Identification of the proton pathway in bacterial reaction centers: Inhibition of proton transfer by binding of Zn\textsuperscript{2+} or Cd\textsuperscript{2+}  
(bacterial photosynthesis/Rhodobacter sphaeroides/metal binding/proton-coupled electron transfer)  
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ABSTRACT  The reaction center (RC) from Rhodobacter sphaeroides converts light into chemical energy through the light induced two-electron, two-proton reduction of a bound quinone molecule Q\textsubscript{B} (the secondary quinone acceptor). A unique pathway for proton transfer to the Q\textsubscript{B} site had so far not been determined. To study the molecular basis for proton transfer, we investigated the effects of exogenous metal ion binding on the kinetics of proton-coupled electron transfer based on the dependence of the stoichiometric binding of the metal ion shows that there is one large change in the rate of proton transfer caused by the binding of Cu\textsuperscript{2+}, which serves as a mobile electron and proton carrier (3–5), transferring electrons and protons from the RC to other components of the bioenergetic cycle.  
The first electron transfer to Q\textsubscript{B} (k\textsubscript{AB(1)}) does not involve direct protonation of the quinone (Eq. 1).

\[
Q_{\text{A}}^{-}Q_{\text{B}}^{-} + H^{+} \rightarrow Q_{\text{A}}(Q_{\text{B}}H)^{-}.
\]  

However, the second electron transfer (k\textsubscript{AB(2)}) is coupled to the direct protonation of the quinone (Eq. 2a). Subsequent protonation (Eq. 2b) leads to the formation of quinol.

\[
\begin{align*}
\text{fast} & \quad Q_{\text{A}}^{-}Q_{\text{B}}^{-} + H^{+} \rightarrow Q_{\text{A}}^{-}Q_{\text{B}}H^{-} \\
\text{slow} & \quad Q_{\text{A}}^{-}Q_{\text{B}}H^{-} + H^{+} \rightarrow Q_{\text{A}}Q_{\text{B}}H_{2}.
\end{align*}
\]  

In native RCs from Rh. sphaeroides, the proton-coupled electron transfer reaction k\textsubscript{AB(2)} (Eq. 2a) was shown to be a two-step process in which fast protonation precedes rate-limiting electron transfer (6). The value of k\textsubscript{AB(2)} in native RCs is, therefore, not a direct measure of the rate of proton transfer. However, when the proton transfer rate is sufficiently reduced, proton transfer becomes the rate-limiting step as has been observed in the site-directed mutation of Asp-L213 to Asn (7). To determine which of the two steps in Eq. 2a is rate limiting, a driving-force assay, based on the free-energy dependence of k\textsubscript{AB(2)}, has been used (6).

The pathways for proton transfer have been studied by a number of groups (8–13). Results of k\textsubscript{AB(2)} measurements on site-directed mutants had shown the importance of several amino acid residues, e.g., Glu-L212, Ser-L223, and Asp-L223, for the proton transfer reactions (Eq. 2) (reviewed in refs. 14 and 15). These residues can be connected to the external surface through a number of possible proton transfer pathways and an internal carboxylic acid cluster that have been observed in the crystal structures of the RC (16–19). Which of these is functionally the most important pathway had not been determined.

A complementary approach to site-directed mutagenesis, to identify residues involved in proton transfer, is to assess the effect of metal binding on the kinetics of proton transfer (Eq. 2). In solution, metal ions bind to acidic and uncharged amine or imidazole groups (20). In a protein, these groups are provided by carboxylic acids and histidine residues. For example, the binding of Cu\textsuperscript{2+} has an inhibitory effect on proton transfer in carbonic anhydrase (21–23).

