In reaction centers of *Rhodobacter sphaeroides*, site-directed mutagenesis has implicated several acidic residues in the delivery of protons to the secondary quinone (Q_b) during reduction to quinol. In a double mutant (Asp^L210_→_Asn + Asp^M17_→_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_a acids, the total concentration of salt required near neutral pH was high. The ionic strength effect of added salts stimulated the rate of proton-coupled electron transfer at pH < 7, but decreased it at pH > 7.5, indicating an effective isoelectric point between these limits. In this region, a substantial rate enhancement by weak acids was clearly evident. A Bronsted plot of activity versus pK_a of the rescuing acids was linear, with a slope of -1, and extrapolated to a diffusion-limited rate at pK_a = 1. However, the maximum rate at saturating concentrations of acid did not correlate with pK_a, indicating that the acid and anion species compete for binding, both with weak affinity. This model predicts that pK_a represents a true pK_a = 4–5, similar to that for a carboxylic acid or Q_b itself. Only rather small, neutral acids were active, indicating a need to access a small internal volume, suggested to be a proton channel to the Q_b domain. However, the on-rates were near the diffusion limit. The implications for intraprotein proton transfer pathway design are discussed.

Light absorption by photosynthetic reaction centers (RCs) drives the formation of an electrochemical gradient of H^+ ions (protons) across the coupling membrane, the thylakoid membrane in chloroplasts and cell membrane in bacteria, and the generation of mobile reducing power. The transfer of electrons in the primary photosynthetic events generates most of the electrical component, whereas electron-coupled proton uptake and release accompanying the redox reactions of secondary donors and acceptors is largely responsible for the proton concentration gradient (ΔpH) (1).

In *Rhodobacter sphaeroides*, reducing equivalents are stored in the double reduction of the secondary ubiquinone, Q_b, via the primary quinone, Q_a, 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^+, 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_b, which accompanies the second electron transfer to form Q_aH_2, 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_b in the structure, and there is general agreement that Glu^L213^ (18–20), Asp^L213^ (19, 21), and Ser^L223^ (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_a 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_b. These include Glu^H173^ (24), His^H173^ and His^H178^ in the H subunit (25), Asp^M17^ in the M subunit, and Asp^L210^ (26–28), Arg^L207^, and Arg^L217^ in the L subunit (29).

In mutant RCs with the mutation Asp^L213_→_Asn (mutant L213DN), the second electron transfer rate was inhibited by at least 10^4-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 ≥10^4-fold in the mutant (30). Interestingly, the mutant RC activity could be partially reactivated ("rescued") by small weak acid anions, such as azide, N_3^- (31). In RCs with the mutation Glu^H173_→_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_3^- (31), or an electrostatic effect of the bound anion, N_3^- (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^+ 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^H126^ and His^H1128^, that had been identified in the binding site region of certain divergent transition metal ions that effectively blocked proton uptake (38, 39). Both the first and the second
Chemical Rescue of Proton-coupled Electron Transfer

The RC complementation vector, pLMX415, contains pufLM, -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 QuickChange 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) dimethylamino-N-oxide (LDAO) (Fluka), followed by ammonium sulfate precipitation, and column purification using DEAE-Sephaloc (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 A260/A680 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, QA → QH, 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 QA in the nearby bacteriopheophytin and is close to isosbestic for P/Q. The kinetics were fit to a single exponential representing the first electron transfer, k1(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, QA → QH, 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, k2(T), and the 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 QA → QH electron transfer kinetics from those of P re-reduction. At 2–4 μM ferrocene, P re-reduction was reduced with a half-time 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, QA → QH —The second electron transfer, with rate constant k2(T), is substantially (>99%) inhibited in L210DN/M17DN mutant RCs (28). We measured k2(T) ~ 14 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...
Chemical Rescue of Proton-coupled Electron Transfer

