Mechanism of Proton Transfer Inhibition by Cd\textsuperscript{2+} Binding to Bacterial Reaction Centers: Determination of the pK\textsubscript{A} of Functionally Important Histidine Residues\textsuperscript{7}

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ABSTRACT: The bacterial photosynthetic reaction center (RC)\textsuperscript{1} from Rhodobacter sphaeroides is a transmembrane protein complex that catalyzes the light-induced electron and proton transfer reactions leading to reduction and protonation of a bound quinone molecule Q\textsubscript{B} (eq 1) (1, 2). Light absorbed by the

\[ Q_B + 2e^- + 2H^+ + 2hv \rightarrow Q_BH_2 \]  

RC initiates the photoionization of the primary donor, D, a bacteriochlorophyll dimer. Electrons are transferred via a bacteriochlorophyll and bacteriopheophytin to the primary quinone Q\textsubscript{A} and then to the secondary quinone Q\textsubscript{B}. In cell membranes, the protons required for the reduction of the quinone to quinol (eq 1) come from the cytoplasm. The Q\textsubscript{B}H\textsubscript{2} leaves the RC (3–6) and is oxidized by the cytochrome bc\textsubscript{1} complex, thereby releasing the quinol protons into the periplasm. This creates a proton gradient across the membrane that drives ATP synthesis (3).

The importance of His side chains for enzyme catalysis has been well documented (7–8). In the bacterial RC, two surface His have been shown to be important for catalysis of proton transfer associated with quinol formation (eq 1) (9). The determination of the proton affinities (i.e., pK\textsubscript{A} values) of these groups by measuring the pH dependence of the binding of the proton transfer inhibitor Cd\textsuperscript{2+} which has been shown to bind to these surface His (10). Thus, information of the thermodynamics of proton and metal binding can be obtained from the pH dependence of the dissociation constant, K\textsubscript{D} (11–13).

The overall protonation reaction takes place in two sequential light-induced electron transfer reactions. The first light-induced reaction results in electron transfer to Q\textsubscript{B} (k\textsubscript{AB}(\textsuperscript{1})) that is coupled to the protonation of a nearby carboxylic group Glu-L212 (Glu\textsuperscript{−} in eq 2):

\[ k_{AB}^{(1)} \]

\[ (Q_A^+\overset{K_{d(1)}}{\rightarrow}\overset{K_{e(1)}}{\rightarrow}Q_B^+\overset{K_{d(2)}}{\rightarrow}\overset{K_{e(2)}}{\rightarrow}Q_B^+\overset{K_{d(3)}}{\rightarrow}\overset{K_{e(3)}}{\rightarrow})Glu^+ \]  

where k\textsubscript{H\^{+}(1)} and k\textsubscript{out(1)} are the rate constants for proton and electron transfer, respectively. The second light-induced reaction results in electron transfer (k\textsubscript{AB}(\textsuperscript{2})) that is coupled to the first direct protonation of the semiquinone. The mechanism of the proton-coupled electron transfer reaction
(eq 3) was shown to be a two-step process in which fast protonation precedes rate-limiting electron transfer (14):

\[
Q_A^+Q_B^- + H^+ \xrightarrow{k_{H+}^{(2)}} Q_A^+Q_B^H \xrightarrow{k_{eq(2)}} Q_AQ_B^H^-(3)
\]

where \(k_{H+}^{(2)}\) and \(k_{eq(2)}\) are the rate constants for proton and electron transfer, respectively. Following the finding that the RC could be copurified with an externally bound Zn\(^{2+}\) (15), a systematic study of transition metal binding showed that Zn\(^{2+}\) and Cd\(^{2+}\) bound stoichiometrically (pH 7.8) to the RC surface. The binding of Cd\(^{2+}\) inhibited the protonation step, \(k_{H+}^{(2)}\) (eq 3), which made proton uptake the rate-controlling step (16). The exact mechanism of the inhibition has been a matter of debate (9, 13, 16).

Subsequent internal proton transfer of H\(^+\) from Glu-L212 (eq 4) leads to the formation of quinol. Note that although

\[
(Q_AQ_B^H^-)\rightarrow\text{GluH}\rightarrow(Q_AQ_B^H)\rightarrow\text{Glu}^-(4)
\]

this H\(^+\) is taken up from solution during the first electron transfer step (eq 2), it is not transferred to reduced Q\(_B^+\) until after the second electron transfer step (eq 3).

