A New Metal-Binding Site in Photosynthetic Bacterial Reaction Centers That Modulates Q_A to Q_B Electron Transfer†

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ABSTRACT: Isolated reaction centers (RCs) from *Rhodobacter sphaeroides* were found to bind Zn(II) stoichiometrically and reversibly in addition to the 1 equiv of non-heme Fe(II). Metal and EPR analyses confirm that Zn(II) is ligated to a binding site that is distinct from the Fe site. When Zn(II) is bound to this site, electron transfer between the quinones Q_A and Q_B ($Q_A^- Q_B \rightarrow Q_A Q_B^-$) is slowed and the room-temperature kinetics become distributed across the microsecond to millisecond time domain. This effect of metal binding on the kinetics is similar to the more global effect of cooling RCs to 2 °C in the absence of Zn(II). This suggests that Zn(II) binding alters localized protein motions that are necessary for rapid $Q_A^- Q_B \rightarrow Q_A Q_B^-$ electron transfer. Inspection of the RC crystal structure suggests a cluster of histidine ligands located beneath the Q_B binding pocket as a potential binding site.

The structurally characterized (*1*−*3*) photosynthetic bacterial reaction center (RC) provides a useful model system to address fundamental questions concerning biological electron transfer. In RCs, electron transfer occurs sequentially after photoexcitation of a bacteriochlorophyll dimer (P), through one set of cofactors, terminating in the electron transfer between two quinones Q_A and Q_B. The electron transfer between Q_A and Q_B is temperature activated (*4*−*6*), coupled to proton movement (*7*, *8*), and believed to be rate limited by protein motion (*9*, *10*). While it is widely accepted that protein conformational changes play an important part in biological electron transfer (*11*−*13*), there is little experimental information on the nature of protein motion coupled to electron transfer. We have discovered a metal-binding site on the RC that regulates electron transfer between Q_A and Q_B possibly by altering the dynamics of conformational changes.

We have found that RCs from *Rb. sphaeroides* R26 stoichiometrically bind Zn(II) and this Zn(II)-binding slows the $Q_A^- Q_B \rightarrow Q_A Q_B^-$ electron transfer. Interestingly, Zn(II) regulates electron transfer from a remote position on the RC as the Zn(II) site is spatially distinct from the non-heme high-spin Fe(II) site which is buried in the protein interior between Q_A and Q_B. The Fe site has been the focus of many studies, yet no definitive role for this Fe(II) has been established. Apparently, the Fe(II) does not facilitate electron transfer between Q_A and Q_B, and substitution of different divalent metal ions into the Fe site does not significantly alter the electron-transfer characteristics (*14*). We have found, however, that divalent metal ions can modulate electron transfer between the quinones when bound to a previously unidentified metal site. Furthermore, we propose that Zn(II) is influencing electron transfer by altering the conformation of a local protein domain, thereby limiting protein motions which are necessary for efficient electron transfer.

EXPERIMENTAL PROCEDURES

Preparation of Zn−*RCs.* RCs from the photosynthetic bacterium *Rb. sphaeroides* R-26 were isolated according to the procedure of Wraight (*15*), adapted by Tiede (*6*) and Utschig (*16*). EDTA was present in all buffers throughout the preparation at a concentration of 10 μM. For the final step of the purification procedure, RCs were eluted from the DEAE-Sephacel column with 10 mM Tris-HCl at pH 7.9, 10 μM EDTA, 280 mM NaCl, and 0.045% lauryldimethylamine *N*-oxide (LDAO) as the detergent. Final 280 nm/802 nm ratios were 1.2/1.3. Protein concentrations were determined with the extinction coefficient $\epsilon_{802} = 288\ \text{mM}^{-1}\ \text{cm}^{-1}$ (*17*).

Typically, the RC protein ($OD_{800} \approx 10-15\ \text{cm}^{-1}$) was incubated at ice temperature with 4−10 mol equiv of $ZnSO_4$ (from a 50 mM stock solution) for 12 h. The incubation buffer contained 10 mM Tris-HCl at pH 7.9, 10 μM EDTA, 150−280 mM NaCl, and 0.045% LDAO. The free Zn(II) was separated from bound metal ion by gel filtration chromatography through a Sephadex G-25 (Pharmacia) column equilibrated with 10 mM Tris-HCl at pH 7.9, 50 mM NaCl, and 0.045% LDAO. Alternatively, the detergent 0.8% octyl-β-glucoside (OG) can be used in place of LDAO. Zn(II) reproducibly binds stoichiometrically in the presence of OG after incubation with 15 equiv of $ZnSO_4$.