Utschig et al. (24) have shown that Zn\textsuperscript{2+} binds to the RC and affects the rate of transfer of the first electron k\textsubscript{AB(1)} (Eq. 1). We have confirmed their findings and have extended the work

Abbreviations: D, primary donor; Q\textsubscript{A}, primary quinone acceptor; Q\textsubscript{B}, secondary quinone acceptor; Q, quinone molecule; Q\textsubscript{U10}, coenzyme Q\textsubscript{U10} (2,3-dimethoxy-5-methyl-6-decasoprenyl-1,4-benzquinone); RC, reaction center; NQ, napthoquinone; UQ, ubiquinone.  
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to an investigation of the effect of Zn$^{2+}$ and Cd$^{2+}$ binding on the proton-coupled electron transfer $k_{AB}^{(2)}$ (Eq. 2a). In addition, we have investigated the driving-force dependence of $k_{AD}^{(2)}$, to establish which of the two steps in Eq. 2a is rate limiting. Furthermore, we have measured the effects of the metal ions on the charge recombination rate constant $k_{BD}$ (D$^+$Q$_A$Oh$^{-}$ $\rightarrow$ DO$_A$Q$_B$, where D is the primary donor), which is sensitive to the electrostatic potential near Q$_A$. By localizing the position of the bound cation, the location of the proton entry into the RC from which proton transfer to Q$_B$ occurs.

**METHODS**

**Reagents and Quinones.** The quinones Q$_{10}$ (coenzyme Q$_{10}$; 2,3-dimethoxy-5-methyl-6-decaisoprenyl-1,4-benzoquinone) and M$_3$Q$_4$ (menatetrenone; 2-methyl-3-tetraisoprenyl-1,4-naphthoquinone) were obtained from Sigma. ADMNQ (2,6-dimethyl-3-undecyl-1,4-naphthoquinone) and ATMNQ (2,6,7-trimethyl-3-undecyl-1,4-naphthoquinone) were kindly provided by Andreas Labahn (Albert-Ludwigs Universität, Freiburg, Germany) (25). All quinones were prepared in ethanol before their use. The QA site inhibitors terbutryne and stigmatellin were obtained from Chem Service (West Chester, PA) and Fluka, respectively, and were prepared in ethanol. Cytochrome c from horse heart was obtained from Sigma and was reduced (95%) by hydrogen on platinum black (Aldrich) and filtered (0.2-μm pore size acetate filter). All other reagents were of analytical grade.

**Isolation and Preparation of RCs.** RCs from *Rh. sphaeroides* R26 were isolated in 15 mM Tris-HCl, pH 8, 0.025% lauryl dimethylamine-N-oxide (LDAO), 1 mM EDTA following published procedures (26) as modified by Utschig et al. (27). Both QA and Q$_B$ were removed as described (28, 29) to yield RCs with $\leq$ 10% residual Q$_A$/RC and $\leq$ 0.2% residual Q$_B$/RC, as measured at 865 nm from the charge recombination rate and amplitude (28). The ratio of absorbance, $A_{280}^{\infty}$/A$_{800}^{\infty}$, was 1.20. Substitution of a naphthoquinone (NQ) for the native UQ at the Q$_A$ site was performed as described (6, 30).

Reconstitution of the Q$_B$ site was achieved by incubation of the solubilized RC solution with Q$_{10}$ adsorbed to celite (diatomaceous earth, Fisher) for $\sim$20 min at 25°C while stirring (6) or by addition of Q$_{10}$ ($\sim$3 Q$_{10}$/per RC) solubilized in 1% LDAO. Occupancy of the Q$_B$ sites varied from 50% to 80% over the range of pH studied.

**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 (Phase R DL 2100c, 590 nm, $\sim$0.2 μJ/pulse, 0.4-μs full-width-half-max) by using a single-beam spectrophotometer (31). All measurements were performed at 21°C. To determine the D$^+$Q$_A$Oh$^{-}$ $\rightarrow$ DO$_A$Q$_B$ recombination rate ($k_{BD}$), the observed absorption decays were fitted to multiple exponentials by using procedures previously described (30). To measure the D$^+$Q$_A$O$_{3B}$ $\rightarrow$ DO$_A$Q$_B$ recombination rate ($k_{AD}$), electron transfer from Q$_A$ to Q$_B$ was blocked by the addition of 100 μM terbutryne or 10 μM stigmatellin. For studies of the driving-force dependence of rates, NQs were substituted into the QA site. The relative occupancies of the NQ and UQ in the QA site were determined from a deconvolution of the charge recombination kinetics (6), because $k_{AD}$ is different for NQ and UQ (6, 30).