The protons involved in the reactions described by eqs 1–4 are taken up from the cytoplasm. Several key components of these pathways have been determined from studies of the effects of site-directed mutations in isolated RCs (17–22). Three residues (Glu-L212, Ser-L223, and Asp-L213), located near Q\(_B^+\) (±5 Å) were shown to be crucial (reviewed in ref 27). The entry point of the protons was more recently identified to be near His-H126, His-H128, and Asp-H124 (Figure 1), which is the binding position of the proton transfer inhibitors Zn\(^{2+}\) and Cd\(^{2+}\) (10). The importance of His-H126 and His-H128 in facilitating proton transfer into the RC was subsequently established through site-directed mutagenesis (9).

The binding of metal ions inhibits proton transfer reactions in other membrane-bound systems such as voltage-gated proton channels (11) and the cytochrome bc\(_1\) complex (12). Studies of the pH dependence of metal binding in these systems suggested that the metal ion ligates to a site with a pK\(_A\) near that of a His side chain (11, 12). In the isolated RC, the proton transfer inhibitors Zn\(^{2+}\) and Cd\(^{2+}\) bind at the protein surface to His-H126, His-H128, and Asp-H124 (10).

In this work, we extend previous reports on the pH dependence of metal ion binding in the RC (13, 24) to obtain information on the proton affinities of the metal ion ligands, i.e., pK\(_A\) values. The determination of the pK\(_A\) of the His is important for understanding the rate and energetics of the process of proton transfer through the protein to the catalytic Q\(_B^+\) site. We report on the pH dependence of Cd\(^{2+}\) binding to the native RC and to mutant RCs lacking one of the side chains of the residues that form ligands to Cd\(^{2+}\), His-H126, His-H128, and Asp-H124. We used Cd\(^{2+}\) rather than Zn\(^{2+}\) for these studies because of the greater solubility of Cd\(^{2+}\) and the tendency of the RC to precipitate at high Zn\(^{2+}\) concentrations. This allowed us to determine \(K_0\) over a pH range of ~5 units. A preliminary account of this work has been presented (24).

**MATERIALS AND METHODS**

**Reagents and Quinones.** Q\(_{10}\) (2,3-dimethoxy-5-methyl-6-decaisoprenyl-1,4-benzoquinone; Sigma) was prepared in ethanol, dried under nitrogen, and solubilized in 1% DDAO (lauryldimethylamine N-oxide; Fluka). To maintain pH, we used a mixture of buffers consisting of 2 mM Ches [2-(N-cylohexylamino)ethanesulfonic acid; Sigma], Tris [tris(hydroxymethyl)aminomethane; Boehringer Mannheim], Hepes [N-(2-hydroxyethyl)piperazine-N\(^-\)2-ethanesulfonic acid; Merck], and Mes (4-morpholinoneethanesulfonic acid; Calbiochem). The pH was adjusted by adding NaOH or HCl. Different amounts of Cd\(^{2+}\) were added to the RC samples as follows: CdCl\(_2\) was dissolved in water to make a stock solution (80 mM) whose concentration was determined (±3%) using atomic absorption, and a series of dilutions were made down to 0.1 mM. Typically, less than 10 \(\mu\)L was added to a 1 mL sample. All reagents were of analytical grade.

**Site-Directed Mutagenesis and Preparation of Reaction Centers.** The His-H126 \(\rightarrow\) Ala [HA(H126)], His-H128 \(\rightarrow\) Ala [HA(H128)] and Asp-H124 \(\rightarrow\) Asn [DN(H124)] mutant RCs were constructed as described (9, 25). RCs from \(Rb.\) *sphaeroides* R26.1 and mutant strains were purified to a ratio \(A_{800}/A_{800}^{\text{so}}\) of \(\leq\)1.3 in DDAO, and the RC concentration was determined from the \(A_{802}^{\text{so}}\) using \(\epsilon = 288\) mM\(^{-1}\) cm\(^{-1}\) as described (26). The Q\(_B\) site was reconstituted by addition of a 3–4 times molar excess of Q\(_{10}\) in 1% DDAO, followed by dialysis against 15 mM Tris, 0.1 mM EDTA, and 0.04% dodecyl \(\beta\)-d-maltoside (Dojindo, Gaithersburg, MD).

