb\textsuperscript{H+}, with \( E_m \approx +240 \text{ mV} \). This would pull the reaction to completion in spite of the unfavorable, initial protonation.

The distinction between the two possible routes for the second electron transfer and coupled proton uptake was made by Graige et al., who used artificial quinones as Q\textsubscript{A} to alter the driving force for the ET without changing the properties (\( pK_a \) or \( E_m \)) of Qb\textsubscript{2+}.\textsuperscript{136} This “driving force assay” showed clearly that the observed rate, \( k_{AB}^{(2)} \), was responsive to the ET driving force, indicating that the reaction was ET limited. A full analysis showed the results to be compatible only with the PT/ET sequence, with rate limiting ET preceded by fast PT equilibrium. Thus, the observed reaction \( Q\textsubscript{A}Qb\textsuperscript{−} \rightarrow Q\textsubscript{A}Qb\textsuperscript{H+} \) proceeds with a rate given by:

\[
k_{AB}^{(2)} = k_{ET}^{(2)} f(Qb\textsubscript{H}) \tag{10}
\]
The measured rate is pH dependent because the population of $Q_B^-$ is pH dependent. In the simplest case, one would expect the pH dependence to follow the Henderson-Hasselbalch equation: 

$$f(Q_B^-H) = \frac{10^{pK_a - pH}}{1 + 10^{pK_a - pH}}.$$ 

However, the complex electrostatics of the protein interior do not allow such a simple response, but result in a $pK_a$ that is pH-dependent with an operational $pK_a \approx 4.5$ at pH 7.5 (see\textsuperscript{100,147}). The essential mechanism seems well founded and is summarized in Scheme 4.

\begin{equation}
\begin{array}{c}
\text{Q}_A\text{Q}_B^-
\end{array}
\end{equation}

\begin{equation}
\begin{array}{c}
\text{Q}_A\text{Q}_B^- \quad \text{pK}_a(Q_B^-) \quad k_{ET} \quad \Delta G_{ET} \\
\text{Q}_A\text{Q}_B^H \\
\Delta Q_{AB}^2
\end{array}
\end{equation}

\text{(Scheme 4)}

7.3 Proton transfer to the $Q_B$ pocket

An important result of the mechanism of Scheme 4 for the 2nd ET is the implied rate of proton transfer to $Q_B$. In the wild type, the observed rate, $k_{AB}^{(2)}$, is about $3 \times 10^3$ s$^{-1}$ at pH 7.0. If the $pK_a$ for $Q_A Q_B^−/Q_A Q_B H$ is 4.5, this corresponds to $k_{ET}^{(2)} = 10^6$ s$^{-1}$, as estimated by Graige et al. (1999). Since this is rate limiting, the PT equilibrium must be established faster than $10^7$ s$^{-1}$. The speed of equilibration is the sum of forward and reverse rates, $k_{eq} = k_{on} - k_{off}$. In solution, this can be readily satisfied by a low $pK_a$, i.e., $k_{off} = k_{on}10^{-pK_a}$; so, for $k_{on} = 10^{10} - 10^{11}$ M$^{-1}$ s$^{-1}$, $k_{eq} \approx k_{off} = 10^7$ s$^{-1}$ when $pK_a = 3 - 4$. In the RC, however, equilibration with the bulk pH must fully span the protein between $Q_B$ and the surface, which does raise the question of how it is achieved and how it maintains substantial robustness in the face of possible mutational lesions.

