Small Weak Acids Reactivate Proton Transfer in Reaction Centers from *Rhodobacter sphaeroides* Mutated at Asp<sup>L210</sup> and Asp<sup>M17</sup><sub>*</sub>

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In reaction centers of *Rhodobacter sphaeroides*, site-directed mutagenesis has implicated several acidic residues in the delivery of protons to the secondary quinone (Q<sub>b</sub>) during reduction to quinol. In a double mutant (Asp<sub>L210</sub> → Asn + Asp<sub>M17</sub> → Asn) that is severely impaired in proton transfer capability over a wide pH range, proton transfer was “rescued” by added weak acids. For low pK<sub>a</sub> acids the total concentration of salt required near neutral pH was high. The ionic strength effect of added salts stimulated the rate limit, and saturated concentrations of acid did not correlate with pK<sub>a</sub> values of other residues, is not straightforward.

In *Rhodobacter sphaeroides*, reducing equivalents are stored in the double reduction of the secondary ubiquinone, Q<sub>b</sub>, via the primary quinone, Q<sub>a</sub>, and quinol is released into the membrane after two light-activated turnovers of the RC (2, 3). Each turnover results in transfer of an electron to the quinones from the primary donor, P, a special pair of bacteriochlorophylls (4–7). The oxidized primary donor, P<sup>+</sup>, is reduced by a secondary donor after each photoactivation, and the events in the acceptor quinone complex can be summarized as (8–10) in Scheme 1. The proton stoichiometric factors, a, b, etc., indicate the variable influence that the different quinone states have on nearby ionizable amino acid residues (10–14).

The RC quinones are well buried in the protein, and proton transfer to Q<sub>b</sub>, which accompanies the second electron transfer to form Q<sub>b</sub>H<sub>2</sub>, must extend over a distance of 13–15 Å. The delivery pathway has been partially mapped out by site-directed mutagenesis (reviewed in Refs. 9, 10, and 15) and involves several members of a large cluster of ionizable, predominantly acidic, residues that have been identified by inspection of the x-ray structures (Fig. 1) (16, 17). Some are identifiable as likely proton carriers from their proximity to Q<sub>b</sub> in the structure, and there is general agreement that Glu<sub>H173</sub> (18–20), Asp<sub>M17</sub> (19, 21), and Ser<sub>1</sub> (22, 23), in the L subunit, are terminal members of the pathway. However, in other cases, distinguishing a true proton-carrying role for an amino acid from a less active function, such as setting the local electrostatic potential and pK<sub>a</sub> values of other residues, is not straightforward.

In addition to the terminal members of the pathway, other residues have been identified by site-directed mutagenesis to have highly significant influences on proton-coupled electron transfer to Q<sub>b</sub>. These include Glu<sub>H173</sub> (24), His<sub>H126</sub> and His<sub>H128</sub> in the H subunit (25), Asp<sub>M17</sub> in the M subunit, and Asp<sub<L210</sub> (26–28), Arg<sub>L107</sub>, and Arg<sub>L127</sub> in the L subunit (29).

In mutant RCs with the mutation Asp<sub>L213</sub> → Asn (mutant L213DN), the second electron transfer rate was inhibited by at least 10<sup>3</sup>-fold at pH > 7.21. The proton transfer rate was even more strongly inhibited, because it is not rate-limiting in the wild-type but is rate-limiting in the mutant, leading to estimates that proton transfer is inhibited by ≥10<sup>3</sup>-fold in the mutant (30). Interestingly, the mutant RC activity could be partially reactivated ("rescued") by small weak acid anions, such as azide, N<sub>3</sub> (31). In RCs with the mutation Glu<sub>H173</sub> → Gln (mutant H173EQ), the second electron transfer was also strongly inhibited but could be fully rescued by azide (24). However, it was not clear in these cases if the recovery was due to a proton delivery function of the added acid species, N<sub>3</sub>H (31), or an electrostatic effect of the bound anion, N<sub>3</sub> (24), as has been proposed for the mechanism of many second site revertants to this and other primary lesions (32–36).

Paddock and coworkers have studied RCs mutated at a putative H<sup>+</sup> entry site on the protein surface, which are substantially impaired in proton uptake at elevated pH (25, 37). The mutation neutralized two surface histidine residues, His<sub>H126</sub> and His<sub>H128</sub>, that had been identified in the binding site region of certain divalent transition metal ions that effectively blocked proton uptake (38, 39). Both the first and the second
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The RC complementation vector, pLMX415, contains pufL, -M, and -X but lacks pufB and -A, which were eliminated using an engineered BamHI restriction site located upstream of pufB, and a naturally occurring BssHII site, located downstream of pufA. The codon changes for L210DN/M17DN double mutation were introduced using a protocol based on the QuikChange mutagenesis method (Stratagene, La Jolla, CA).

Cells were initially grown aerobically in the dark in 2-liter flasks placed in a rotary shaker (320 rpm) with 400 ml of Sistrom’s medium supplemented with 10% Luria Broth (42) and in the presence of 2 μg/ml tetracycline. Cultures were then allowed to develop full pigmentation under semi-aerobic conditions with the addition of 800 ml of Sistrom’s medium (43) containing yeast extract (0.4%) and tetracycline (2 μg/ml), with 200 rpm agitation. RCs were isolated by detergent fractionation of the membranes with 0.7% lauryl (dodecyl) dimethylamine-N-oxide (LDAO) (Fluka), followed by ammonium sulfate precipitation, and column purification using DEAE-Sephacel (Sigma). The RCs were washed extensively on the column with 60 and 80 mM NaCl (in 0.06% LDAO, 10 mM Tris, pH 8.0), with short, additional washes at 100–140 mM NaCl, and then eluted from the column with 180–200 mM NaCl. Isolated RCs typically displayed a 2:\text{max}/\text{A}_{450} ratio of 1.25–1.35.