**Electron Transfer Measurements.** Absorbance changes in response to a laser flash were measured using a setup of local design (27). Actinic illumination was provided by a Nd:YAG laser (Optek, Carlsbad, CA). The pseudo-first-order rate constant \(k_{AB}^{(2)}\) for electron transfer (eq 3) was determined from transient absorbance changes monitored at 450 nm following the second of two single laser flashes (\(\lambda = 800\) nm). The transient spectral changes resulting from the decay of the semiquinone absorbance at 450 nm (10–15, 25) were fitted to the sum of two exponential terms:
\[ \Delta A(t) = A_F e^{-k_F t} + A_S e^{-k_S t} \]  

(5)

where \( A_F \) and \( k_F \) are the amplitude and rate constants, respectively, for the fast component (given by the observed rate in the absence of added metal) and \( A_S \) and \( k_S \) are the amplitude and rate constant for the slow component (given by the observed rate in the presence of saturating amounts of metal). In this work we are mainly interested in the slow component. The absorbance at times longer than \( 1/k_{AB}^{(2)} \) is due to the absorbance change of horse heart cyt c, which is used as an external reductant for the light-induced oxidized RC donor, D$^+$. Conditions were 1 \( \mu \)M RCs, 30 \( \mu \)M horse heart cyt c, and 0.1% dodecyl \( \beta \)-D-maltoside at 21 °C.

**Fittings**. All fittings were performed using the nonlinear fitting algorithm Origin 6.1 (OriginLab Corp.).

**Model for the Dependence of \( k_{AB}^{(0)} \) on Cd$^{2+}$ Concentration.**

The dependence of \( k_{AB}^{(0)} \) on the concentration of Cd$^{2+}$ (eq 4) was modeled with a simple binding curve equation (eq 6):

\[ f(\text{RC Cd}^{2+}) = \frac{A_S}{A_S + A_F} = \frac{[\text{Cd}^{2+}]}{[\text{Cd}^{2+}] + K_D} \]  

(6)

where \( f(\text{RC Cd}^{2+}) \) is the fraction of the RC population with a bound Cd$^{2+}$. \([\text{Cd}^{2+}]\) is the concentration of free Cd$^{2+}$, and \( K_D \) is the dissociation constant (7). At the higher concentrations required to bind Cd$^{2+}$ at lower pH, there is likely some nonspecific binding to the RC surface. This would result in a small correction of the concentration of free Cd$^{2+}$ in solution. However, since \([\text{RC}] \ll K_D\), this correction is negligibly small.

**Model for the pH Dependence of \( K_D \).**

To fit the observed pH dependence of \( K_D \), we first propose a simple model based on the known crystal structure of the Cd$^{2+}$-bound complex (10). This model entails the Cd$^{2+}$ interacting with one titratable nitrogen atom in each of His-H126 and His-H128 as illustrated in the scheme:

\[
\begin{align*}
\text{RC + Cd}^{2+} + 2H^+ & \rightleftharpoons \text{RCCd}^{2+} + 2H^+ \\
\text{RCH}^+ + \text{Cd}^{2+} + H^+ & \rightleftharpoons \text{RCH}^+ \text{Cd}^{2+} + H^+ \\
\text{RCH}^+ \text{H}^+ + \text{Cd}^{2+} & \rightleftharpoons \text{RCH}^+ \text{Cd}^{2+}
\end{align*}
\]

(7)

where \( K_A(1) \) and \( K_A(2) \) are the proton dissociation constants for each of the two titrating nitrogen atoms [having \( pK_A(1) \) and \( pK_A(2) \), respectively] and \( K_D^{(0)} \) is the dissociation constant with both titrating sites deprotonated. The simplest model to fit our data includes direct competition between Cd$^{2+}$ and \( H^+ \) for the same site. From an analysis of the reactions, we derive an expression for \( K_D^{\text{obs}}(\text{Cd}^{2+}) \):

\[
K_D^{\text{obs}}(\text{Cd}^{2+}) = (1 + 10^{pK_A(1)-pH} + 10^{pK_A(1)-pH}10^{pK_A(2)-pH})K_D^{(0)}
\]  