Because the terminal points are well established on the quinone, the second electron transfer-coupled proton delivery is somewhat better defined than the protonation events on the first electron transfer, although the intervening pathway itself is subject to the same complex interactions. The general flavor of the pathway is shown in Figure 10, derived from studies on mutants and on the serendipitous finding that certain divalent transition metal ions, which bind in the vicinity of a histidine cluster on the surface of the H subunit,\textsuperscript{148-151} dramatically inhibit proton entry.\textsuperscript{151,152}

7.4 Outline of the PT elements in the $Q_B$ domain

The effects on the 2nd ET of site directed mutations in the immediate vicinity of $Q_B$ have identified Asp$^{1213}$ and Ser$^{1223}$ as critical for the delivery of the first proton to C$_1$-O, and Glu$^{1212}$ as essential for delivery of the second proton to C$_4$-O.\textsuperscript{117,118} Glu$^{H173}$ was also found to have a powerful influence on both proton deliveries.\textsuperscript{155}
Figure 10 Proton transfer pathway to $Q_B$. Side chains only are shown, and the prenyl tail of $Q_B$ is truncated. Residues His$^{H126}$, His$^{H128}$ and Asp$^{M17}$ are at the surface of the protein. The path from the bulk phase to Asp$^{L213}$ is shared by both protons delivered to $Q_B$. Accompanying the 1st electron to $Q_B$, Glu$^{L212}$ becomes fully protonated and subsequently donates the 2nd $H^+$ to $Q_B^-$, after the 2nd electron. The 1st $H^+$ is delivered in the $Q_A^-Q_B^-$ state via Ser$^{L223}$, prior to the 2nd electron transfer – see text. Water molecules (green) fill some but not all gaps in the putative $H^+$ pathway. Different positions are occupied in other structures indicating the possibility of water dynamics in the proton delivery. (Coordinates from 1aig.pdb). (Figure prepared in VMD using perspective display mode. Glu$^{H173}$ and Asp$^{M17}$ are shown dimmed to accentuate their positions relatively in the background.)

No other single mutants have dramatic effects on the proton-coupled ETs to $Q_B$, but it is important to remember that substantial change in PT kinetics may be necessary before any readily observable effect can be seen on ET. For example, second site revertants have lent some qualification to the conclusion of absolute functional requirement for all of these residues. In many cases, the rate of the 2nd ET in suppressor mutants is still 10-100 smaller than in the wild type. However, when the primary mutation is PT limited, even quite feeble restoration of the observed ET kinetics, if no longer PT limited, indicates several orders of magnitude restoration of the PT rates.

Rather minor changes in the behavior of two single mutants, Asp$^{L210}$→Asn and Asp$^{M17}$→Asn, led Paddock and coworkers to make the double mutant Asp$^{L210}$→Asn + Asp$^{M17}$→Asn (mutant L210DN/M17DN), which was dramatically inhibited. The 2nd ET rate constant was inhibited by more than 200-fold ($k_{AB}^{(2)} \approx 10 \text{ s}^{-1}$) and was insensitive to the driving force for ET (the $\Delta E_m$ between $Q_A$ and $Q_B$), i.e., the reaction is PT limited.
Since, in the wild type, PT is faster than ET and estimated to be at least \(10^7\) s\(^{-1}\), the implication is that PT in this mutant is decreased by a factor of \(10^6\) or more.

Inhibition of the 2\(^{nd}\) ET in the L213DN mutant is even more dramatic, with the observed rate as slow as 0.1 s\(^{-1}\).\(^{153}\) The rate is not ET limited and the inhibition of PT is therefore apparently on the order of \(10^8\) fold.\(^{160,164}\) In contrast, in H173EQ mutant RCs, which exhibit observed rates similar to those seen in the L210DN/M17DN double mutant,\(^{155}\) the rate was modified in the driving force assay,\(^{102}\) indicating that it is ET limited and that PT is still fast. This mutant may be marginal with respect to its PT capabilities, but it is at least roughly described by Equation 10, implying an altered \(pK_a\) value for \(Q_B^{-}\). This illustrates how misleading unqualified reaction rates can be. Thus, if one wishes to study the proton transfer events through the lens of the coupled ET, it is important that the PT limited nature of the process be established clearly.