To investigate the reversibility of Zn(II) binding, purified RCs and Zn−RCs prepared by gel filtration were dialyzed for 48 h at 4 °C vs Chelex 100 metal-chelating resin (Bio-Rad) in a buffer containing 10 mM Tris-HCl at pH 7.9, 10 μM EDTA, and 0.045% LDAO. Approximately 5 g of Chelex 100 resin/100 mL of buffer was used. The pH of the buffer was adjusted at room temperature after addition and equilibration with the Chelex 100 resin.

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Metal Analysis. Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was used to determine the amount of Fe and Zn bound to the RC. ICP-AES enables rapid sequential multielement determination with minimal sample volume. A Thermo Jarell Ash Auto Scan 25 spectrometer was used for these measurements. The metal content of the RCs was analyzed before and after addition of Zn(II). In addition, Sephadex G-25 column fractions before, during, and after protein elution were analyzed to obtain a complete column profile of the metal content. This ensured that a good separation was obtained between free and protein-bound metal ion and that no contaminating metal ions were present in the column running buffer. The analytical standard deviation for each ICP-AES measurement was at most ± 0.01 metal/RC. The standard deviation of the mean detailed in the results section describes sample-to-sample variations resulting from differences between sample preparations. These values should not be confused with the analytical standard deviation of each individual ICP-AES measurement.

EPR Spectroscopy. Low-temperature Q-band ($\sim$34 GHz) EPR spectra were obtained to determine the amount of $P_{865}^{+}[Fe^{2+}Q_A]^{-}$ or $P_{865}^{+}Q_A^{-}$ formed under continuous illumination. The Q-band data were collected using a Varian E9 EPR spectrometer with a Bruker Q-band ER051QG bridge. Spectra were measured at 12 K with illumination using a Xenon lamp. The dark signal before and after illumination was measured.

Measurement of Optical Absorption Changes Associated with the $Q_A^{-}Q_B \rightarrow Q_AQ_B^{-}$ Electron Transfer. Optical absorption changes associated with quinone anions in transient $P^{+}Q^{-}$ states were identified by recording transient spectra as a function of time between a 30 ns laser excitation pulse at 614 nm and a weak 0.65 μs xenon probe pulse, using a 1024 element, single diode array, multiwavelength detector. The experimental setup has been described previously (*6*).

RESULTS

Metal analysis reveals that RCs isolated from the chromatophore membranes of *Rb. sphaeroides* R-26 (*16*) contain a significant amount of Zn(II), in addition to the 1 equiv of Fe(II) occupying the quinone Fe site. Fourteen different RC preparations were analyzed, resulting in an average ratio of 0.3 ± 0.3 Zn/RC, with 0.3 being the standard deviation due to variations between sample preparations. No additional Zn(II) was added during the purification procedure. Thus, even after several ammonium sulfate precipitations and purification in the presence of EDTA, purified RCs retain a significant amount of Zn(II). We have observed preparations with Zn(II) contents ranging anywhere from 0 to 1 mol equiv of Zn(II)/RC. The Zn(II) that purifies along with the RC is stable to gel filtration chromatography.

Stoichiometric Zn binding (Zn to RC mole ratio of 1) can be obtained by incubating purified RCs with several equivalents of $ZnSO_4$ followed by gel filtration chromatography to remove unbound metal ions.(Figure 1A) Metal analysis of 12 separate chromatography experiments yielded an average value of 1.0 ± 0.2 Zn/RC, with 0.2 being the standard deviation between sample preparations. Five of the above preparations were simultaneously analyzed for Fe content and found to contain 0.9 ± 0.1 Fe/RC in addition to the Zn(II). Thus, a total of 2 mol equiv of metal ion, with 1 mol equiv each of Fe(II) and Zn(II) are bound to the RC. Thus, metal analysis indicates that the Zn(II) does not displace Fe(II). This result is reasonable as it is unlikely that the buried Fe(II) would be displaced simply by incubating intact native RCs with excess Zn(II). Stringent conditions of chaotropic treatment followed by time-specific addition of Zn(II) are needed to remove the Fe(II) from and insert Zn(II) into the Fe site (*16*). Furthermore, treatment with the metal-chelating resin Chelex 100 does not remove metal ions (Fe, Mn, or Zn) bound to the buried Fe site in native RCs (*16*). Dialysis of the Zn−RCs (prepared by the gel filtration procedures detailed above) against Chelex 100, however, does remove Zn(II) bound to the RC. Typically, dialyzing Zn−RCs (1.0 ± 0.2 Zn/RC) vs Chelex 100 resin in buffer for over 48 h reduces the Zn(II) content to <0.3 Zn/RC. Thus, Chelex accessibility suggests that the metal