(8)

This is the same equation as used to fit similar data in other studies (11-13), except that we neglect binding of the metal ion to the singly and doubly protonated states (RCH$^+$Cd$^{2+}$ or RCH$^+$H$^+$(Cd$^{2+}$) which are not observed in our experiments (see Results).²

**RESULTS**

**Effect of Cd$^{2+}$ on Reaction Kinetics.**

The first step in obtaining a dissociation constant \( K_D \) for Cd$^{2+}$ is to determine the fraction of RCs with a bound Cd$^{2+}$. For the RC, the addition of Cd$^{2+}$ decreases the rate of the second electron transfer (eq 3). As shown previously, the rate of the overall reaction decreases as the concentration of Cd$^{2+}$ increases (Figure 2) and is described by an increase in the fraction of RCs displaying a slow decaying component (eq 5) (13, 16, 24). It was also shown that the slower rate for the fraction of RCs with a bound Cd$^{2+}$ is a consequence of a decrease in the rate of proton uptake (16) caused by Cd$^{2+}$ binding to surface His (10). Thus, the observed reaction was fitted to the sum of two exponentials as shown in eq 5. From the relatively amplitude of the slower decaying component (eq 6), the fraction of RCs with a bound Cd$^{2+}$ was determined.

**Determination of the Dissociation Constant \( K_D \).**

From a study of the fraction of RCs with a bound Cd$^{2+}$ as a function of the Cd$^{2+}$ concentration, we obtained a binding curve (Figure 3). The data were fitted to a classic binding curve equation (eq 6), yielding a value for the dissociation constant \( K_D \). The fit of the data at pH 7 yielded a value for \( K_D^{(0)} \) of \( 0.4 \pm 0.1 \mu M \).

These measurements were repeated at different values of pH ranging from 4.5 to 8.5 (Figure 4). Over this range the values for \( K_D \) and \( k_f \) (eq 5) were sufficiently different (Figure 4) to allow the determination of \( K_D \) from fits to the binding data. Below pH 4.5 and above pH 8.5, \( K_D \) became difficult to determine because \( K_D \) approached the solubility limit of Cd$^{2+}$ (lower pH) or of its hydroxide and carbonate forms (higher pH).

**Fitting the Observed pH Dependence.**

The values for \( K_D \) at different values of pH are shown in Figure 5. We analyzed the pH dependence using eq 8 which was obtained from the

² The \( pK_A \) values in eq 8 are for the first and second protons, respectively. Each value is a weighted average of the intrinsic \( pK_A \) values of the imidazole groups of the two His that share the proton.
reaction scheme of eq 7. From the fit to the data, we obtain for the three free parameters the following values:

\[
\begin{align*}
K_D^0 &= 0.4 \pm 0.1 \mu M \\
pK_A(1) &= 6.8 \pm 0.5 \\
pK_A(2) &= 6.3 \pm 0.5 \\
pK_A(1) - pK_A(2) &= 0.5 \pm 0.3
\end{align*}
\]  

Attempts to fit the data to one titrating site, e.g., by eliminating the third term in eq 8, did not reproduce the steep pH dependence of \(K_D\) observed below pH 6 (see dashed line in Figure 5). In other words, the steep dependence was adequately described only by a competition between Cd\(^{2+}\) and two H\(^+\). Inclusion of the singly and doubly protonated Cd\(^{2+}\)-bound states (RCH\(^+\)Cd\(^{2+}\) or RCH\(^+\)H\(^-\)Cd\(^{2+}\)) would result in a curve that is less pH dependent at lower pH, which is in contradiction with the data; \(K_D\) values for Cd\(^{2+}\) binding to either RCH\(^+\) or RCH\(^+\)H\(^-\) would be greater than 10 mM (see highest measured value for native RC at low pH in Figure 5).

