Identification of the proton pathway in bacterial reaction centers: Replacement of Asp-M17 and Asp-L210 with Asn reduces the proton transfer rate in the presence of Cd^{2+}

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The reaction center (RC) from Rhodobacter sphaeroides converts light into chemical energy through the reduction and protonation of a bound quinone molecule Q_{b} (the secondary quinone electron acceptor). We investigated the proton transfer pathway by measuring the proton-coupled electron transfer, $k_{AB}^{(2)}$ [Eq. 1], in native and mutant RCs in the absence and presence of Cd^{2+}. Previous work has shown that the binding of Cd^{2+} decreases $k_{AB}^{(2)}$ in native RCs ~100-fold. The preceding paper shows that bound Cd^{2+} binds to Asp-H124, His-H126, and His-H128. This region represents the entry point for protons. In this work we investigated the proton transfer pathway connecting the entry point with Q_{b}^{•−} by searching for mutations that greatly affect $k_{AB}^{(2)}$ (~10-fold) in the presence of Cd^{2+}, where $k_{AB}^{(2)}$ is limited by the proton transfer rate ($k_{p}$). Upon mutation of Asp-L210 or Asp-M17 to Asn, $k_{p}$ decreased from ~60 s⁻¹ to ~7 s⁻¹, which shows the important role that the proton transfer chain plays in electron transfer. By comparing the rate of proton transfer in the mutants ($k_{p} \sim 7$ s⁻¹) with that in native RCs in the absence of Cd^{2+} ($k_{p} \geq 10^{3}$ s⁻¹), we conclude that alternate proton transfer pathways, which have been postulated, are at least 10³-fold less effective.

**bacterial photosynthesis | Rhodobacter sphaeroides | metal binding | proton-coupled electron transfer**

The conversion of light into chemical energy in photosynthetic bacteria is initiated within a membrane-bound pigment–protein complex called the reaction center (RC). The isolated RC from Rhodobacter (Rb.) sphaeroides is composed of three polypeptide subunits (L, M, and H); four bacteriochlorophylls; two bacterioferritin; one internally bound nonheme Fe^{2+}; and two ubiquinone (UQ₁₀) molecules (reviewed in refs. 1 and 2). Light induces electron transfer from the primary donor (a bacteriochlorophyll dimer) through a series of electron donor and acceptor molecules (a bacteriochlorophytin and a quinone molecule Q_{A}) to a loosely bound secondary quinone Q_{b}. Q_{b} accepts two electrons, sequentially transferred through the electron transfer chain, and two protons, through the proton transfer pathway(s), to form quinol. The first electron transfer to Q_{b} ($k_{AB}^{(1)}$) does not involve direct protonation of the quinone (Eq. 1).

$$Q_{A}^{•−}Q_{b} \rightarrow Q_{A}Q_{b}^{•−} \quad [1]$$

However, the second electron transfer ($k_{AB}^{(2)}$) is coupled to the direct protonation of the quinone (Eq. 2). The mechanism of the proton-coupled electron transfer reaction $k_{AB}^{(2)}$ (Eq. 2) was shown to be a two-step process in which fast protonation precedes rate-limiting electron transfer (3). Subsequent protonation (Eq. 3) leads to the formation of quinol.

$$Q_{A}^{•−}Q_{b}^{−} + H^{+} \rightarrow Q_{A}Q_{b}H^{−} \quad [2]$$

$$Q_{A}Q_{b}H^{−} + H^{+} \rightarrow Q_{A}Q_{b}H_{2} \quad [3]$$

The quinol, Q_{b}H_{2}, serves as a mobile electron and proton carrier (4–6) transferring electrons and protons from the RC to other components of the bioenergetic cycle.