All kinetics assays were performed on samples with 1–2 μM RC, 0.02% LDAO, 40 μM ubiquinone-10 (Q-10) (Sigma), and 1 mM each of the following buffers, MES, MOPS, Tricine, CHES, and CAPS. The kinetics of the first electron transfer, \( Q_A^{-} \rightarrow Q_A^{+} \), were measured at 397 nm in the absence of donor to \( P^+ \), and 32 kinetic traces were averaged to achieve the desired signal-to-noise ratio. This wavelength (397 nm) corresponds to a distinctive electrochromic effect of \( Q_A^{-} \) on the nearby bacteriopheophytin and is close to isosbestic for \( P/P^+ \). The kinetics were fit to a single exponential representing the first electron transfer, \( k_{AB}^{(1)} \). This adequately describes the major component, although the spectral response is known to be more complex and still poorly understood (44–46). The kinetics of the second electron transfer, \( Q_A^{-} \rightarrow Q_A^{+} \), were measured at 450 nm, following the second of two short flashes 0.5 s apart, in the presence of ferrocene as a donor to \( P^+ \), as previously described (12). The kinetics were analyzed by a two-component exponential fit, representing the second electron transfer, \( k_{AB}^{(2)} \), and the re-reduction of \( P^+ \) by the exogenous donor, ferrocene, which is seen at the same wavelength. Because the observed electron transfer rate varied widely with experimental conditions, the ferrocene concentration was adjusted to optimally separate the \( Q_A^{-} \rightarrow Q_A^{+} \) electron transfer kinetics from those of \( P/P^+ \) re-reduction. At 2–4 μM ferrocene, \( P^+ \) was reduced with a halftime of ~200 ms, and at 150–200 μM ferrocene the \( P^+ \) re-reduction halftime was ~2 ms. The second electron transfer kinetics were well fit by a single component. All kinetic measurements were made at 21 °C.

RESULTS

The Second Electron Transfer, \( Q_A^{-} \rightarrow Q_A^{+} \) — The second electron transfer, with rate constant \( k_{AB}^{(2)} \), is substantially (>99%) inhibited in L210DN/M17DN mutant RCs (28). We measured \( k_{AB}^{(2)} \approx 14 \text{ s}^{-1} \), compared with 1600 s\(^{-1}\) in wild-type, in low salt (2.5 mM KCl) at pH 7.0. However, the rate in the mutant RCs was greatly enhanced by addition of various salts of weak acids. Because the concentrations required were rather high, we examined the influence of ionic strength using salts of strong acids.

Salt Dependence — To test the effect of weak acid salts independently of any ionic effect, our interest was to find a regime in which the ionic response was minimal. We initially tested various symmetrical univalent and divalent electrolytes, alone or in combination, but found
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FIGURE 2. Salt and pH dependence of the rate of the second electron transfer to Qb (k_AB (2)) in L210DN/M17DN mutant RCs, determined from kinetics measured at 450 nm. Main figure: salt dependence (Na2SO4 added as indicated) at various pH. Inset: pH dependence of k_AB (2) in 2.5 mM KCl (closed circles) and in 150 mM Na2SO4 (open squares). Conditions: 1–2 μM RCs in 1 mM each of MES, MOPS, Tricine, CHE, and CAPS buffers, 2.5 mM KCl, 40 μM Q-10, 0.02% Triton X-100, 4 or 200 μM ferrocene (depending on rate of k_AB (2)).

Na2SO4 to give the simplest behavior. The Na2SO4 concentration dependence of the measured rate, k_AB (2), is shown in Fig. 2 for pH values from 5.5 to 8.5. At acidic pH, the rate was quite sensitive to salt, e.g. at pH 5.5, it increased 4–5 fold as the concentration of Na2SO4 was raised from 0 to 100 mM. At pH 7.0 and 7.5 the effect of salt was minimal and, after a small increase at concentrations below 20 mM, the rate was almost constant up to 600 mM Na2SO4. Addition of 300 mM NaCl on top of 600 mM Na2SO4 had no significant effect. At pH 8 and higher, the rate actually decreased with added salt. Over the same concentration range, the rate in wild-type reaction centers was relatively much less sensitive, e.g. it increased from 1600 to 2000 s⁻¹ at pH 7.0 (not shown).

The pH dependence of k_AB (2) in L210DN/M17DN mutant RCs, in the presence and absence of 150 mM Na2SO4, is shown in the inset to Fig. 2. At pH values below 7.0 the rate was significantly enhanced by added salt, whereas above pH 7.5 addition of salt decreased the rate. At pH 7.0–7.5, the rate was largely unresponsive to salt, and 150 mM Na2SO4, pH 7.0, was chosen as the standard condition for testing the activity of weak acid salts; under these conditions, NaCl was titrated on top of the 150 mM Na2SO4 (see Fig. 5A).