\[3 \text{ This is equivalent to a } pK_A \text{ shift greater than 5 units upon binding of Cd}^{2+} \text{ [i.e., } \log(K_D/K_D^0) > 5 \text{]. Such a large shift cannot be explained by an electrostatically induced } pK_A \text{ shift of a more central amino acid (e.g., Asp-L213) due to Cd}^{2+} \text{ binding since the Coulombic interaction } (r = 14.4 \text{ eV/e} \Delta G) \text{ would require the distance } r \text{ to be } <0.5 \text{ Å even for a dielectric constant } e \text{ of 80 (or an even shorter distance for a lower value of } e). \text{ Therefore, the proton displaced upon binding of Cd}^{2+} \text{ comes from one of its ligands (e.g., His-H126, His-H128, or Asp-H124) rather than from a farther amino acid.}\]

FIGURE 3: Binding curves generated from the concentration dependence of the observed kinetics at different pH values as indicated. The fraction bound was determined from the relative amplitude of the slow component (eq 6). The data were fitted to a single binding site model (eq 6) from which the value for \(K_D\) was determined. Note that the binding curves are shifted to the right with decreasing pH resulting in larger \(K_D\) values at lower pH. This is due to a competition between the metal binding and proton binding to the same sites; i.e., as the proton concentration increases at lower pH, higher concentrations of metal ions are needed to achieve 50% binding. (Conditions: same as in Figure 2.)

FIGURE 4: Comparison of the pH dependence of \(k_{AB}^{(2)}\) for the native RC in the absence of Cd\(^{2+}\) and the presence of saturating Cd\(^{2+}\). The rate constant in the absence of Cd\(^{2+}\) is \(k_f\) and that + Cd\(^{2+}\) is \(k_S\) (see eq 5). Note that the ratio of the rate constants is \(\sim 10\) over the pH range from 4.5 to 8.5 over which the \(K_D\) data were determined and modeled. (Conditions: same as in Figure 2.)

FIGURE 5: Dissociation constant of Cd\(^{2+}\), \(K_D\), as a function of pH for the native RC. The data were fitted to a model with one titrating metal binding ligand (dashed line, eq 8, \(pK_A = 7.3, K_D^0 = 0.4 \mu M\)) and two titrating metal binding ligands (solid line, eq 8, \(pK_A(1) = 6.8, pK_A(2) = 6.3, K_D^0 = 0.4 \mu M\)). Note that only the model with two titrating ligands fits the observed data at all pH values, whereas the single titrating ligand model fits only above pH 6. Above pH \(\sim 7.5\) the values for \(K_D\) remain essentially unchanged as the pH is increased. (Conditions: same as in Figure 2.)

**DISCUSSION**

The major function of the RC is to catalyze the light-induced reduction of a quinone molecule to a quinol in the QB site of this membrane-bound protein. This process requires the uptake of two protons from the aqueous solution (eq 1) and transports them through proton transfer pathways
that originate near the surface at two His side chains (Figure 1) (9, 10, 16). To obtain a complete understanding of the proton transfer process, a determination of the energy level of the various protonation states (i.e., \( pK_A \) values) is required. The identification of surface His at the proton pathway entry point (9, 10) led us to determine their \( pK_A \) values by studying the thermodynamic competition between the proton transfer inhibitor \( \text{Cd}^{2+} \) and \( \text{H}^+ \). To prove that the His side chains contribute to the observed pH dependence, measurements were made using site-directed mutant RCs in which each His (i.e., \( \text{Cd}^{2+} \) ligand) was changed to a nonionizable side chain.

\( pK_A \) of the Surface His. The fit of the pH dependence of \( K_D \) for \( \text{Cd}^{2+} \) (Figure 5) indicates that two titrating groups, each with an \( pK_A \) value near 7, are important for the binding of \( \text{Cd}^{2+} \). Furthermore, at low pH the slope of the logarithm of \( K_D \) versus pH approached 2, showing that two protons were released upon \( \text{Cd}^{2+} \) binding.

The apparent \( pK_A \) values of 6.8 and 6.3 for the two protons (eq 9) (approximately that of a proton bound to a His imidazole group) and the crystal structure of the metal-bound complex (Figure 1) suggested the involvement of His-H126 and His-H128. To confirm this assignment, the pH dependence of \( K_D \) (Figure 5) indicates that two titrating groups, each His binds one \( \text{H}^+ \) that is displaced by the binding of \( \text{Cd}^{2+} \) (Figure 7). This results in the slope of \( \sim 2 \) for the pH dependence of \( \log(K_D) \) (see pH \(<6 \) in Figure 5) and shows that \( \text{Cd}^{2+} \) binding is electrostatically compensated. At \( pK_A \) values close to 7, the slope of the pH dependence of \( K_D \) varies in response to the protonation states of the His. We assume that Asp-H124 remains ionized throughout this pH range.