In the RC embedded in the bacterial membrane, the protons, taken up to form quinol at the Q_{b} site, come from the cytoplasm. The pathways for these proton transfer events have been studied in isolated RCs by a number of groups (7–12). Large decreases (≥10³-fold) in $k_{AB}^{(2)}$ and/or the rate of proton uptake in RCs with mutations at Glu-L212, Ser-L223, and Asp-L213, located near Q_{b}^{•−} (≤5 Å), had shown that these amino acid residues are important for the proton transfer reactions (Eqs. 2 and 3) (reviewed in refs. 13 and 14). The effect of mutations at sites located further from Q_{b}^{•−} (≥10 Å) are much smaller. Because $k_{AB}^{(2)}$ (Eq. 2) is in most mutant RCs not a direct measure of the rate of proton transfer (15–17), the observed decrease in $k_{AB}^{(2)}$ does not necessarily represent a reduction in the rate of proton transfer. The lack of identification of a single amino acid that is clearly more important than any other led to the proposal of many possible pathways for proton transfer (7–12). Furthermore, the crystal structure revealed that Glu-L212, Asp-L213, and Ser-L223 could be connected to the surface through a number of routes (18–22), supporting the idea of several possible proton transfer pathways, each of them having a distinct surface entry point. The recent finding that binding of a single Zn^{2+} (23, 24) or Cd^{2+} metal ion to the RC surface (24) slows the rate of proton transfer ≥100-fold shows that there is a unique proton entry point—i.e., that not all possible pathways are equally effective (24).

An important consequence of the decrease in the rate of proton transfer is a change in the mechanism of the proton-coupled electron transfer $k_{AB}^{(2)}$. In the presence of a bound metal ion, the first step becomes rate limiting (Eq. 4) (24).

$$Q_{A}^{•−}Q_{b}^{−} - \cdot \cdot - M^{2+} + H^{+} \rightarrow (Q_{A}^{•−}Q_{b}H^{−}) - \cdot \cdot - M^{2+} \quad [4]$$

**Abbreviations:** D, primary donor; Q_{A}, primary quinone electron acceptor; Q_{b}, secondary quinone electron acceptor; Q, quinone molecule; Q₁₀, coenzyme Q₁₀ (2,3-dimethoxy-5-methyl-6-decaisoprenyl-1,4-benzoquinone); RC, reaction center; Cyt c, cytochrome c.

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Reconstitution of the QB site was achieved by incubating the RC (26). The final ratio of absorbance, \( A \), followed by dialysis against TL buffer (10 mM Tris-HCl, pH 8.0/0.025% LDAO) followed by dialysis against TL buffer (10 mM Tris-HCl, pH 8.0/0.025% LDAO). Occupancy of the QB sites was 70% to 80%.

where M²⁺ is either Zn²⁺ or Cd²⁺. Thus, measurement of \( k_{AB(2)} \) in the presence of a bound M²⁺ provides us with an assay to determine directly the effect of a mutation on the rate of proton transfer as opposed to inferring it from a change in the observed rate (Eq. 2).

In this study, we investigated the pathway for proton transfer from solution to the bound semiquinone Q_B⁻. We measured \( k_{AB(2)} \) in the presence of Cd²⁺, where proton transfer is the rate-limiting step (24), to determine the effect of a mutation on the rate of proton transfer. We searched for mutant RCs that decreased the rate of proton transfer without affecting other kinetic rates to eliminate mutations that affect the properties (e.g., the redox potential and pKₐ) of the proton acceptor Q_BH⁺. This approach ensures that the mutations affect the proton pathway between the entry point (25) and Q_B⁻. Two mutant RCs, DN(L210) [Asp-L210 → Asn] and DN(M17) [Asp-M17 → Asn], satisfied the above criteria and are the focus of this work. The effects of these mutations on the transfer rate of the first electron, \( k_{AB(1)} \) (Eq. 1), and on the rate of the proton-coupled second electron transfer, \( k_{BD(2)} \) (Eq. 2), in the absence and presence of Cd²⁺ were measured. In addition, the rates of charge recombination \( k_{AD(1)}(D^-Q_A^+) \rightarrow Q_A^{-} \) and \( k_{BD(2)}(D^-Q_A^+ \rightarrow Q_A^+Q_B) \), which are sensitive probes of the electrostatic and structural changes near Q_B⁻, were determined.