Stimulation of k_AB (2) by Weak Acid Salts—In contrast to the slight effect of NaCl (up to 600 mM), added on top of 150 mM Na2SO4, addition of similar concentrations of various, weak monoprotic acids caused a marked acceleration (Fig. 3). The most effective of these was azide (N₃⁻), which was able to restore a significant fraction of the wild-type rate, at sufficiently high concentrations (>1 mM). Several other weak acids significantly accelerated the rate, and titrations for formate, fluoride, and phosphate are also shown in Fig. 3 (the small effect of NaCl has been subtracted in all panels). A smaller number of experiments were done with 300 mM Na2SO4 as the background salt, with identical results.

The stimulation by weak acid salts was from the initial slope of the salt concentration dependence, yielding a second order rate constant in terms of total salt added (k(2)). The small effect of NaCl was subtracted, and this slope was multiplied by (1 + 10⁻³⁶ pKₐ), where pKₐ is the pKₐ of the added weak acid, to express k(2) in terms of the concentration of the protonated form [AH]. On this basis, the efficacy of the acids was clearly correlated with their solution pKₐ values (see Fig. 6, below), indicating that the protonated weak acid was responsible for overcoming a rate-limiting step in the proton transfer pathway within the RC. However, for those acids that showed curvature, indicating saturation of their effect on the measured rate, k_AB (2), the extrapolated maximum rate did not correlate with the acid pKₐ. For example, several acids with lower pKₐ than azide (pKₐ = 4.72) gave significantly smaller rates at apparent saturation (see, e.g. Fig. 3).

pH Dependence of the Second Order Rate Constant—To strengthen the justification for expressing the second order rate constant in terms of the protonated acid form, we determined k(2) over the usable pH range, 6.5–7.5. At higher pH the rates were too slow for reliable measurement and at lower pH the rate was already quite fast and was more sensitive to the purely ionic effect of added salts. For those acids tested, the second order rate constant, when based on the concentration of the AH species, was independent of pH (Fig. 4).

The results for all weak acids that were tested are shown in Table 1. Included are three cationic acids, triazole (pKₐ = 2.3) and pyridine (pKₐ = 5.4), which were ineffective, and ammonium (pKₐ = 9.3), which was very weakly effective in stimulating k_AB (2), and two dibasic acids, oxalate (pKₐ = 1.3 and 4.2) and phosphate (pKₐ = 2.15 and 7.0). Oxalate gave very weak stimulation, whereas phosphate was effective (see Fig. 3). Nitrite (pKₐ = 3.45) and cyanate (pKₐ = 3.45) gave significant stimulation at low concentrations, from which a value of k(2) could be determined, but were less effective at high concentrations (cyanate) or even inhibitory (nitrite). Bicarbonate (pKₐ = 3.68) could only be tested at low concentrations (up to 20 mM) due to instability of the pH and bubble formation from released CO₂. Cyanide (pKₐ = 7.0) severely and irreversibly inhibited RC activity and appeared to destroy the acceptor quinone complex.

Effect of Cd²⁺ Ions on the Second Electron Transfer Reactivated by Weak Acids—Various divalent transition metal ions have been found to substantially inhibit proton uptake by reaction centers, thereby inhibiting the observed electron transfer (47–50). We tested the effect of cadmium on RCs of the L210DN/M17DN double mutant in the presence of weak acids to reanimate the second electron transfer to varying degrees.
Cadmium had no effect on the measured rate in unrescued L210DN/M17DN mutant RCs or when reactivated with 100 mM azide or 100 mM fluoride (Fig. 5A). However, Cd$^{2+}$/H11001 did partially inhibit the rate when reactivated by 100 mM formate (Fig. 5B). The kinetics were monophasic at all concentrations of Cd$^{2+}$, and slowed progressively as the cadmium concentration was raised. This indicates that the unbinding of metal ion is substantially faster than the electron transfer rate in the unbound state, i.e. $k_{\text{off}} > 80$ s$^{-1}$. Half inhibition occurred at 100 $\mu$M, with a maximum effect of $\sim$45% inhibition (after correction for the cadmium-insensitive baseline rate of the double mutant RCs). The same degree of inhibition was seen in the initial slope (i.e. $k_{\text{(2)}}$), when formate was titrated in the presence of 400 $\mu$M Cd$^{2+}$.

In wild-type RCs, in low salt, subsaturating concentrations of Cd$^{2+}$ induce biphasic kinetics of the second electron transfer, reflecting titration of the site and slow unbinding of the metal ion, and the slow phase amplitude has been used to quantify the binding of metal ion (49, 50). In the presence of 150 mM Na$_2$SO$_4$, however, the affinity was weaker and we found that separation of the two components was less reliable. To ensure that the monophasic kinetic analysis of the mutant reflected the same properties as the assay of slow phase amplitude in the wild-type, we compared the two assays in wild-type RCs. In low salt (2.5 mM KCl), the dissociation constant, $K_d$, was 2 $\mu$M when assayed by slow phase amplitude, while retardation of the single component (average) rate constant gave $K_d = 3$ $\mu$M. In 150 mM Na$_2$SO$_4$, the two assays gave dissociation constants of 8 and 10 $\mu$M, respectively (data not shown). Thus, we expect the value obtained for the double mutant ($K_d = 100$ $\mu$M) to be a reliable estimate of the affinity for Cd$^{2+}$, which is about 10-fold weaker than in wild-type RCs.