The rate constant ($k_{BD}^{(1)}$) for the transfer of the first electron to Q$_B$ (Eq. 1) was measured by monitoring the bacteriopheophytin bandshift at 750 nm, which is sensitive to the reduction state of the quinones QA and Q$_B$ (31, 32). To improve the signal-to-noise ratios, 9–36 traces were averaged.

The proton-coupled electron transfer $k_{AB}^{(2)}$ (Eq. 2a) 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 cytochrome c) (33). In RCs with a NQ added to occupy the Q$_A$ site (see above), biphasic kinetics were observed with one rate corresponding to the rate observed in RCs with UQ$_{10}$ occupying the Q$_A$ site ($k_{AD}^{(2)}$ $\approx$ 1.200 s$^{-1}$, pH 7.5, native RCs without a heavy metal) and the other rate corresponding to RCs with a NQ occupying the Q$_A$ site ($k_{AB}^{(2)}$ $\approx$ 6,000 s$^{-1}$, pH 7.5, native RCs without a heavy metal). The relative occupancies of UQ$_{10}$ and NQ determined in this manner agreed with the relative occupancies determined from the deconvolution of biphasic kinetics for $k_{AD}$.

**EPR Spectroscopy.** The light-induced EPR spectra of RC samples in the presence and in the absence of 10 mM ZnSO$_4$ and CdSO$_4$ were obtained on a spectrometer of local design at a microwave frequency of 9 GHz (34). The samples were concentrated to $A_{800}^{\infty}$ $\approx$ 77, frozen in a 15×6×1-mm flat quartz cell and illuminated in the frozen state by a 500-W projector lamp (34).

**RESULTS**

**Binding of Exogenous Metal Ions to the RC.** The measured value of $k_{AB}^{(2)}$ (Eq. 2a) was used as functional assay for heavy metal binding to the RC. Most of the heavy metals compounds that were tested (MnSO$_4$, CuSO$_4$, ZnSO$_4$, CdSO$_4$, HgCl$_2$) caused a decrease in the observed rate at 1 mM concentrations, except for FeSO$_4$, which had no effect at this concentration. However, only the addition of CdSO$_4$ and ZnSO$_4$ caused a significant decrease in the observed rate at 10 μM concentrations (Fig. 1). These two metals became, therefore, the focus of our investigation. The disparate results obtained with different compounds show that the observed effect on $k_{AB}^{(2)}$ is not caused by the anionic counterion (i.e., SO$_4^{2-}$), which was the same for all of the metals tested, except HgCl$_2$.

**Measurements of Charge Recombination.** The charge recombination rates for the reactions D$^+$Q$_A$O$_{3B}$ $\rightarrow$ DO$_A$Q$_B$ ($k_{AD}$) and D$^+$Q$_A$O$_{3B}$ $\rightarrow$ DO$_A$Q$_B$ ($k_{BD}$) were measured at 865 nm in the presence and absence of an exogenous cation. The measured values of $k_{AD}$ ($\approx$9 s$^{-1}$) and $k_{BD}$ ($\approx$0.8 s$^{-1}$) were the same with or without exogenous Zn$^{2+}$ or Cd$^{2+}$ (Table 1). The amplitude of the $k_{BD}$ phase remained unchanged upon addition of Zn$^{2+}$ or Cd$^{2+}$. Similarly the pH profile of $k_{BD}$ was essentially the same with or without Zn$^{2+}$ or Cd$^{2+}$ (data not shown).

**Measurements of the First Electron Transfer Rate $k_{AB}^{(1)}$.** The measured rates of transfer for the first electron to Q$_B$ ($k_{AB}^{(1)}$, Eq. 1), measured at 750 nm, were reduced $\sim$10-fold upon addition of 10 μM Zn$^{2+}$ or Cd$^{2+}$ (Table 1). The slower observed rate constant was independent of the metal concentration above 10 μM. At cation concentrations below 10 μM, we could deconvolute the observed kinetics into two phases, one phase at 7,000 s$^{-1}$ (the rate observed without exogenous cations) and one phase at 700 s$^{-1}$ (the rate observed at $\geq$10 μM concentration). From the dependence of the amplitude of the slow phase with cation concentration, we estimated a dissociation constant ($K_D$) of $\approx$0.5 μM for Zn$^{2+}$ and Cd$^{2+}$ (data not shown).