Materials and Methods

Reagents and Quinones. Coenzyme Q₁₀ (2,3-dimethoxy-5-methyl-6-decaisoprenyl-1,4-benzoquinone) was obtained from Sigma, and the Q₀ site inhibitor stigmatellin (prepared in ethanol) was from Fluka. Horse heart cytochrome c (Cyt c) was obtained from Sigma, reduced (>95%) by hydrogen gas on platinum black (Aldrich), and filtered (0.2-µm pore size acetate filter). All other reagents were of analytical grade.

Modification, Isolation, and Preparation of RCs. The site-directed mutations Asp-L210 → Asn [DN(L210)] and Asp-M17 → Asn [DN(M17)] were constructed as previously described (7). RCs from *Rh. sphaeroides* R26 and the mutant strains were isolated in 15 mM Tris-HCl, pH 8.0/0.025% lauryldimethylamine N-oxide (LDAO)/0.1 mM EDTA by following published procedures (26). The final ratio of absorbance, \( A_{280}/A_{600} \), was ≤1.25. Reconstitution of the Q₀ site was achieved by incubating the RC solution with Q₁₀ (5 Q₁₀ per RC) solubilized in 1% LDAO followed by dialysis against TL buffer (10 mM Tris-HCl, pH 8.0/0.025% LDAO). Occupancy of the Q₀ sites was 70% to 80%.

Table 1. Measured rate constants for native and mutant RCs in the presence and absence of Cd²⁺ (pH 7.7; 21°C)

<table>
<thead>
<tr>
<th>RC*</th>
<th>( k_{AD} ) s⁻¹</th>
<th>( k_{BD} ) s⁻¹</th>
<th>( k_{AB(1)} ) s⁻¹</th>
<th>( k_{AB(2)} ) s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>8.8</td>
<td>0.8</td>
<td>7,000</td>
<td>1,200</td>
</tr>
<tr>
<td>DN(M17)</td>
<td>9.0</td>
<td>0.6</td>
<td>1,000</td>
<td>500</td>
</tr>
<tr>
<td>DN(L210)</td>
<td>8.9</td>
<td>1.0</td>
<td>900</td>
<td>600</td>
</tr>
<tr>
<td>Native + Cd²⁺</td>
<td>9.0</td>
<td>0.8</td>
<td>700</td>
<td>60</td>
</tr>
<tr>
<td>DN(M17) + Cd²⁺</td>
<td>8.9</td>
<td>0.8</td>
<td>300</td>
<td>8</td>
</tr>
<tr>
<td>DN(L210) + Cd²⁺</td>
<td>9.0</td>
<td>2.2</td>
<td>150</td>
<td>8</td>
</tr>
</tbody>
</table>

Transient Optical Spectroscopy. Charge recombination rates were measured by monitoring the recovery of the donor band at 865 nm after bleaching with a single laser flash (PhaseR DL2100c, 590 nm, ~0.2 J per pulse, 0.4-µs full-width-half-maximum) using a single-beam spectrophotometer (27). All measurements were performed at 21°C. To determine the recombination rate, \( k_{BD} \) (\( D^-Q_AQ_B^- \rightarrow Q_AQ_B \)), the observed absorption decays were fitted to two exponentials by using procedures previously described (28). The recombination rate, \( k_{AD}(D^-Q_A^+Q_B^- \rightarrow Q_D^+A) \), was measured in the presence of 10 µM stigmatellin, which blocks electron transfer to Q_B⁻.

The rate constant, \( k_{AB(1)} \), for the transfer of the first electron to Q_B⁻ (Eq. 1) was measured by monitoring the bacteriopheophytin bandshift at 750 nm, which is differentially sensitive to the reduction state of the quinones Q_A and Q_B (27, 29). To improve the signal-to-noise ratios, 9 to 36 traces were averaged.