The First Electron Transfer, $Q_A^- Q_B^+ \rightarrow Q_A Q_B^-$ — The first electron transfer to $Q_B$ is weakly coupled to proton transfer, with H$^+$ uptake driven by relatively small changes in the p$K_a$ values of many ionizable residues in and around the quinone binding sites (for review, see Ref. 10). In RCs of the L210DN/M17DN double mutant, the rate of the first electron transfer, $k_{\text{AB}}$, is also greatly inhibited in comparison to the
The isoelectric point for the whole RC has been reported as pI 7.0–7.5 is a clear indicator that this is an ionic effect that reflects the surface charge of the RC, with a functional isoelectric point in this range. The existence of a cross-over point at pH 7.0 (data not shown). We observed a very similar response of wild-type (28), we measured $k_{AB}^{\text{AP}} = 320 \text{ s}^{-1}$ versus 7000 s$^{-1}$, in low salt, at pH 7.0 (data not shown). We observed a very similar response of $k_{AB}^{\text{AP}}$ to added weak acids, with similar values of $k_{AB}^{\text{AP}}$ calculated from the initial slopes of the concentration dependence. This work will be presented in more detail in a subsequent paper.

**DISCUSSION**

**Salt Effects**—The effect of salts like NaCl and Na$_2$SO$_4$ on $k_{AB}^{\text{AP}}$ in RCs of the L210DN/M17DN double mutant is substantial but qualitatively different at low and high pH, accelerating with increased salt at low pH and decelerating at high pH. The existence of a cross-over point at pH 7.0–7.5 is a clear indicator that this is an ionic effect that reflects the surface charge of the RC, with a functional isoelectric point in this range. The isoelectric point for the whole RC has been reported as pI = 6.1, for R. sphaeroides, strain R26 (51), but a discrepancy between this net pI and the cross-over pH for $k_{AB}^{\text{AP}}$ is not surprising. Proton uptake and transfer coupled to Q$_B$ reduction is likely to be under the electrostatic influence of a sub-domain of the cytoplasmic surface rather than the global potential. Furthermore, the mutation of two aspartic acids in the L210DN/M17DN double mutant is expected to raise the pI of the mutant RCs compared with wild-type. The isoelectric points for proteins can often be estimated from the amino acid composition and the N and C termini. Using standard pK$_a$ values, and correcting for the presence of the iron atom (Fe$^{2+}$) and the non-ionizability of the histidine ligands to the iron and to the bacteriochlorophylls, we used the Biology Workbench Protein Tools facility (workbench.sdsc.edu/) to calculate the pI. The value obtained was identical to the measured value (6.1). Although such good agreement is undoubtedly partly fortuitous, it gives confidence to calculations of other pI values. The pI of the cytoplasmic domain of the RC was calculated using only the cytoplasmic loops of the L and M subunits and the globular domain of the H subunit, with the same constraints for

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**FIGURE 4. pH independence of $k_{AB}^{\text{AP}}$.** For each acid, $k_{AB}^{\text{AP}}$ was determined from the initial slope of the titration of rate versus total salt added, $A_r$, as in Fig. 3, and converted to the free acid, [AH], using the acid pK$_a$, and the prevailing pH: $k_{AB}^{\text{AP}} = k_{AB}^{\text{AP}}(1 + 10^{	ext{pH} - pK_a})$. Conditions: as for Fig. 2, with 150 mM Na$_2$SO$_4$.

**FIGURE 5. Influence of cadmium (Cd$^{2+}$) on the rescue activity of weak acids.** A, reactivation of $k_{AB}^{\text{AP}}$ by fluoride in the presence (open circles) and absence (closed circles) of 800 μM CdCl$_2$. Curve through data points is derived from the Model B described in the Supplementary Materials, with the parameters given in Table 2. Lower curve is for the salt response, NaCl added on top of 150 mM Na$_2$SO$_4$. B, Cd$^{2+}$ titration of $k_{AB}^{\text{AP}}$ partially rescued by 100 mM formate. Conditions for both panels are as those for Fig. 2, with 150 mM Na$_2$SO$_4$, pH 7.0.

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**TABLE 1**

| Acid/salt$^a$ | pK$_a$ | $k_{AB}^{\text{AP}}$ | $k_{AB}^{\text{AP}}$ | $k_{AB}^{\text{AP}}$ |
|--------------|--------|----------------------|----------------------|----------------------|
|              |        | $k_{AB}^{\text{AP}}$ | $k_{AB}^{\text{AP}}$ | $k_{AB}^{\text{AP}}$ |
| Nitrite      | 3.37   | 1615                 | 6.90 × 10$^6$        |                      |
| Cyanate      | 3.46   | 1685                 | 5.85 × 10$^6$        |                      |
| Bicarbonate  | 3.58   | 805                  | 2.12 × 10$^6$        |                      |
| Formate      | 3.72   | 675                  | 1.29 × 10$^6$        |                      |
| Oxalate (1)  | 2.15   | 715                  | 5.07 × 10$^6$        |                      |
| Phosphate (1)| 2.3    | (−)$^b$              |                      |                      |
| Oxalate (2)  | 4.15   | (2)$^b$              |                      |                      |
| Azide        | 4.72   | 4420                 | 8.43 × 10$^5$        |                      |
| Acetate      | 4.76   | 265                  | 4.61 × 10$^4$        |                      |
| Phosphate (2)| 7.00   | 715                  | 7.15 × 10$^3$        |                      |
| Ammonium     | 9.25   | 15                   | 1.50 × 10$^1$        |                      |
| Water        | 15.74  | 22                   | 2.20 × 10$^4$        |                      |

$^a$ (1) and (2) indicate first and second ionization equilibria (phosphate and oxalate). $^b$ $k_{AB}^{\text{AP}}$ values are from experimental initial slopes, corrected for the NaCl control (85 M$^{-1}$ s$^{-1}$).