**Measurements of the Proton-Coupled Electron Transfer Rate $k_{AB}^{(2)}$.** The rate of transfer for the second electron to Q$_B$ ($k_{AB}^{(2)}$, Eq. 2a), after the second saturating laser flash at 450 nm, was measured in native RCs to be 1,200 s$^{-1}$ at pH 8 (Table 1). Upon addition of 10 μM Zn$^{2+}$ or Cd$^{2+}$, $k_{AB}^{(2)}$ decreased to a limiting value of 120 s$^{-1}$ and 60 s$^{-1}$, respectively (Table 1). The effect of the metal on the rate was observed immediately after addition without an incubation period. The fraction of the sample exhibiting the slower rate depended on the concentration of the cations (Fig. 2) and allowed us to estimate a dissociation constant ($K_D$) of $\approx$0.5 μM for Zn$^{2+}$ and Cd$^{2+}$, which is, within experimental error, the same as that determined from the $k_{AB}^{(1)}$ measurements.
Fig. 1. Absorbance decay of the semiquinones at 450 nm as a function of time after the second of two laser flashes in the presence of various concentrations of ZnSO₄ (a) and CdSO₄ (b). From the decay, the rate constant k_AB(2) was determined. Note the slowing of the kinetics with increasing cation concentrations. The pedestal at long times after the laser flash is caused by the absorbance change of the cytochrome c used to reduce the primary donor (see Materials and Methods). Conditions were: 2 μM RCs in 10 mM Tris-HCl (pH 7.7), 0.25% lauryl dimethylamine-N-oxide with the concentration of ZnSO₄ or CdSO₄ as indicated in the figure.

The decrease in the value of k_AB(2) caused by the addition of exogenous Zn²⁺ or Cd²⁺ was eliminated upon addition of EDTA (a strong chelator of cationic metals) to a concentration of twice the exogenous metal concentration. Further addition of Zn²⁺ or Cd²⁺ to twice that of the EDTA concentration led to a reduced value of k_AB(2) to 120 s⁻¹ or 60 s⁻¹, respectively.

The reduced value of k_AB(2) to 60 s⁻¹ upon addition of 10 μM Cd²⁺ could be increased to 120 s⁻¹ upon addition of 50 μM Zn²⁺, showing that Zn²⁺ had replaced Cd²⁺.

The pH profile of k_AB(2) was measured in a mixture of 2 mM Hepes, 2 mM Ches, 2 mM Mes in 0.025% lauryl dimethylamino-N-oxide buffer from pH 7 to 9.5 and in 0.04% maltoside either detergent at the same pH. In the presence of Zn²⁺ or Cd²⁺, the value of k_AB(2) decreased with increasing pH with a slope proportional to [H⁺]⁰.⁵ over the measured pH ranges (data not shown). This pH dependence differs from the native behavior where k_AB(2) decreases with pH proportional to [H⁺]¹.⁸ below pH 5 and [H⁺]¹.⁵ above pH 8.5.

Dependence of the Proton-Coupled Electron Transfer Rates on the Driving Force for Electron Transfer. The driving force for electron transfer was changed by replacing the native Q₁₀ in the Q₉ site with a series of NQs that have different redox protentials while retaining the native Q₁₀ in the Q₉ site. The experimental results on native RCs without a bound Zn²⁺ or Cd²⁺ showed an increase in the observed rate upon increasing the driving force for electron transfer. In the presence of Zn²⁺ and Cd²⁺, k_AB(2) became approximately independent of the driving force (Eq. 3). The dependence of k_AB(2) on the driving force was restored to either sample by addition of EDTA to twice the concentration of that of the exogenous metal. Upon further addition of Zn²⁺ and Cd²⁺ to twice the EDTA concentration, k_AB(2) became again approximately independent of the driving force.

Table 1. Measured rate constants for RCs in the presence and absence of metal ions (pH 8.0, T = 21°C)

<table>
<thead>
<tr>
<th>RCs*</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>Native + Zn²⁺</td>
<td>8.8</td>
<td>0.7</td>
<td>700</td>
<td>120</td>
</tr>
<tr>
<td>Native + Cd²⁺</td>
<td>9.0</td>
<td>0.8</td>
<td>700</td>
<td>60</td>
</tr>
</tbody>
</table>

Errors in the rates are estimated to be ~8% for the charge recombination rate constants k_AD and k_BD and ~15% for the forward electron transfer rate constants k_AB(1) and k_AB(2).