NaSO₄ to give the simplest behavior. The NaSO₄ concentration dependence of the measured rate, \( k_{AB}^{(2)} \), is shown in Fig. 2 for pH values from 5.5 to 8.5. At acid pH, the rate was quite sensitive to salt, e.g., at pH 5.5, it increased 4–5 fold as the concentration of NaSO₄ 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 NaSO₄. Addition of 300 mM NaCl on top of 600 mM NaSO₄ 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 NaSO₄, 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 NaSO₄, pH 7.0, was chosen as the standard condition for testing the activity of weak acid salts; under these conditions, \( k_{AB}^{(2)} = 23 \pm 2 \) s⁻¹. As a control for the effect of additional weak acid salts, most of which were monoprotic, NaCl was titrated on top of the 150 mM NaSO₄ (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 NaSO₄, addition of similar concentrations of various, weak monoprotic acids caused a marked acceleration (Fig. 3). The most effective of these was azide (\( N_3^- \)), 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 NaSO₄ as the background salt, with identical results.

The stimulation by weak acid salts was determined 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 \( (1 + 10^{pK_a-pK_a}) \), where \( pK_a \) is the \( pK_a \) of the added weak acid, to express \( k_{(2)}^{(2)} \) in terms of the concentration of the protonated form [AH]. On this basis, the efficacy of the acids was clearly correlated with their solution \( pK_a \) 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 \( pK_a \). For example, several acids with lower \( pK_a \) than azide (\( pK_a = 4.72 \)) 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 (\( pK_a = 2.3 \)) and pyridine (\( pK_a = 5.4 \), which were ineffective, and ammonium (\( pK_a = 9.3 \)), which was very weakly effective in stimulating \( k_{AB}^{(2)} \), and two dibasic acids, oxalate (\( pK_a = 1.3 \) and 4.2) and phosphate (\( pK_a = 2.15 \) and 7.0). Oxalate gave very weak stimulation, whereas phosphate was effective (see Fig. 3). Nitrite (\( pK_a = 3.45 \)) and cyanate (\( pK_a = 3.45 \)) 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 (\( pK_a = 3.58 \)) could only be tested at low concentrations (up to 20 mM) due to instability of the pH and bubble formation from released CO₂. Cyanide (\( pK_a = 7.0 \)) 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\(^{2+}\) 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. \(k_{off} > 80\) 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 (23 s\(^{-1}\)) of the double mutant RCs). The same degree of inhibition was seen in the initial slope (i.e. \(k_{(2)}\)), 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 \(pK_a\) 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, \(k_{AB}^{(1)}\), 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 cross-over point at pH 7.0 (data not shown). We observed a very similar response 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 rates with triazole and pyridine were less than for the NaCl control. The rates with oxalate could not be considered reliably above the NaCl control. Using standard pKₐ values, and correcting for the presence of the iron atom (Fe²⁺) and the non-ionizability of the histidine ligands to the iron atom 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

**TABLE 1**

<table>
<thead>
<tr>
<th>Acid/salt</th>
<th>pKₐ</th>
<th>k(1)</th>
<th>k(2)</th>
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<tr>
<td></td>
<td></td>
<td>μM⁻¹ s⁻¹</td>
<td>μM⁻¹ s⁻¹</td>
</tr>
<tr>
<td>Hydronium</td>
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<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Oxalate (1)</td>
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<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Phosphate (1)</td>
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<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Fluoride</td>
<td>2.37</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Nitrite</td>
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<td>0.40</td>
</tr>
<tr>
<td>Cyanate</td>
<td>2.58</td>
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<td>0.40</td>
</tr>
<tr>
<td>Bicarbonate</td>
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<td>0.40</td>
</tr>
<tr>
<td>Water</td>
<td>2.80</td>
<td>0.40</td>
<td>0.40</td>
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</tbody>
</table>

**FIGURE 4. pH Independence of k(AB)**

For each acid, k(AB) was determined from the initial slope of the titration of rate versus total salt added, A₇, as in Fig. 3, and converted to the free acid, [AH], using the acid pKₐ, and the prevailing pH: k(AB) = k(AB)¹ × (1 + 10pH-pKₐ). Conditions: as for Fig. 2, with 150 mM Na₃SO₄.