Finally, several divalent transition metal ions are now known to inhibit proton uptake and delivery to \(Q_B\).\(^{151,165,166}\) The binding site for these ions has been identified by crystallography,\(^{150}\) and also by EPR methods to be on the surface of the H-subunit.\(^{148,149}\) Depending on the identity of the metal, the ligands include one or both of two histidine residues (H126 and H128) and one of two aspartic acid residues (H124 or M17). The extent of inhibition is significant, although not as great as for the H173EQ mutant, but \(k_{AB}^{(2)}\) is not responsive to the ET driving force so the kinetics are clearly PT limited. Identification of the metal ion binding site led to construction of a site directed mutant lacking both histidines - His\(^{H126}\)→Ala + His\(^{H128}\)→Ala, referred to as mutant 2xHis in the original work.\(^{167}\) This mutant was not inhibited at pH ≤ 7 but it was progressively slowed at pH > 7.\(^{152}\) The inhibition of the 2\(^{nd}\) ET was only 4 fold, even at pH 8.5, but the driving force assay showed it to be PT limited, as for the metal-bound wild type, which means that PT had been very substantially slowed down.

### 7.5 The Proton Conduction Pathway to \(Q_B\) and the \(Q_B\) Domain

The results from metal ion binding and the 2xHis mutant establish a unique entry point for H\(^+\) ions that is active for proton delivery to internal protein residues on the 1\(^{st}\) ET and to \(Q_B\) on the 2\(^{nd}\) ET.\(^{151,152,165}\) At pH ≥ 8, the inhibition of proton entry by divalent metal ions inhibits the 1\(^{st}\) ET in WT but not in L212EQ mutant RCs,\(^{151}\) indicating that proton uptake on the first turnover leads to protonation (neutralization) of Glu\(^{L212}\), which is required to favor the ET equilibrium. As Glu\(^{L212}\) was titrated, RCs in which the residue was already protonated were not dependent on light induced H\(^+\) uptake.

On the other hand, Paddock et al.\(^{165}\) found that the 2\(^{nd}\) ET was greatly inhibited by divalent metal ions, showing that the first H\(^+\) delivered to \(Q_B^{-}\) in the \(Q_A\) state came along the same pathway. The second H\(^+\) to \(Q_B\) is considered to be delivered internally, from Glu\(^{L212}\) protonated on the first turnover. Thus, the Pt path is common to both protons, with a bifurcation near Asp\(^{L213}\) that allows delivery to Glu\(^{L212}\), on the one hand, and \(Q_B\) on the other.

With the identification of the surface histidines as the metal binding site, the major impact of mutating Glu\(^{H173}\), Asp\(^{L210}\) and Asp\(^{M17}\), and the terminal residues near \(Q_B\) (Glu\(^{L212}\), Asp\(^{L213}\) and Ser\(^{L223}\)), a proton conduction path is roughly mapped out (Figure 10). However, it is noteworthy that these residues do not define a continuous path, and significant gaps exist in the crystal structures. Although some gaps are bridged by water molecules in some structures, the appearance of water molecules does not seem to be a
simple matter of resolution and structure refinement. More likely they are transient entities that are relatively stabilized in some states or mutants and less so in others.

7.6 Investigations of Intraprotein PT Coupled to the First and Second ETs

The nature of the PT coupled to the 1st ET has been studied in the 2xHis mutant, at pH 8.5 where PT is rate limiting. Paddock and colleagues found that the inhibition of the 1st ET that is evident in this mutant at elevated pH is reversed by a wide variety of cationic buffers, with $pK_a$ ranging from 2 to 11 – the mutant RCs were “rescued.” From the slope of the rate of ET vs. buffer concentration, a second order rate constant ($k_2$) was obtained for the protonated buffer, and a plot of $\log k_2$ vs. buffer $pK_a$ gave the result expected for a “normal” acid. The rate constant reached diffusion controlled values (approx. $10^{10}$ M$^{-1}$ s$^{-1}$) when the buffer $pK_a \approx 2$. In a simple solution PT between an acid and a base, this would indicate the $pK_a$ of the acceptor (base) (see Section 6 above). This is clearly too low to correspond to the expected terminal acceptor (Glu$^{L212}$ with $pK_a \approx 8.5$), which prompted the inclusion in the model of an intermediate. This is very reasonable in view of the known structure. The existence of an intermediate changes the interpretation of the limiting $pK_a$ value, which now reflects an acceptor $pK_a$ of about 4. This is fully consistent with the carboxylic acids - for example, Asp$^{H24}$ or Asp$^{M17}$ - immediately beneath the presumed cation binding site (where the two histidines (H126 and H128) would be in wild type RCs).