*Conditions for kinetic measurements as described in Materials and Methods. The samples labeled Native + Zn²⁺ and Native + Cd²⁺ were measured in the presence of 10μM ZnSO₄ and CdSO₄, respectively.

†This is the observed rate constant using a single exponential fit to the data. This reaction can be better fitted with a sum of two exponentials (24), but the effect of the metal binding is clearly shown by using this simple analysis.

DISCUSSION

We investigated the effects of Zn²⁺ and Cd²⁺ binding to RCs on the transfer rate of the first electron, k_AB(1) (Eq. 1), and on the rate of the proton-assisted second electron transfer, k_AB(2)
Fig. 3. The rate constant of proton-coupled second electron transfer, \( k_{AB} \), in RCs as a function of the change in redox free energy (driving force) for electron transfer in the absence and in the presence of 10 \( \mu \)M Zn\(^{2+}\) and Cd\(^{2+}\). Note that \( k_{AB}^{(2)} \) in the absence of added metals shows a “Marcus”-like dependence on the electron driving force characteristic of a rate-limited electron reaction as has been reported (6), whereas in the presence of Zn\(^{2+}\) or Cd\(^{2+}\), \( k_{AB}^{(2)} \) is approximately independent of the electron driving force, showing that proton transfer (Eq. 3) has become rate limiting. Quinones substituted into the QA site were from left to right: MQt, menadione (2-methyl-1,4-naphthoquinone); QO, coenzyme Q; MQ4, menatetrenone (2-methyl-3-tetraisoprenyl-1,4-naphthoquinone); ADMNQ, 2,6-dimethyl-3-undecyl-1,4-naphthoquinone; and ATMNQ, 2,6,7-trimethyl-3-undecyl-1,4-naphthoquinone. Conditions were the same as in Fig. 1.

(Eq. 2a). By localizing the binding site of Zn\(^{2+}\) and Cd\(^{2+}\) we identified the point of entry of the protons and the start of the proton transfer pathway(s) to QB\(^{-}\).

The Effect of Zn\(^{2+}\) and Cd\(^{2+}\) Binding on \( k_{AB} \). The rate of the first electron reduction \( k_{AB}^{(1)} \) (Eq. 1) was reduced \( \approx 10\) fold upon the binding of Zn\(^{2+}\) or Cd\(^{2+}\). These findings confirm the results of Utschig et al. (24) on the effect of Zn\(^{2+}\) binding and show that a similar effect is found upon binding of Cd\(^{2+}\).

The reaction mechanism of \( k_{AB}^{(1)} \) in isolated RCs involves a slow rate-limiting gating step that involves the movement of QB (18) before electron transfer (35). Thus, the decreased rate upon binding Zn\(^{2+}\) or Cd\(^{2+}\) implies a slowing down of the conformational gating step. The movement of QA into the active position requires a rotation of the quinone head group and a displacement of several water molecules (17–19). The possibility that bound Zn\(^{2+}\) or Cd\(^{2+}\) directly hindered the quinone rotation and movement is excluded because a decrease in \( k_{AB} \) would be expected as discussed in a later section. A possible explanation advanced by Utschig et al. (24) is that cation binding alters protein conformation, thereby affecting protein dynamics that are necessary for QB\(^{-}\) formation. One possibility is that bound Zn\(^{2+}\) or Cd\(^{2+}\) hinders the movement of water out of the RC through a contiguous water chain that was observed in the crystal structures of Rb. sphaeroides (16, 18, 19), thereby indirectly hindering the movement of QA into its active position. Another possible explanation is that QB reduction is slowed as a consequence of a slowing of the rate of proton uptake and/or redistribution of the protons that stabilize the semiquinone (36–40).