**DISCUSSION**

Salt Effects—The effect of salts like NaCl and Na₂SO₄ on k(AB) 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) is not surprising. Proton uptake and transfer coupled to Qₐ 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 pKₐ values, and correcting for the presence of the iron atom (Fe²⁺) and the non-ionizability of the histidine ligands to the iron atom 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

**FIGURE 5. Influence of cadmium (Cd²⁺) on the rescue activity of weak acids.** A, reactivation of k(AB) by fluoride in the presence (open circles) and absence (closed circles) of 800 μM Gd(³⁺). 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 Na₂SO₄. B, Cd²⁺ titration of k(AB) partially rescued by 100 mM formate. Conditions for both panels are as those for Fig. 2, with 150 mM Na₂SO₄ pH 7.0.
Chemical Rescue of Proton-coupled Electron Transfer

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 AspM17 and AspL210 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 shows 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 local surface pH will be higher than the bulk value. 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 H + at the surface, thereby enhancing the protonation state of all ionizable groups and stimulating the 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 will again screen the surface charge and diminish the magnitude of the surface potential, but in this case the potential is negative and the surface H + concentration will decrease with increasing ionic strength, resulting in a decrease in the proton limited electron transfer rate.

A related effect of salt, on H + uptake kinetics in wild-type RCs, was shown by Maróti and Wraith (54). Salts caused a substantial slowing of H + uptake by the $P^+Q_A^-$ state, i.e. in the absence of any rate-limiting electron transfer. High salt concentrations were required for this effect at alkaline pH, and this was taken to indicate a substantial charge density, qualitatively consistent with the large number of ionizable residues in the RC cytoplasmic domain.

In the related L210DA/M17DA double mutant (i.e. with Asp → Ala), Paddock and coworkers also reported a stimulation of the second elec-
dation (such as via ferrocenium or O2), further indicating a more local nature of the electrostatic domain influencing the proton-coupled electron transfer. In the wild-type, with AspM17 and AspL210 both ionized, this domain will be much more negative, with a lower pl.

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 was significantly active. On the other hand, phosphate was active, and reasonable $k_{AB}^{(2)}$ values could be calculated for either the neutral or monoanion as active donor (discussed further, below). Two cationic acids with seemingly suitable pKa values, triazole (pKa = 2.3) and pyridine (pKa = 5.4), gave no significant response at all, so the rate was actually less than at equivalent concentrations of NaCl. This is consistent with these species being predominantly neutral at pH 7.0.

Categorizing tested acids as active was done conservatively, because the involvement of the pKa 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 $k_{2}$ versus pKa). The potential for an artifactual influence of the pKa on the catalytic rate was therefore evaluated by plotting $k_{AB}^{(2)}$ versus pKa (Fig. 6). The effect of oxalic acid was also very small, with a slope barely greater than the NaCl control. However, with pKa = 1.3, and hence a very small concentration of protonated species, any finite difference from the background rate yields a datum that fits reasonably well on the trend line. Between these extremes, the

![Diagram](https://www.jbc.org/content/281/7/4418/F6)

**FIGURE 6. Relationship between the second order rate constant for rescue (log $k_{AB}$) and acid pKa (Brønsted plot).** Acid salts shown: phosphate (pK$_{H_2PO_4}$ = 2.15, pK$_{HPO_4^+}$ = 7.0), fluoride (pK$_F$ = 3.16), nitrite (pK$_N$ = 3.37), cyanate (pK$_C$ = 3.46), formate (pK$_H$ = 3.72), bicarbonate (pK$_B$ = 3.58), azide (pK$_A$ = 4.72), acetate (pK$_A$ = 4.76), and ammonium (pK$_N$ = 9.25). Dotted fine shows diffusion limit at $k_{cat} = 10^{-9}$ s$^{-1}$. The fitted line (slope = −1.01) excludes ammonium and the second pKa of phosphate (pK$_{H_2PO_4}$ = 7.0); see text. For ammonium, the open circle shows the largest experimental value of $k_{AB}$ obtained, and the arrow indicates the range (see text). $k_{AB}$ for each acid was determined as described in Fig. 4, using the acid pKa and the prevailing pH 7.0. For phosphate, fluoride, formate, azide, acetate, and ammonium, $k_{AB}$ values were also obtained by fitting the full titration curve, according to the kinetic model described in the text (closed circles). Points are also shown for H$_2$O$^+$ (pK$_H$ = 1.74) and H$_2$O (pK$_H$ = 15.74), calculated assuming concentrations of 10$^{-5}$ M and 55 M, respectively.