$^c$ $k_{AB}^{\text{AP}} = k_{AB}^{\text{AP}}(1 + 10^{	ext{pH} - pK_a})$.

$^d$ The rates with oxalate could not be considered reliably above the NaCl control.

$^e$ The rates with triazole and pyridine were less than for the NaCl control.

$^f$ The rates with oxalate could not be considered reliably above the NaCl control.

$^g$ The rates with triazole and pyridine were less than for the NaCl control.
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the iron ligands. The calculated pls were 8.0 for wild-type RCs and 8.7 for the L210/M17 double mutant. These values are consistent with the “cis positive rule” for membrane proteins, whereby a positively charged cytoplasmic domain is expected, complementing the negative polarity of the cytoplasmic compartment (52, 53). However, they are clearly out of the range indicated by the cross-over pH for \( k_{AB}^{(2)} \), further indicating a more local nature of the electrostatic domain influencing the proton-coupled electron transfer. In the wild-type, with Asp\(^{172} \) and Asp\(^{210} \) both ionized, this domain will be much more negative, with a lower pl.

The response of \( k_{AB}^{(2)} \) for the L210DN/M17DN mutant RCs to the bulk phase ionic conditions is as expected from the pH dependence of the reaction, which slows progressively as the pH is raised (28). The mutations make proton transfer rate-limiting, and any enhancement or depression of proton delivery will affect the measured electron transfer rate. At low pH, when the relevant surface area is positively charged, the depression of proton delivery will affect the measured electron transfer rate. Conversely, at higher pH, above the local (effective) pl, the protein is net negatively charged and the pH at the surface will be lower than in the bulk phase. Addition of salt to screen the surface charge (and possibly change it by weak adsorption) will diminish the surface potential and allow higher concentrations of

\[
\frac{1}{k_{AB}} = \frac{1}{k_{AB}^{(0)}} + \frac{1}{k_{AB}^{(0)}} - 1.01
\]

indicating that neither the neutral acid nor the monoanion were significantly active. On the other hand, phosphate was active, and reasonable \( k_{AB}^{(0)} \) values could be calculated for either the neutral or monoanion as active donor (discussed further, below).

We tested a variety of acids for stimulatory activity, and found that only a limited selection were indisputably active. These were all small and monoprotic, with the exception of phosphate. Acetic acid was the largest acid that showed sufficient activity to be clearly discriminated above the residual ionic effect. Oxalate exhibited a very marginal ability to rescue \( k_{AB}^{(2)} \), indicating that neither the neutral acid nor the monoanion were significantly active. On the other hand, phosphate was active, and reasonable \( k_{AB}^{(0)} \) values could be calculated for either the neutral or monoanion as active donor (discussed further, below).

Categorizing tested acids as active was done conservatively, because the involvement of the \( k_{AB}^{(0)} \) value in determining both the concentration and the intrinsic reactivity of the protonated form presents a strong bias toward apparent activity. Even a tiny enhancement of \( k_{AB}^{(2)} \) above the control rate with NaCl can translate into a significant datum in the double logarithmic representation of a Brønsted plot (log \( k_{AB}^{(2)} \) versus \( k_{AB}^{(2)} \)). The potential for an artifactual influence of the pKa of sodium was demonstrated (54), and showed that the second order rate constant, based on the concentration of protonated acid species, was pH independent. If the active species were the salt (anion), the correction to protonated species would generate an artificial pH dependence, with a slope of \(-1\) for log \( k_{AB}^{(2)} \) versus pH. We therefore conclude that the observed rate enhancement is a genuine “rescue” effect of the protonated form of the weak acid.
effects of weak acids were substantial and could be determined with confidence. The data used for our analysis and for the linear fit of the Brønsted plot are therefore limited to those acids that were indisputably active, for which the uncorrected rate enhancement \( k_{\text{ET}} \) (the initial slope with respect to total salt concentration) was at least 5-fold greater than the NaCl control.

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p_{K_a} \text{ Dependence of Recovery—A Brønsted plot (log } k_{\text{ET}} \text{ versus } p_{K_a} \text{) for all active acids was linear (Fig. 6). Although the } p_{K_a} \text{ range was limited by the availability of suitable materials, small, neutral weak acids, it was sufficient to clearly indicate a slope very close to } -1 \text{ (the line drawn in Fig. 6 is a least squares fit to only the most conservatively active acids). This is strongly indicative of a direct role for the weak acids as proton donors to the mutant RCs. However, there were some apparent anomalies in the data. In particular, azide and acetate have almost identical solution } p_{K_a} \text{ values, but azide was significantly more active and acetate somewhat less active than predicted by the simple linear relationship. This suggested that size might be a limiting factor and may explain the failure of some candidate acids to exhibit activity (see below).}
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For a simple process of proton-coupled electron transfer, once the proton donor has sufficient acidity for the proton transfer to be non-rate-limiting, one expects the maximum electron transfer rate (at saturating concentrations of acid) to approach a common value that is independent of the acid concentration. However, the experimental concentration dependencies revealed a wide range of maximum rates at saturation that did not correlate with the acid concentration. For example, fluoride (p\(K_a\) = 3.16) and phosphate (p\(K_a\) = 2.15) were unable to fully restore the rate, or even to attain the same level as azide (p\(K_a\) = 4.72). However, this is readily accounted for if we allow for the anion (A\(^-\)) to interact at the same site as the acid (AH) species, i.e. as a competitive inhibitor.