The Effect of Zn\(^{2+}\) and Cd\(^{2+}\) Binding on \( k_{AB}^{(2)} \). The rate of the proton-coupled electron transfer \( k_{AB}^{(2)} \) (Eq. 2a) was reduced \( \approx 10\) fold and \( \approx 20\) fold upon addition of Zn\(^{2+}\) or Cd\(^{2+}\), respectively. The mechanism of the \( k_{AB}^{(2)} \) reaction was deduced from the dependence of the observed rate on the driving force for electron transfer (6). For RCs in the absence of cations, \( k_{AB}^{(2)} \) depends on the electron transfer driving force (Fig. 3) consistent with a rate-limiting electron transfer after a fast proton transfer \( k_{i+} \approx k_{AB}^{(2)} \), i.e., \( \approx 10^4 \text{s}^{-1} \) as was previously reported (6). In the presence of Zn\(^{2+}\) or Cd\(^{2+}\), \( k_{AB}^{(2)} \) is approximately independent of the driving force (Fig. 3), implying a change in the mechanism of the proton-coupled electron transfer. This conclusion is further supported by the change in the pH dependence of \( k_{AB}^{(2)} \). The rate of proton transfer (first step of Eq. 2a) now has become the rate-limiting step for the reaction (i.e., \( k_{AB}^{(2)} = k_{i +}^{(2)} \approx 10^2 \text{s}^{-1} \)), i.e.,

\[
\begin{align*}
\text{slow} & \quad (Q_A^- \text{Q}_B^-) \rightarrow M^2+ + H^+ \rightarrow (Q_A^- \text{Q}_B^-H) \rightarrow M^2+ \\
\text{fast} & \quad (Q_A^- \text{Q}_B^-H) \rightarrow M^2+ \rightarrow (Q_A^- \text{Q}_B^-H) \rightarrow M^2+,
\end{align*}
\]

where M\(^{2+}\) is either Zn\(^{2+}\) or Cd\(^{2+}\). Thus, the rate of proton transfer to QB\(^{-}\) is reduced from \( k_{i+} \approx 10^4 \text{s}^{-1} \) without a bound metal ion to \( k_{i+} \approx 10^2 \text{s}^{-1} \) with a bound metal ion (i.e., a \( \approx 10\) fold reduction). The reduced rate of proton transfer upon stoichiometric binding of Zn\(^{2+}\) or Cd\(^{2+}\) implies that there is one dominant site of proton entry into the RC that is blocked by the bound metal ion.

In RCs with a bound Zn\(^{2+}\) or Cd\(^{2+}\), \( k_{AB}^{(2)} \) is a measure of the rate of proton transfer, which enables one to trace the proton transfer pathway from QB\(^{-}\) to the surface of the RC by measuring \( k_{AB}^{(2)} \) in a series of site-directed mutant RCs. These studies should provide insight into the rates of proton conduction in the RC.

Characterization and Localization of the Binding Site of Zn\(^{2+}\) and Cd\(^{2+}\): Identification of the Dominant Proton Transfer Pathway. The competitive replacement of Cd\(^{2+}\) by Zn\(^{2+}\) shows that both metal ions bind to the same or to nearby positions on the RC. The stoichiometry of the kinetic effects found was to be \( \approx 1 \) metal cation per RC. The dissociation constants for Zn\(^{2+}\) and Cd\(^{2+}\) were determined to be \( K_D \approx 0.5 \mu \text{M} \).

We now turn to a discussion of the location of the Zn\(^{2+}\) and Cd\(^{2+}\) binding site(s). The light-induced, low-temperature EPR spectrum, which is characteristic of the Fe\(^{2+}\)–Q\(^{-}\) complex, excludes the possibility that either Zn\(^{2+}\) or Cd\(^{2+}\) displaces the Fe\(^{2+}\) in the interior of the RC. The measured recombination kinetics, \( k_{RB} \), in the presence of Zn\(^{2+}\) and Cd\(^{2+}\) show that there are no electrostatic or structural changes near QB. Thus, a direct interaction between QB\(^{-}\) and the bound Zn\(^{2+}\) or Cd\(^{2+}\) is excluded.

The most likely location(s) of the metal ions would be a surface accessible region that is rich in His, Glu, and/or Asp residues. There are three surface accessible His residues (H68, H126, H128) forming a cluster located \( \approx 20 \) Å from the QB binding site at the surface of the H subunit (Fig. 4); this cluster previously was proposed as a possible Zn\(^{2+}\)–binding site location by Utschig et al. (24). His-H68 is located near one of the termini of the possible proton transfer pathway P1 (Fig. 4). His-H126 and His-H128 are located closer to P3 (Fig. 4).