The proton-coupled electron transfer \( k_{AB(2)} \) (Eq. 2) was determined by monitoring the decay of the semiquinone absorption at 450 nm after a second saturating laser flash in the presence of an external reductant (10 µM horse heart Cyt c) (30). The kinetic decay was fitted to the sum of two exponentials. The observed Cd²⁺ concentration dependence of \( k_{AB(2)} \) was fitted to the kinetic model (Eq. A4) by using ORIGIN 6.0 (Microcal).

Results

Charge Recombination Rates. The charge recombination rates for the reactions \( D^-Q_A^+ \rightarrow Q_D^+A \) (\( k_{AD} \)) and \( D^-Q_AQ_B^- \rightarrow Q_AQ_B \) (\( k_{BD} \)) were measured at 865 nm. In the absence of CdSO₄, the measured values of \( k_{AD} \) (~9 s⁻¹) and \( k_{BD} \) (~1 s⁻¹) were approximately the same for native and mutant DN(M17) [Asp-M17 → Asn] and DN(L210) [Asp-L210 → Asn] RCs at pH 7.7 (Table 1). The addition of 1 mM CdSO₄ produced only small changes in \( k_{AD} \) and \( k_{BD} \) for native and mutant RCs (Table 1).

The First Electron Transfer Rate, \( k_{AB(1)} \). In the absence of CdSO₄, the transfer rates for the first electron to Q_B⁻ (\( k_{AB(1)} \), Eq. 1), measured at 750 nm, were reduced ~7-fold in the DN(M17) and DN(L210) compared with native RCs at pH 7.7 (Table 1). Upon addition of 1 mM CdSO₄, \( k_{AB(1)} \) was decreased ~10-fold in native RCs, ~3-fold in the DN(M17), and ~6-fold in the DN(L210) RCs (Table 1). The observed rate constant was essentially independent of the metal concentration above 10 µM.

The Proton-Coupled Electron Transfer Rate, \( k_{AB(2)} \). The rate of transfer of the second electron to Q_B⁻ (\( k_{AB(2)} \), Eq. 2), after a second saturating laser flash, was measured at 450 nm in native RCs to
Table 2. Parameters obtained from the fit of the CdSO₄ concentration dependence on \( k_{\text{on}}\) (see Fig. 2) to Eqs. 5 and A4

<table>
<thead>
<tr>
<th>RC</th>
<th>( K_d )</th>
<th>( k_{\text{on}}) (Cd²⁺), s⁻¹</th>
<th>( k_{\text{on}}) (0), s⁻¹</th>
<th>( k_{\text{off}}), s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0.3 µM</td>
<td>60</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DN(M17)</td>
<td>3.1 µM</td>
<td>7.1 (3)</td>
<td>630</td>
<td>30</td>
</tr>
<tr>
<td>DN(L210)</td>
<td>1.2 µM</td>
<td>7.5 (6)</td>
<td>690</td>
<td>90</td>
</tr>
</tbody>
</table>

Errors are 10% for \( K_d \), 20% for \( k_{\text{on}}\), and 5% for \( k_{\text{off}}\). ND, not determinable (in view of the Cd²⁺ concentration independence). Conditions: same as in Fig. 1.

Discussion

In this study, we investigated the pathway for proton transfer from solution to the bound semiquinone \( \text{Q}^\text{B}^+ \) in isolated RCs from \( Rb. \text{sphaeroides} \) by measuring the proton-coupled electron transfer rate \( k_{\text{on}}\) in the presence of \( \text{Cd}^2\)⁺ in the absence and presence of \( \text{Cd}^2\)⁺. We searched for mutant RCs in which \( k_{\text{on}}\) in the presence of \( \text{Cd}^2\)⁺, where proton transfer is the rate-limiting step (24), was decreased significantly (≥10-fold). This occurred in two mutant RCs, DN(L210) and DN(M17). The effect of these mutations on the conformationally gated step of the first electron transfer, \( k_{\text{on}}\) (Eq. 1), on the protonation step
of the proton-coupled second electron transfer, $k_{AB}^{(2)}$ (Eqs. 2 and 4), and the implications of these results for the identification of the proton transfer pathways are discussed below.