Paddock et al. used a steady state kinetic analysis similar to that employed widely in enzyme kinetics. From this, they obtained a steady state maximum rate (effectively $k_{cat}$) of $10^5$ s$^{-1}$ for imidazole, as a close analogue of the native histidine. The system is underdetermined but reasonable estimates translate to an elementary PT rate constant for transfer from imidazole to the intermediate (uphill by 5 pK units) of $10^6$ s$^{-1}$ and from the intermediate to Glu$^{L212}$ (energetically highly favorable) of $10^{10}$ s$^{-1}$.

In the L213DN mutant, which has the most severely inhibited 2nd ET of any known mutant, Takahashi and Wright observed partial rescue by weak neutral acids, including azide, and speculated that these agents act as proton carriers through the mutational block - as had been suggested for the effect of azide on the Asp$^{S98}$$\rightarrow$Asn mutant of bacteriorhodopsin. Subsequently, we found that H173EQ mutant RCs, also with severely inhibited 2nd ET, could be fully rescued by azide, and noted that the effect could be due to the anion binding and restoring a functional $pK_a$ that had been depressed by the loss of charge in the Glu$^{H173}$$\rightarrow$Gln mutation. Given the evident strength and complexity of the interactions within the acid cluster, distinguishing between these two modes of action is difficult. Recently, we have examined the effect of weak acids on the 2nd ET using the PT-limited L210DN/M17DN double mutant.

The L210DN/M17DN double mutant is readily rescued by weak neutral acids, including azide, acetate, nitrite, formate, bicarbonate, fluoride, and phosphate. The plot of $\log k_2$ for the neutral acids vs. $pK_a$ was linear with a slope of $-1$ (Figure 11), although azide was more effective and acetate was less effective than predicted by the linear fit (both with $pK_a \approx 4.75$). Results with other buffers suggested a size limitation on the activity of the weak acids, and a probable exclusion of cationic species.

Notably, the maximum level of restoration of activity did not correlate simply with the acid $pK_a$, e.g., high concentrations of azide ($pK_a \approx 4.72$) restored $k_{cat}$ to almost wild
Figure 11  Dependence on pK\textsubscript{a} of the second order rate constant, $k_2$, for weak acids to rescue the second electron transfer in the L210DN/M17DN double mutant (Bronsted plot). Values for $k_2$ were obtained from the initial slope of the titration of measured rate ($k_{AB}^{(2)}$) as a function of total salt added, $A_T$, and converted to the free acid, [AH], using the acid pK\textsubscript{a} and the prevailing pH 7.0: $[AH] = A_T 10^{pK_{a}^{(1)}}/(1+10^{pK_{a}^{(1)}})$. Acids shown: phosphate (pK\textsubscript{a} = 2.15), fluoride (pK\textsubscript{a} = 3.16), nitrite (pK\textsubscript{a} = 3.46), formate (pK\textsubscript{a} = 3.72), bicarbonate (pK\textsubscript{a} = 3.58), azide (pK\textsubscript{a} = 4.72), acetate (pK\textsubscript{a} = 4.76), phosphate (pK\textsubscript{a} = 7.0), ammonium (pK\textsubscript{a} = 9.25). The point for ammonium is an upper limit and has been omitted from the fitted line (slope = -1.0). Points are added for H\textsubscript{3}O\textsuperscript{+} (pK\textsubscript{a} = -1.74) (triangle) and H\textsubscript{2}O (pK\textsubscript{a} = 15.74) (square) as potential donors, calculated for the inhibited rate (unrescued), with [H\textsubscript{3}O\textsuperscript{+}] = 10^{-7} M (pH 7.0) and [H\textsubscript{2}O] = 55.5 M. Dashed line indicates the diffusion limit at $k_{on} \approx 10^9$ s\textsuperscript{-1} (see text).