There are also several surface accessible carboxylic acid residues (Asp-L210, Asp-M17, Asp-H124, Glu-H224, Asp-M240). Three of these carboxylic acid groups are components of a larger cluster of carboxylic acid residues located \( \approx 10 \) Å from QB and near P3 (Fig. 4), which was proposed to act as a local proton reservoir (13).

The most direct way of determining the binding location of Zn\(^{2+}\) and Cd\(^{2+}\) is by x-ray diffraction. Preliminary x-ray diffraction results have been obtained by H. L. Axelrod, E. C. Abresch, M. L. P., M. Y. O., and G. F. (unpublished work), which show that Cd\(^{2+}\) and Zn\(^{2+}\) are ligated to His-H126, His-H128, and Asp-H124 (see Fig. 4). Thus, this region of the RC surface
Possible proton transfer pathways (P1–P3) proposed by Abresch sphaeroides in a drastic reduction (on the H-subunit (His-H126, His-H128, and Asp-H124) results from a structural point (Fig. 4). Yet another explanation is that the metal ion creates a barrier for proton entry into one of the proton transfer pathways that have been proposed (8–15, 19, 41–43). The simplest explanation for the inhibition of proton transfer is that one or more of these three residues function as proton donors.

The Mechanism of Proton Conduction in the Bacterial RC. The binding of Cd²⁺ or Zn²⁺ to the surface accessible region on the H-subunit (His-H126, His-H128, and Asp-H124) results in a drastic reduction (≥ 10²-fold) in the rate of proton transfer. The simplest explanation for the inhibition of proton transfer is that one or more of these three residues function as proton donors. The binding of Cd²⁺ in the vicinity of P3 reduces their effectiveness as a source of protons.

An alternate explanation is that the binding of the metal ion creates a barrier for proton entry into one of the proton transfer pathways that have been proposed (8–15, 19, 41–43). In this view, the bound cation may electrostatically hinder proton uptake. The pathway most likely to be involved is P3 (19) (Fig. 4). Yet another explanation is that the metal ion binding affects the protein dynamics as have been postulated to be the cause for the changed kinetic for k_{AB}⁻(1) (24).

**SUMMARY**

The results of the binding of Zn²⁺ and Cd²⁺ to RCs from *Rh. sphaeroides* can be summarized as follows:

(i) Zn²⁺ and Cd²⁺ bind nearly stoichiometrically at or near the same position on the RC with a dissociation constant K_{D} ≤ 0.5 µM.

(ii) The first electron transfer rate, k_{AB}⁻(1) (Eq. 1), is reduced ~10-fold, implying a slowing down of the conformational gating step.

(iii) The proton transfer rate to Q'B⁻ is reduced ~10²-fold, making it the rate-limiting step in the k_{AB}⁻(2) reaction (Eq. 2a).

(iv) The large reduction (≥ 10²-fold) in the rate of proton transfer upon stoichiometric binding implies that there is one dominant proton entry point into the RC.

(v) Preliminary X-ray studies localized the Cd²⁺ and Zn²⁺ binding site near His-H126, His-H128, and Asp-H124.

(vi) The simplest explanation of the inhibitory effects of Zn²⁺ and Cd²⁺ on the proton transfer rate is that their binding to the histidine and aspartic acid residues reduces their effectiveness as proton donors.

**Note Added in Proof.** The proposal that the slow rate of proton transfer (Eq. 3) is caused by disruption of the proton donor(s) or blockage of the proton transfer pathway is supported by recent results from the effect of mutations on the observed rate. Replacement of either Asp-M17 or Asp-L210 with Asn resulted in an additional ~10-fold reduction in the observed rate (at 1 mM CdCl₂) from that observed in native RCs. Because the mutation sites are located close to P3 (Fig. 4), these results show that proton transfer in the presence of Cd²⁺ proceeds through P3 or a pathway near P3.

In addition to Zn²⁺ and Cd²⁺, we found that Co²⁺ and Ni²⁺ reduced k_{AB}⁻(2) by ~40-fold and ~100-fold, respectively.

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