**Effect of the DN(L210) and DN(M17) Mutations and of Cd$^{2+}$ on the Conformationally Gated Step of $k_{AB}^{(1)}$.** The rate of the first electron transfer reaction $k_{AB}^{(1)}$ (Eq. 1) was reduced ≈5-fold in the DN(L210) and DN(M17) mutant RCs (Table 1) compared with the native rate. Upon addition of Cd$^{2+}$, $k_{AB}^{(1)}$ was decreased ≈10-fold in native RCs (20, 21), ≈3-fold in the DN(M17) RCs, and ≈6-fold in the DN(L210) RCs compared with their respective rate in the absence of Cd$^{2+}$ (Table 1). Thus, mutant RCs with bound Cd$^{2+}$ have a smaller $k_{AB}^{(1)}$ than their native counterpart.

The reaction mechanism of $k_{AB}^{(1)}$ in isolated RCs involves a slow rate-limiting gating step, which involves the movement of Qb (20) before electron transfer (31, 32). Thus, the decrease rate observed in the mutant RCs and upon binding Cd$^{2+}$ implies a slowing of the conformationally gated step (23, 24). The effects of the mutation and the binding of Cd$^{2+}$ produce similar effects on $k_{AB}^{(1)}$. Because the mutations and the binding of Cd$^{2+}$ both result in a local charge change, the decrease in $k_{AB}^{(1)}$ is attributed to a change in the electrostatic environment (more positive) near the mutation and Cd$^{2+}$ binding sites. The further reduction in $k_{AB}^{(1)}$, produced by the binding of Cd$^{2+}$ to the mutant RCs, shows that the effects of the two changes in the electrostatic potential are partially additive.

A possible candidate for the involvement in the gating process is Glu-H173. It is located near the mutation sites (∼4 Å) and becomes more disordered when Qb is reduced, indicating its role in the proton transfer pathways are discussed below.

$$
\frac{\text{O}_{A\text{Qb}}\text{H}^{-}}{\text{O}_{A\text{Qb}}\text{Qb}^{+}} \rightarrow \text{Cd}^{2+} \rightarrow k_{\text{off}} \left( Q_{A}\text{Qb}^{+} \right) + \text{Cd}^{2+} \rightarrow k_{\text{on}} \text{Cd}^{2+} \left( \text{O}_{A}\text{Qb}^{+} \right) + \text{O}_{A\text{Qb}}\text{H}^{-}$$

where the upper states represent RCs with a bound Cd$^{2+}$ (left) and unbound Cd$^{2+}$ (right). The proton-coupled electron transfer rate proceeds with a bound Cd$^{2+}$ at a rate $k_{AB}^{(2)}$(Cd$^{2+}$) (Eq. 4) and without a bound Cd$^{2+}$ at a rate $k_{AB}^{(2)}$(0) (Eq. 2). The upper bound and unbound states equilibrate with a dissociation constant $K_{d} = k_{\text{off}}/k_{\text{on}}$.

For the mutant RCs $k_{AB}^{(2)}$(0) $>$ $k_{\text{off}}$ $>$ $k_{AB}^{(2)}$(Cd$^{2+}$). Therefore, at low Cd$^{2+}$ concentrations, ((O$_{A}$Q$b$)$^{+}$) - Cd$^{2+}$ decays at the rate $k_{\text{off}}$ $<$ $k_{AB}^{(2)}$(0), where the rate-limiting step for this decay is $k_{\text{off}}$, which is independent of Cd$^{2+}$ concentration. It is responsible for the flat region of the slow phase of $k_{AB}^{(2)}$ at low Cd$^{2+}$ concentration (Fig. 2). Thus, the intercept of the ordinate corresponds to $k_{\text{off}}$. As the Cd$^{2+}$ concentration increases, $k_{\text{off}}$(Cd$^{2+}$) starts to compete with $k_{AB}^{(2)}$(0), resulting in an increase in the steady-state concentration of the ((O$_{A}$Q$b$)$^{+}$) - Cd$^{2+}$ state with a concomitant decrease in the observed rate. This is responsible for the region that depends on Cd$^{2+}$ concentration. At high Cd$^{2+}$ concentration (i.e., [Cd$^{2+}$] $>$ $K_{d}$), essentially all RCs have a bound Cd$^{2+}$ and the observed rate is $k_{AB}^{(2)}$(Cd$^{2+}$), which is independent of Cd$^{2+}$ concentration. It corresponds to the asymptotic value of $k_{AB}^{(2)}$ at high Cd$^{2+}$ concentration (Fig. 2).