**A Model for Weak Acid Rescue of Proton Transfer**—Scheme 2 shows a kinetic diagram for the binding of weak acids and functional proton donation coupled to the second electron transfer to QbH \((Q_{\alpha}^- Q_{\beta}^+ \leftrightarrow Q_{\alpha}^- Q_{\beta}^+ H \rightarrow Q_{\alpha}^- Q_{\beta}^+ H^+)\). A\(^-\) represents a key group in the proton transfer pathway to QbH in the RC. \(K_{D} \) and \(K_{A} \) are the dissociation constants for binding of the protonated (AH) and deprotonated (A\(^-\)) forms of the acid, \(k_{on} \) and \(k_{off} \) are the on and off rates for acid (AH) binding and release.

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k_{on} \text{ and } k_{-1} \text{ are the rate constants for forward and backward proton transfer within the RC, and } k_{off}/k_{on} = K_{D} = 10^{(p_{K_a}-p_{K_a}^*)} = 10^{p_{K_a}^*}, \text{ where } p_{K_a}^* \text{ and } p_{K_a} \text{ are the } p_{K_a} \text{ values for the exogenous weak acid, AH, and the endogenous intermediate, RH, respectively. } k_{ET} \text{ is the rate constant for electron transfer to QbH and is determined by the free energy drop in the purely electron transfer step, } Q_{\alpha}^- Q_{\beta}^+ H \rightarrow Q_{\alpha}^- Q_{\beta}^+ H^+, \text{ and by the distance between the two quinones (see, e.g. Ref. 56). It has been estimated at } \sim 10^6 \text{ s}^{-1} \text{ in wild-type RCs} \text{ (57, 58) and is largely unaffected by conditions and mutations that induce significant electrostatic changes in the acid cluster of the Qb domain} \text{ (28, 30). We therefore take the wild-type value of } k_{ET} = 10^6 \text{ s}^{-1}. \text{ In fact, } k_{ET} \text{, as used here, could include proton transfer events between RH and QbH, but, so long as the proton transfer from RH is fast and not unfavorable, the effective rate constant approaches the true } k_{ET}. \text{ Alternatively, } R \text{ could be QbH itself.}
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The data fits by the model show that AH and A\(^-\) both bind very weakly, with \(K_{D} \) and \(K_{A} \) in the molar range, so \(k_{on} \) and \(k_{off} \) are of the same order. This is superficially apparent from the concentration range of the rate enhancement. Also, the observed kinetics of electron transfer were monophasic at all concentrations of rescuing acid, indicating that the binding equilibrium is fast (say, 10-times greater) compared with the step that is rate-limiting when proton transfer is restored, i.e. electron transfer, with \(k_{ET} \approx 10^6 \text{ s}^{-1}. \text{ The equilibration rate for binding is the sum of on and off rates. Thus, fast equilibrium will be established for}
\]

\[
\text{Scheme 2}
\]

\[
\begin{align*}
A^- \cdot R^- & \quad \text{\(K_I\)} \quad \uparrow \quad R^- \\
& \quad \text{\(k_{on}\)} \quad \uparrow \quad \text{\(k_{off}\)} \\
AH \cdot R^- & \quad \text{\(k_{H}\)} \quad \downarrow \quad A^- \cdot RH \\
& \quad \text{\(k_{HT}\)} \\
k_{ET} & \\
\end{align*}
\]

The available data did not reach the maximum rate of \(10^6 \text{ M}^{-1} \text{ s}^{-1} \) expected for the diffusion limit, but extrapolation to this limit indicates that such a value would be reached for \(p_{K_a} \approx 1 \text{ M}. \text{ This is well borne out by the experimental data, which extend over a range of } p_{K_a} \text{ from 2 to 9. Because of the potential for systematic bias for acids with extreme } p_{K_a} \text{ values (discussed above), the linear fit in Fig. 6 is only to the data between } p_{K_a} = 2-5 \text{, where the measured rate enhancements were 10- to 50-fold greater than the NaCl control. The fitted slope in this range is } -1.0.
\]

The available data did not reach the maximum rate of \(10^6 \text{ M}^{-1} \text{ s}^{-1} \) expected for the diffusion limit, but extrapolation to this limit indicates that such a value would be reached for \(p_{K_a} \approx 1 \text{ M}. \text{ In the simplest case, this would be equal to the } p_{K_a} \text{ of the acceptor species, } R^-, \text{ within the RC, i.e. } p_{K_a} = p_{K_a} \text{. However, the role of } R \text{ as an intervening proton carrier between the donor (AH) and the assay function (electron transfer) leads to an offset of the apparent } p_{K_a} \text{ from the true } p_{K_a} \text{ of RH/RH}. \text{ The offset is equal to log } (k_{ET}/k_{off}), \text{ which is } -3. \text{ Thus, the actual } p_{K_a} \text{ of the acceptor group should be in the ballpark of 4. This is consistent with } R^- \text{ being a carboxylic acid, but it could also be QbH itself, for which a } p_{K_a} \text{ value of 4.5 has been estimated (10, 58). In fitting the data, we have used } p_{K_a} = 4.3 \text{. This value does not enter into the assessment of } k_{(2)} \text{. The}
\]
precise value of $pK_a$ influences the magnitude of the binding affinities, $K_D$ and $K_r$, but does not affect the qualitative conclusions to be drawn.