type values, but formate (pK\textsubscript{a} ≈ 3.72) restored it only to about 30%. This can be accounted for by considering the protonated (neutral acid) and deprotonated (anion) forms to bind competitively:

$$\begin{align*}
    & \quad A^{-} \cdot R^{-} \\
    K_I & \quad \uparrow \\
    & \quad R^{-} \\
    K_D & \quad k_{on} \leftrightarrow k_{off} \quad \downarrow \quad k_2 \\
    \quad \text{(Scheme 5)}
\end{align*}$$

$$\begin{align*}
    & \quad AH \cdot R^{-} \quad \xrightleftharpoons[k_{H^{-}}]{k_H} \quad A^{-} \cdot RH \quad \xrightarrow{k_{ET}} \\
    & \quad \xrightarrow{k_H} \\
\end{align*}$$

R' represents a group in the proton transfer pathway to Q\textsubscript{b} in the RC. $K_D$ and $K_I$ are the dissociation constants for the protonated (AH) and deprotonated (A\textsuperscript{-}) forms of the
rescuing acid - binding of AH and A⁻ are both very weak. \( k_{\text{on}} \approx 10^9 - 10^{10} \text{ M}^{-1} \text{ s}^{-1} \) and \( k_{\text{off}} \approx 10^7 - 10^{11} \text{ s}^{-1} \) are the on and off rates for acid (AH) binding and release. \( k_{\text{f}} \) and \( k_{\text{b}} \) are the rate constants for forward and backward proton transfer within the RC \( (k_{\text{f}}/k_{\text{b}} = K[H] = 10^{6\text{pK}}) \). \( k_{\text{ET}} \) is the rate constant for electron transfer to QₐH \( (k_{\text{ET}} \approx 10^6 \text{ s}^{-1} \) from estimates in wild type RCs - see above \(^{100,147}\)).

The data extrapolate to the maximum expected (diffusion controlled) rate of about \( 10^7 - 10^{10} \text{ M}^{-1} \text{ s}^{-1} \), and an acid pKₐ = 0-1. In the simplest case, this would be equal to the pKₐ of the acceptor species (R⁻), within the reaction center. However, the model predicts that the apparent pKₐ will be offset from the actual pKₐ of RH/R⁻ by log \( (k_{\text{ET}}/k_{\text{off}}) \), which is on the order of -4. Thus, the actual pKₐ of the acceptor group should be in the ballpark of 4-5. This is consistent with R⁻ being a carboxylic acid, but it could also be Qₐ⁻ itself, for which pKₐ \( \approx 4.5 \) has been estimated.

All rescuing acids shared similar features – small size, neutral acid form, very weak binding of the acid, and slightly stronger binding of the anion. This relative order of binding strengths could account for the relative inactivity of the cationic acids tested. In the absence of any added acids, the inhibited rate in this mutant could reflect the activity of H₂O⁺ as donor, with pKₐ = -1.74. At pH 7, the calculated bimolecular rate constant would be about 2x10⁵ \text{ M}^{-1} \text{ s}^{-1}. This is significantly low on the plot of Figure 11, but it would be consistent with a disfavored electrostatic environment for cations and might provide some rationalization for the existence of a PT pathway at all. The natural Qₐ site engineering appears to be primarily directed at stabilizing the anionic semiquinone. To the extent that this is achieved by a positive electrostatic potential, this would be incompatible with the equally important function of delivering H⁺ ions to Qₐ for full reduction. Thus, free access by H₂O⁺ is not a satisfactory solution, in spite of its strong proton donor potential, and the pathway is based on neutral and anionic carboxylic acid/carboxylate functions.