In native RCs, the more negative potential due to the Asp residues increases $k_{AB}^{(2)}$(Cd$^{2+}$) and decreases $k_{\text{off}}$ with respect to the mutant RCs such that $k_{AB}^{(2)}$(Cd$^{2+}$) $>$ $K_{d}$. Consequently, ((O$_{A}$Q$b$)$^{+}$) - Cd$^{2+}$ does not decay via the (O$_{A}$Q$b$)$^{+}$ state and no concentration dependence is observed (Fig. 2).

The qualitative considerations discussed above are borne out by the exact mathematical solution of Eq. 5 presented in the Appendix (Eq. A4). The parameters used to fit the experimental results are summarized in Table 2.

**Proton Transfer Pathway to the Qb Site.** We have previously shown (25) that the binding of Cd$^{2+}$ to the surface accessible region on Cd$^{2+}$ concentration (Figs. 1 and 2). At the concentration of Cd$^{2+}$ (1 mM) above which no further changes in $k_{AB}^{(2)}$ occur, $k_{AB}^{(2)}$ was decreased $\approx$10-fold compared with native RCs (Fig. 3).1 Because in RCs with a bound Cd$^{2+}$, $k_{AB}^{(2)}$ is a measure of proton transfer (24), this large decrease of $k_{AB}^{(2)}$ in the mutant RCs points to the important role of Asp-L210 and Asp-M17 in the proton transfer.

To gain further insight into the binding and proton transfer rates, we analyzed the Cd$^{2+}$ concentration dependence of the slow component of $k_{AB}^{(2)}$ using the kinetic scheme given by Eq. 5.
between the Cd\textsuperscript{2+} binding region and Q_B\textsuperscript{−}, ineffective, thereby allowing an alternate pathway to take over.\textsuperscript{5} The maximum rate of this alternate pathway, which does not involve Asp-L210 or Asp-M17, such as P1 (11) or P2 (20) (see figure 1 of ref. 21 or figure 4 of ref. 24), cannot be larger than the observed rate of \( \approx 10 \text{ s}^{-1} \). This rate is at least \( 10^3 \)-fold smaller than the physiological transfer rate in native RCs (\( k_1 \approx 10^4 \text{ s}^{-1} \)) (3).

The results presented above address the pathways for the transfer of the first proton to Q_B\textsuperscript{−} (Eq. 2). Although it has not been established whether these pathways are also dominant for the transfer of the second proton (Eq. 3) that ends up at a spatially different oxygen (O4) of Q_BH\textsuperscript{−} located near His-L190 (Fig. 4) (13, 14).

The greater rate of proton transfer (\( \approx 10^3 \)-fold) through the pathways near Asp-L210 and Asp-M17 shows that the activation barrier for proton transfer is smaller than for the other possible pathways, such as P1 and P2 (see, e.g., refs. 21 and 24). This smaller activation energy could be a consequence of the relatively short length of the pathway and the high density of carboxylic acid groups (Asp-L210, Asp-M17, and Asp-L213), which electrostatically stabilize the proton in the interior of the protein (see, e.g., ref. 41). The involvement of carboxylic acid groups in proton conduction has also been suggested in several other membrane-bound proteins. These include bacteriorhodopsin (reviewed in refs. 42 and 43), terminal oxidases (reviewed in refs. 44 and 45), lactose permease (reviewed in ref. 46), and the ubiquinol:Cyt c oxidoreductases (47–49). Thus, the involvement of carboxylic acid residues may represent a general strategy used to lower the activation energy to facilitate fast proton conduction through proteins.