The changes in the values for \( K_D \) of the mutant RCs compared to the native RC reflect the different binding of \( \text{Cd}^{2+} \). The slowing down of the kinetics observed upon addition of \( \text{Cd}^{2+} \) in the mutant RCs shows that \( \text{Cd}^{2+} \) binds near the same proton entry point. However, we do not observe a pH dependence indicative of ligation to a protonated His. Therefore, in the single His mutant RCs, \( \text{Cd}^{2+} \) either

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**Figure 6:** (a) Dissociation constant \( K_D \) of \( \text{Cd}^{2+} \) as a function of pH in the His-H126 → Ala [HA(H126)] and His-H128 → Ala [HA(H128)] mutant RCs (■) compared to the native RC (○). Note that the pH dependence of \( K_D \) is significantly altered in the mutant RCs lacking the imidazole group of His-H126, His-H126 → Ala [HA(H126)], or of His-H128, His-H128 → Ala [HA(H128)]. (b) Dissociation constant \( K_D \) of \( \text{Cd}^{2+} \) as a function of pH for the Asp-H124 → Asn [DN(H124)] mutant RC (■) compared to the native RC (○). Note that, in contrast to the approximate pH independence of \( K_D \) observed in the His mutant RCs, the pH dependence of \( K_D \) in the Asp-H124 → Asn RC is similar to that observed for the native RC with an \( \sim 1 \) unit \( pK_A \) shift to lower pH attributable to the change in charge resulting from the amino acid replacement. (Conditions: same as in Figure 2.)

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**Figure 7:** Structural schematic illustrating proton displacement by \( \text{Cd}^{2+} \) binding. Upon binding of \( \text{Cd}^{2+} \) at pH \(<6 \) two protons \( (2\text{H}^+) \) are released, one from His-H126 and one from His-H128. Thus, at \( \text{pH} < pK_A(1) \), \( pK_A(2) \) the binding of \( \text{Cd}^{2+} \) is electrostatically compensated by the release of two protons. Consequently, the binding of \( \text{Cd}^{2+} \) at low pH competes with the binding of two protons, which leads to the strong pH dependence of \( K_D \) (Figure 5).
pK_A of His at Proton Entry Point in RCs

binds at a different (but nearby) site or the His is not protonated near pH 7.

The pK_A of the His at the proton transfer entry point provides an important parameter for refining the energy profile of the proton transfer process (28). It can be summarized as follows: the proton transfer process involves rapid reversible protonation of the His followed by proton transfer uphill by ~240 meV to an intermediate state. The energy barrier reflects the difference in pK_A between the surface His (pK_A of 6.8) and the intermediate (pK_A of ~3; 28). This is followed by proton transfer to the terminal proton acceptor group, which is either Glu-L212 (eq 2) or Qb^- (eq 3).

Mechanism of Inhibition of the Bound Cd^{2+} on H^+ Transfer. The inhibition of proton uptake by the binding of metal ions (e.g., Cd^{2+}) has been previously shown (13, 16, 28, 29). In the native RC, proton transfer k_H^+(2) is not rate controlling for eq 3, and hence k_{AB}^{(2)} was not a direct measure of k_H^+. Recently, using chemical rescue of a mutant RC lacking both His-H226 and His-H128, the rate constant for proton transfer was determined to be ~2 x 10^5 s^{-1} in the pH range from 8.5 to 9.5 (28). Note that, in the native RC, k_H^+ is ~20-fold greater than k_{AB}^{(2)}. In contrast, in the presence of a bound Cd^{2+}, proton transfer became rate controlling, and hence k_{AB}^{(2)} = k_H^+(2) ~ 40 s^{-1} (Figure 4). Hence, upon Cd^{2+} binding the observed change in k_{AB}^{(2)} of ~20-fold (pH 8) is the result of an even larger ~400-fold change in k_H^+.