In addition, the very low concentration of H⁺ ions under physiological conditions necessitates some design functions to enhance the availability of the H⁺ from solution.\(^{171-174}\) The net charge of the protein is negative, which will enhance the concentration of H⁺ at the surface.\(^{130}\) However, the behavior of the 2xHis mutant indicates that this is insufficient and the cluster surface histidines, H126 and H128, and possibly H68, greatly enhance the availability of protons by providing a local reservoir. The functional pKₐ of residues involved in inhibitory metal binding appear to range from 5.9-7.4.\(^{175,176}\) This spread of values may indicate electrostatic interactions, which serve to widen the pH range over which at least one of them is protonated.

8 Concluding Remarks

The establishment of a unique H⁺ ion entry point and a defined pathway for proton delivery in RCs are worth discussing in terms of preconceptions about proteins and small molecules. It has been known for decades that proteins are not readily penetrated by small molecules, although it was a somewhat radical idea when first espoused.\(^{177,178}\) It became clear from studies of myoglobin that even very weakly polar (CO) or apolar (O₂) molecules could not readily reach the heme binding site except through a specific pathway that was designed for flexibility and access.\(^{179,180}\) On the other hand, there are also good indications that water does penetrate into the surface layers of proteins, contributing, among other things, to the non-homogeneous dielectric properties of proteins in a functionally important way.\(^{181-186}\) From the latter, it seemed possible that the
highly polar nature of the $Q_B$ domain could permit proton delivery in a rather non-specific fashion and that no single pathway would be dominant. Water chains, identified in the crystal structures, were suggested as possible proton pathways, but attempts to disrupt these did not result in effects that could be ascribed to proton transfer rates or equilibria. This also seemed to lend some support to a distributed proton delivery network.

However, the identification of the surface histidine site as a unique entry point that serves both the protein residues of the $Q_B$ domain and $Q_B$, itself, shows the pathway to be narrowly defined. Indeed, one can estimate that all other possible routes combined are at least $10^3$-times less effective than the identified path. This will not surprise anyone who is thinking in terms of proton pumps, where a unique gate must control the flow and backflow of protons across the membrane, but it is not a necessary design property for the non-transmembrane proton traffic in cofactor oxidation and reduction. It suggests, perhaps, that the requirements for an effective proton pathway, at least of significant length, are not so easily satisfied. This might include maintaining buried structures of sufficient polarity, in a generic sense, and of establishing specific $pK_a$ values that are accessible to physiological pHs while sufficiently active in internal proton transfer, as well as designing surface properties that can aid in proton collection and injection into the protein.

Having established a functional proton transfer pathway, however, some manner of robustness can be expected from the fact that proton transfer between “normal” acids is intrinsically fast and can be non-rate-limiting over a wide range of $pK_a$ values. In the case of proton-coupled electron transfer in the reaction center acceptor quinone complex, proton transfer must be substantially impaired before it is even noticeable. Of course, loss of an active proton carrier, like Asp, by mutation to a non-ionizable residue can be catastrophic, but the net rate is quite tolerant of significant departures in the $pK_a$ values of the functional proton carriers, due, for example, to mutations in the electrostatic environment. In apparent contrast, alterations in the $pK_a$ of the $Q_B$ semiquinone, as is probably the case in the H173EQ mutant, have direct and large magnitude effect. The difference is that the population of $Q_B$H, which is determined by its $pK_a$, is the multiplier of the rate limiting electron transfer rate constant. A change in the $pK_a$ of an intermediate carrier, however, has its impact on a rate process that is likely to be very fast (pairwise proton transfer).

In a sense, PT pathways may be robust for the same reason as are ET processes – the intervening medium is designed with overkill. For ET, the protein is treated as a homogeneous medium and distances are kept quite short, not pushing the envelope of maximum reach. For PT, the spacing and small scale mobility of the functional groups are sufficient to make the intrinsic rates very fast and the net rate relatively insensitive to the details ($pK_a$ values) of the design.

**Acknowledgements**

Work from the author’s laboratory was supported by the National Science Foundation (MCB 03-44449) and National Institutes of Health (GM 53508).
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