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Chemical Rescue of Proton-coupled Electron Transfer

\[
\begin{align*}
A^- \cdot R^- & \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad 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Chemical Rescue of Proton-coupled Electron Transfer

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, \( K_a \) corresponds to the less negative species, \( K_D \) 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.

<table>
<thead>
<tr>
<th>Charge type</th>
<th>Azide ( pK_a )</th>
<th>Formate ( pK_a )</th>
<th>Fluoride ( pK_a )</th>
<th>Phosphate ( pK_a^{(1)} )</th>
<th>Phosphate ( pK_a^{(2)} )</th>
<th>Acetate ( pK_a )</th>
<th>Oxalate ( pK_a^{(1)} )</th>
<th>Oxalate ( pK_a^{(2)} )</th>
<th>NH(_4^+) ( pK_a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+</td>
<td>0.95</td>
<td>2.4</td>
<td>1.8</td>
<td>1.9</td>
<td>(2)</td>
<td>11</td>
<td>20</td>
<td>(30)</td>
<td>0.5</td>
</tr>
<tr>
<td>1−</td>
<td>0.25</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
<td>(0.17)</td>
<td>0.3</td>
<td>0.4</td>
<td>(0.3)</td>
<td>—</td>
</tr>
<tr>
<td>2−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Fits only limited \( K_D \) to be >0.05 M.

precise value of \( pK_a^{(2)} \) influences the magnitude of the binding affinities, \( K_D \) and \( K_a \), 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), and can be appreciated as follows.

\[
k_{\text{obs}}^{\max} = \frac{k_D K_K}{K_D} = \frac{k_D}{K_D} K_K \times 10^{pK_a - pH} \tag{Eq. 1}
\]

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 \( Q_{BS}^{-} \) (10, 58).

It is noteworthy that, although the protonated forms of the most effective donors (with \( pK_a \ll 7 \)) are active at low concentrations, they all bind very weakly (\( K_D \approx 1 \mathrm{M} \)). This apparent paradox is due to the fast on and off rates (near the diffusion limit) that allow rapid, but low occupancy, binding equilibrium 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, \( K_D \). 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 \( A^- \) and the concentration of free RC available to bind AH is progressively limited. The rapid binding equilibria of both AH and \( A^- \) yields a rate of electron transfer that is proportional to the fraction of RCs with AH bound, which saturates at \( K/K_D \).

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\(_{M17}^{\ddagger}\) and Asp\(_{L210}^{\ddagger}\) 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 \( pK_a^{(2)} \)) 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 > 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 \( Q_B\mathrm{H}^{-}^- \)) (10, 59). Furthermore, the protein appears to allow substantial ionization of the acid cluster near \( Q_B^{-} \), including Asp\(_{210}^{\ddagger}\) and Asp\(_{147}^{\ddagger}\) (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 (\( pK_a^{(2)} = 7.0 \)) 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, \( H_2\mathrm{PO}_4^- \) (\( pK_a^{(1)} = 2.15 \)) and \( H_2\mathrm{PO}^-_4 \) (\( pK_a^{(2)} = 7.0 \)), 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 \( H_2\mathrm{PO}_4^- \) as a competitive inhibitor of \( H_2\mathrm{PO}_4^- \) (\( K_D = 0.3 \mathrm{M} \)) should be the same as its affinity as a proton donor (\( K_D = 2 \mathrm{M} \)). 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 (\( K_a = 0.2-0.4 \mathrm{M} \)), 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 \( pK_a \), however, this translated to an activity that placed it close to the Brensted line for the neutral acids. Also due to the high \( pK_a \) of \( \text{NH}_4^+ \), the shape of the concentration dependence was determined by \( K_D \) rather than \( K_a \) (unlike the neutral acids with \( pK_a < 7 \)), and the apparent affinity of the donor form (\( \text{NH}_4^+ \)) was comparable with other acids (\( K_a \approx 0.5-1 \mathrm{M} \)). It was not possible to estimate \( K_D \) except to say that it was not much smaller than \( K_D \).