Any proposed mechanism of inhibition must account for this amount of reduction.

Two possible explanations for the mechanism of inhibition of proton transfer by binding of Cd^{2+} have been discussed: (1) the metal ion binds to the His, thereby eliminating their function as proton donors/acceptors in the proton transfer step (9, 16); (2) the metal ion electrostatically induces pK_A shift(s) of other functional group(s) (13), as a consequence of which protons are released. Although in principle both (1) and (2) could contribute to the overall decrease of proton transfer, the majority of the effect can be attributed to explanation 1; i.e., the metal ion binding inhibits the His functions as proton donors/acceptors. This conclusion is based on the similarity of the 400-fold decrease in k_H^+ upon Cd^{2+} binding (13, 16, 24) to the 200-fold decrease in RCs in which the function of the His as proton donors was eliminated by removal of the imidazole groups of His-H126 and His-H128 by site-directed mutagenesis (His → Ala) (double His mutant) (9). The 2-fold difference between RCs with a bound Cd^{2+} and the double His mutant is small compared to the main 400-fold effect and may be due to an electrostatic or structural effect.

Additional results from this work provide further evidence that favors mechanism 1 over mechanism 2. Below pH 6, the binding of a Cd^{2+} ion leads to a displacement of approximately two protons (Figure 5), showing that there is no net change in charge upon binding of Cd^{2+}. The results also show that these protons are associated with His-H126 and His-H128. Thus, their ability to function as proton donors/acceptors is severely inhibited as required by mechanism 1. In contrast, the necessary electrostatic change required by mechanism 2 to affect pK_A values of other amino acid groups is inconsistent with the charge compensatory displacement of two protons. Furthermore, the pH dependence of the charge recombination rate constant k_{BD} (D^+Qb^-Qb→D^+Q_A^-Qb→DQ_AQb) is essentially the same in RCs with and without Cd^{2+}, indicating essentially no change in the energy of the D^+Qb^-Qb state upon binding of Cd^{2+} (29). It is difficult to attribute a 10^4-fold decrease in k_H^+(2) to an electrostatically induced pK_A shift of internal groups (required by mechanism 2) in the absence of a change in the energy of the D^+Qb^-Qb state.

Thus, the mechanism of inhibition of proton transfer by Cd^{2+} and other metals is predominantly a consequence of competing with protons for binding to His-H126 and His-H128.

Metal Binding in Other Enzymes. In many other systems, metal ion binding has been shown to inhibit function. Divalent metal ions, in particular Zn^{2+} and Cd^{2+}, have been shown to inhibit the catalytic turnover in other proton-transferring proteins, including several channels, e.g., voltage-gated proton channels (30), the M2 channel (31), and the ligand gated N-methyl-d-aspartate NR1/NR2A channels (32), and several proteins involved in proton transfer/translocation, for example, carbonic anhydrase (where it was shown that Cu^{2+} and Hg^{2+} bind to a histidine that reprotonates the Zn^{2+}–OH^-) (33), the cytochrome bc1 complex (12, 34), and cytochrome c oxidase (35, 36). In the avian cytochrome bc1 complex, two Zn^{2+} binding sites were crystallographically resolved, with the most likely inhibitory site showing ligation of the Zn^{2+} to at least one His (34). In cytochrome c oxidase Zn^{2+} was suggested to bind to surface His close to the entrance of a proton transfer pathway (35) as well as at the exit pathway (36). Inhibition of the exit pathway by Zn^{2+} and Cd^{2+} appears to compete with protons because the Zn^{2+} inhibition is 10-fold weaker at pH 6 than pH 7 (36). However, the metal ligands remain unidentified.

We suggest that the inhibition of catalytic activity by binding of metal ions to many different enzymes is a consequence of the common use of His as either a pH sensor or a proton donor/acceptor group (37). His is a likely candidate for these reactions since its pK_A is near the physiological pH ~7, and its pK_A can be “adjusted” over several pH units by the local environment (7, 8). In such cases, information about the properties of functionally important residues, in particular pK_A values, can be obtained from studies of the pH dependence of metal binding affinity as reported here and elsewhere (11, 12).

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REFERENCES