The variable maximum rate seen for different weak acids, independently of their $pK_a$ and efficacy as proton donors, arises from the competitive binding of the donor acid and inactive base. The two species are present in constant proportion at a fixed pH, and their relative affinities determine the maximum rate that can be recovered at saturating concentrations of added (total) salt (Equation 1) (see Supplementary Materials) as follows.

$$K_{obs}^{max} = K_D K_K / K_D K_K = K_D K_K = \frac{K_D}{K_D} \times 10^{pK_a - \text{pH}} \quad \text{(Eq. 1)}$$

Note that the pH dependence is identical in form to that expected for a simple model of the wild-type rate, where the relevant $pK_a$ is believed to be that for $Q_B^-$ (10, 58).

It is noteworthy that, although the protonated forms of the most effective donors (with $pK_a < 7$) are active at low concentrations, they all bind very weakly ($K_D \approx 1 \text{ m}$). This apparent paradox is due to the fast on and off rates (near the diffusion limit) that allow rapid, but low occupancy, binding equilibria to be established at the low concentrations of free acid prevailing at pH 7.0.

Also somewhat counterintuitively, for weak acids with $pK_a < 7$, the rescue activity titrates with saturation behavior characterized by the anion (inhibitor) dissociation constant, $K_D$, rather than the acid (donor) dissociation constant, $K_r$. This behavior is predicted by the model (see Supplementary Materials), and can be appreciated as follows. For such acids, at pH 7.0, the concentration of the protonated species is small and the range of concentrations is entirely in the linear region of the hyperbolic binding dependence. Conversely, the anion is the overwhelming species present, and the total salt concentration is very high. Thus, even for the weak binding affinities involved, the RCs become substantially occupied by $\text{A}^-$, and the concentration of free RC available to bind AH is progressively limited. The rapid binding equilibria of both AH and $\text{A}^-$ yields a rate of electron transfer that is proportional to the fraction of RCs with AH bound, which saturates at $K_D/K_{ET}$.

Competition for binding between donor acid and inactive base was not considered by Paddock et al. (37) in their study of acid rescue of the 2xHis mutant, where the donor site was at the protein surface. However, in the 2xHis mutant, only cationic acids were active donors, all with weak affinity for a negatively charged site, i.e., with Asp$^{pK_{a1}}$ and Asp$^{pK_{a2}}$ ionized. It is likely that the neutral bases bound much more weakly than the acid forms, in which case any effect of competition would be slight.

Properties of the Rescuing Species—In this work, with the L210DN/M17DN double mutant, almost all rescuing acids shared similar features: small size, neutral acid form, very weak binding of the acid, and slightly stronger (2- to 5-fold) binding of the anion. The possible exceptions are phosphate and oxalate (negative acid forms for the second $pK_a$) and ammonium (positive acid form), and a more general statement might be that the more negative (or less positive) species binds more strongly, and we now discuss these.

At least for the most active acids, the relative order of binding strengths (anion $> \text{acid}$) suggests a local environment that may favor a negative charge. Electrostatic calculations have indicated that the $Q_B$ domain in wild-type RCs is designed to accommodate approximately one extra negative charge, i.e., to stabilize the $Q_B$ semiquinone (and $Q_B\text{H}^+$) (10, 59). Furthermore, the protein appears to allow substantial ionization of the acid cluster near $Q_B$, including Asp$^{pK_{a1}}$ and Asp$^{pK_{a2}}$ (14, 16, 60, 61). Thus, the L210DN/M17DN double mutant should be at least as hospitable, and a preference for the anion species is consistent with this. We might, therefore, expect the monobasic forms of phosphate ($pK_a^{(1)} = 7.0$) and oxalate ($pK_a^{(2)} = 4.2$) to be effective rescuing acids, but this does not appear to be the case. For phosphate, the titrations can be fit for either acid/base pair with the parameters shown in Table 2. Both potential donors, $\text{H}_2\text{PO}_4^-$ ($pK_a^{(1)} = 2.15$) and $\text{H}_2\text{PO}_4^-$ ($pK_a^{(2)} = 7.0$), are predicted to give good activity, with relative binding affinities that favor the more negatively charged species. However, if both donor forms are indeed active, then the affinity for $\text{H}_2\text{PO}_4^-$ as a competitive inhibitor of $\text{H}_2\text{PO}_4^-$ ($K_D = 0.3 \text{ m}$) should be the same as its affinity as a proton donor ($K_D = 2 \text{ m}$). These values are sufficiently different as to be incompatible with this simple model. For oxalate, the distinction is dramatic, although the activity data are very weak in the first place. We conclude that, if oxalic acid is active at all, it is through the neutral diacid and is very weakly so, perhaps because of its size, which is larger than that of acetic acid (62, 63).

To account for the apparent lack of activity by anionic acids, we speculate that the binding site for all anions is subtly distinct from that for the neutral species. Thus, although binding of the conjugate base (anion) blocks the neutral acids from donating the proton, it does not position the anion to be able to donate if it is also an acid. Furthermore, the anion affinities are all quite similar ($K_D = 0.2$–$0.4 \text{ m}$), whereas the acid affinities vary more systematically with efficacy.