**Appendix**

To obtain the kinetic parameters of Eq. 5 we rewrite it in the form:

\[
\begin{align*}
A - \cdots \Cd^{2+} & \rightarrow A + \Cd^{2+} \\
B - \cdots \Cd^{2+} & \rightarrow B + \Cd^{2+}
\end{align*}
\]

where \( A = (\text{Q}_A^\text{−}Q_B^\text{−}), B = (\text{Q}_A^\text{−}Q_BH^\text{−}), k_1 = k_{AB}^{(2)}(\Cd^{2+}) \) and \( k_2 = k_{AB}^{(2)}(0) \). The differential equations describing the decay of \( \{A - Cd^{2+}\} \) and \( \{A\} \) are obtained from Eq. A1:

\[
\begin{align*}
d\{A - Cd^{2+}\}/dt &= -k_1\{A - Cd^{2+}\} - k_{off}\{A - Cd^{2+}\} + k_{on}\{Cd^{2+}\}\{A\} \\
&= \left[ k_{off}\{A\} - k_{on}\{Cd^{2+}\}\right]\{A\}
\end{align*}
\]

\[
\begin{align*}
d\{A\}/dt &= -k_1\{A\} - k_{off}\{A\} + k_{on}\{Cd^{2+}\}\{A\} + k_{off}\{A - Cd^{2+}\}
\end{align*}
\]

\( k_{AB}^{(2)} \) in the absence of Cd\textsuperscript{2+} (Table 1).

\textsuperscript{5}An alternate explanation can, at present, not be excluded. A mutation at one Asp eliminates the pathway involving the mutated residue, but allows proton transfer through the pathway involving the second (unchanged) Asp (Fig. 4), albeit at a diminished rate. The fact that both mutations result in the same transfer rates makes this explanation unlikely.

This point can be settled by constructing a double mutant in which both Asp-L210 and Asp-M17 are changed to Asn.

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\textsuperscript{4}We exclude the possibility that the effects are due to a change in the properties of the proton acceptor Q_B\textsuperscript{−}, which could also reduce the rate of proton transfer (39, 40). This
We are interested in the slow component of the decay of the semiquinones—i.e., the decay of [A- - Cd2+] after the second laser flash. For this process, the initial conditions at time \( t = 0 \) are \([A- - Cd^2+] = 1 \) and \([A(0)] = 0 \) (this state quickly disappears after the second flash because \( k_2 \gg k_{off} \)). The solution for the time dependence of \([A- - Cd^2+]\) is given by:

\[
[A- - Cd^{2+}](t) = [0.5(a - b + c) / a] \exp[-0.5(b + c - a)t] + [0.5(a + b - c) / a] \exp[-0.5(b + c + a)t]
\]

where \( a = \{k_1^2 + 2k_1k_{off} - 2k_2k_1 - 2k_1k_{off}[Cd^2+]\} + k_{off}^2 - 2k_{off}k_2 + 2k_{off}[Cd^{2+}][k_{off} + k_1^2 + 2k_1k_{off}[Cd^2+] + (k_{off}[Cd^{2+}])^{1/2}] \), \( b = k_1 + k_{off} \), and \( c = k_2 + k_{off}[Cd^2+] \). For our situation, \( k_2 > k_1, k_{off} \) (see Tables 1 and 2). This makes the first term in Eq. A3 the dominant term and the observed rate constant is

\[
k_{obs} = 0.5(b + c - a)
\]

We fitted the observed \( Cd^{2+} \) concentration dependence for the two mutants with Eq. A4 by leaving \( k_1, k_2, k_{off}, \) and \( k_{on} \) as free parameters subject to the constraint \( K_d = k_{off} / k_{on} \), where \( K_d \) is the measured dissociation constant (Table 2). The values of the intrinsic rate constants are summarized in Table 2.

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