\(^3\) The real behavior is not “simple,” because the \( pK_a \) of bound \( Q_{BS}^- \) is \( pH \)-dependent, due to changes in the density and distribution of charges in the protein (Graige et al. (58) and Wraith (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 pKₐ = −1.74, or H₂O, with pKₐ = 15.74. For donation by H₂O⁺, at pH 7, the calculated second order rate constant from the single data point would be −2 × 10⁻⁸ M⁻¹ s⁻¹. This is significantly lower than the values (10⁷−10¹¹ M⁻¹ s⁻¹) normally associated with aqueous proton diffusion.⁴

Conversely, if we consider the unrestrained 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 Qₐ and obstructs proton delivery from the entry site (near the surface histidines) to the inner segment consisting of Asp¹⁷⁻¹³, Ser¹²²⁻²³, 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 Qₐ⁻. 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 His¹¹⁰⁻¹¹, His¹²⁻²₀, and Asp¹¹¹⁻¹² (38). Single mutation of either Asp¹¹⁷ or Asp¹²⁰ caused some decrease in affinity (10⁻⁴-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 (kₐᵣᵢᵣ (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 without affecting the tight binding affinity of Cd²⁺ (49). However, when the kinetics were rescued by formate, Cd²⁺ diminished the rate by up to 40%, with a half inhibitory concentration of about 100 μM. We do not know for sure that the site of action of Cd²⁺ indicated here is the same as that identified by crystallography in wild-type RCs, but the low affinity is roughly consistent with the combined effect of the two single mutants (27) and a generally weakened binding due to the high salt concentration used in our work.

We tentatively suggest that these distinct responses to Cd²⁺ reflect the relative sizes of the acids involved, with the smaller ones (HN₃ and HF) readily able to penetrate past the site of cadmium blockage, while passage of formic acid is partially obstructed. Examination of the x-ray structure reveals this as a distinct possibility. Fig. 7 shows a view of the putative proton transfer pathway looking from the Qₐ site toward the protein surface. The contour representation of the surface residues shows a substantial hole through which a small molecule could readily access the putative proton transfer pathway, beginning with Asp¹¹⁻¹¹ (also shown in bond mode), and Pro¹¹²⁻¹ and Gly¹¹³ (in contour mode only). All other residues are buried, except His¹¹⁻¹. Residues are sized to indicate depth of field. The figure was prepared in VMD; structure file was 1dv3.pdb.

FIGURE 7. The putative proton transfer path in reaction centers from R. sphaeroides. The view looks down on the entrance. The white contoured surface encloses surface residues that define a hole sufficient for weak acid entry, Asp¹¹⁻¹¹, His¹¹⁻¹, and Asp¹¹⁻¹¹ (also shown in bond mode), and Pro¹¹²⁻¹ and Gly¹¹³ (in contour mode only). All other residues are buried, except His¹¹⁻¹. Residues are sized to indicate depth of field. The figure was prepared in VMD; structure file was 1dv3.pdb.

REFERENCES
2. Wraith, C. A. (1982) in Function of Quinones in Energy Conserving Systems. ⁴ Any contributions by other components would further lower the estimated rate for H₂O⁺. Carbonic acid is a possible candidate, but the concentration in equilibrium with air is too small (≈2 × 10⁻⁸ M) to contribute significantly to the observed base rate of the mutant.

⁵ This is clearly shown in comparisons of x-ray structures, e.g. 1dv3.pdb (+Cd²⁺) versus 1aig.pdb.
Chemical Rescue of Proton-coupled Electron Transfer