The relative binding affinities of the neutral acids and anionic bases suggest that cations bind even more weakly. This could account for the low or negligible activity of the cationic acids tested, e.g., no reliable activity could be detected for triazole ($pK_a = 2.3$) and pyridine ($pK_a = 5.4$), but size may also be a contributing, or even the overriding, prohibitive factor. Ammonium ($pK_a = 9.3$) was marginally active in terms of our conservative criterion for activity; it was only slightly greater than the general salt effect of NaCl. Because of the high p$K_a$, however, this was translated to an activity that placed it close to the Brensted line for the neutral acids. Also due to the high p$K_a$ of $\text{NH}_4^+$, the shape of the concentration dependence was determined by $K_D$, rather than $K_r$ (unlike the neutral acids with p$K_a < 7$), and the apparent affinity of the donor form ($\text{NH}_4^+$) was comparable with other acids ($K_D \approx 0.5$–$1 \text{ m}$). It was not possible to estimate $K_r$ except to say that it was not much smaller than $K_D$.

### TABLE 2

**Dissociation constants (molar) for donor acids and inhibitory bases (from fits to kinetic model B in Supplementary Materials)**

For each acid/base pair, $K_D$ corresponds to the less negative species, $K_r$ to the more negative species. $pK_a^{(1)}$ and $pK_a^{(2)}$ refer to first and second ionization constants, e.g., of phosphoric acid and oxalic acid.

| Charge Type | Charge | Acid | p$K_a$ | Formate | p$K_a$ | Fluoride | p$K_a$ | Phosphate | p$K_a^{(1)}$ | Phosphate | p$K_a^{(2)}$ | Acetate | p$K_a$ | Oxalate | p$K_a^{(1)}$ | Oxalate | p$K_a^{(2)}$ | NH$_4^+$ | p$K_a$ |
|-------------|--------|------|--------|---------|--------|---------|--------|-----------|-------------|-----------|-------------|--------|--------|--------|-------------|--------|-------------|--------|
| 1+          | 0      | 1.0  | 2.4    | 1.9     | 11     | 20      | 0.3    | 0.4       | (30)        | (0.3)     | (30)        | 0.5    | 9.25    |        |              |        |              |
| 1−          | 0.25   | 0.4  | 0.3    | 0.3     | (0.17) | 0.3     | 0.4    | 0.4       | (30)        | (0.3)     | (30)        |        |        |        |              |        |              |
| 2−          |        |      |        |         |        |         |        |           |             |           |             |        |        |        |              |        |              |

a Fits only limited $K_D$ to be $>0.05 \text{ m}$.

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3 The real behavior is not “simple,” because the p$K_a$ of bound $Q_B^-$ is pH-dependent, due to changes in the density and distribution of charges in the protein (Graige et al. (58) and Wraight (10)).
In the absence of any added acids, the inhibited rate in the mutant RCs (~20 s⁻¹) could reflect donor activity of H₂O⁺, with pKᵣ = -1.74, or H₂O, with pKᵣ = 15.74. For donation by H₂O⁺, at pH 7, the calculated second order rate constant from the single data point would be ~2 × 10⁻⁷ M⁻¹ s⁻¹. This is significantly lower than the values (10⁴–10¹¹ M⁻¹ s⁻¹) normally associated with aqueous proton diffusion.

Conversely, if we consider the unrescued rate of this mutant to be due to donation by H₂O at a concentration of 55 M, the estimated second order rate constant is too large by at least 6 orders of magnitude (see Fig. 6). The intrinsic donor ability of water is evidently so low that almost anything will do better, including H₂O⁺, and possibly even buffers, i.e. species we would consider “inactive.”

**Structural Implications**—The mutational lesion in the L210DN/M17DN double mutant is in the middle of the putative proton transfer pathway from the protein surface to Q₉ and obstructs proton delivery from the entry site (near the surface histidines) to the inner segment consisting of Asp¹²¹³, Ser¹²²³, and possibly one or more water molecules. To circumvent this block, the exogenous weak acids must either donate to one of these inner sites or find a novel route of access to Q₉⁻. In this regard the effect of cadmium is suggestive.

Various divalent transition metal ions have been found to substantially inhibit proton-coupled electron transfer and proton uptake by reaction centers (47–49, 64). The binding site for cadmium (Cd²⁺) was identified, by x-ray crystallography, to consist of His¹¹²⁶, His¹¹²⁸, and Asp¹¹³⁸ (38). Single mutation of either Asp⁴⁴⁷ or Asp¹²¹⁰ caused some decrease in affinity (10- and 4-fold, respectively, at pH 7.7 [27]). However, in addition to the inhibition with relatively high affinity, Cd²⁺ now showed a further slowing of the rate of the proton-coupled electron transfer that required 100-fold higher concentrations of metal ion. This was interpreted to mean that the observed reaction (kₐₑ [2]) seen with Cd²⁺ in the micromolar range proceeded by slow unbinding of the metal, allowing the proton and electron transfer reactions to occur in the uninhibited state. At higher concentrations of metal ion, rebinding of Cd²⁺ progressively diminished the lifetime of the metal-free RCs, so that the only possible electron transfer path was the much slower reaction of the metal-bound RCs. The limiting rate therefore represented the true rate of the proton-limited reaction in the single mutants (27).

In the L210DN/M17DN double mutant RCs, proton transfer is already fully inhibited and showed no additional sensitivity to Cd²⁺. Cadmium also had no effect on the partially recovered kinetics seen in the presence of azide and fluoride. This is consistent with findings in wild-type RCs, where azide could restore second electron transfer activity and fluoride. This is consistent with findings in wild-type RCs, where azide could restore second electron transfer activity.
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