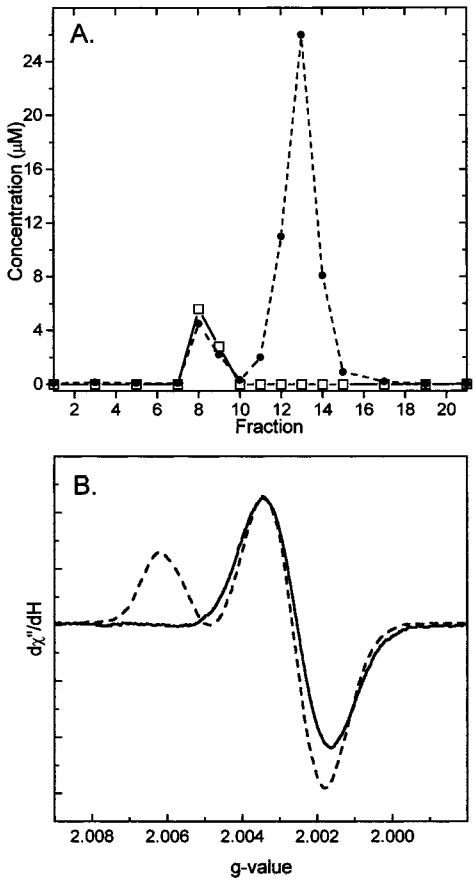

FIGURE 1: Preparation and EPR analysis of Zn−RCs. (A) Zn−RC complexes are readily formed by incubating a 40 μM solution of RCs in 10 mM Tris-Cl, pH 7.8, 10 μM EDTA, 280 mM NaCl, and 0.045% LDAO with 4 equiv of $ZnSO_4$, followed by chromatography through a Sephadex G25 column to separate free Zn(II) from protein bound Zn(II). The running buffer contained 10 mM Tris-Cl, pH 7.8, 50 mM NaCl, and 0.045% LDAO. Zn(II) (circles) and protein concentration (squares) vs fraction collected are displayed. The protein fractions contained 0.8 Zn and 1.2 Fe/RC. (B) The Q-band data were collected at 34 GHz, with 1.5 G modulation amplitude, and 126 μW microwave power. Spectra were measured at 12 K under continuous illumination with a Xenon lamp. The spectra shown are light minus dark signals. The $P^{+}[Zn(II)Q_A]^{-}$ charge-separated state (dashed line) with $g = 2.0026$ (P^{+}) and $g = 2.0049$ (Q_A^{-}) is observed for Fe-removed RCs where Zn(II) has been substituted into the Fe site (*16*). Only the P^{+} signal is observed in the $g = 2$ region for RCs where Zn(II) is bound to Fe-containing RCs (0.8 Zn/RC) by gel filtration (solid line).

ion bound to the newly detected Zn site is more easily exchanged than ions bound to the buried Fe site; hence, the Zn site is possibly located closer to the surface of the protein than the Fe site.

EPR analysis corroborates the metal analysis results: Zn(II) is ligated to a binding site distinct from the quinone Fe site. In native Fe-containing RCs from *Rb. sphaeroides*, both ubiquinones Q_A and Q_B are magnetically coupled to the Fe(II). Upon illumination at 4 K, EPR signals are observed from the radical pair $P^+[Fe(II)Q_A]^-$, with $g = 2.0026$ for P^+ and $[Fe(II)Q_A]^-$ exhibiting a broad resonance centered at $g \approx 1.8$ (*18*, *19*). In the absence of the paramagnetic Fe(II), the quinone signal narrows to that of a typical organic radical with $g = 2.0049$, and at Q-band (34 GHz), the Boltzmann signals from P^+ and Q_A^- partially are resolved (*20*, *21*). When Zn(II) is bound stoichiometrically to native Fe-containing RCs, only a light-induced P^+ signal, and not a Q_A^- signal, is observed in the $g = 2.0$ region (Figure 1B). Thus, when Zn−RCs are prepared by incubation with excess Zn(II) followed by gel filtration chromatography, the Fe(II) is not displaced and remains magnetically coupled to Q_A. For comparison, both P^+ and Q_A^- resonances are observed in the $g = 2.0$ region for chaotropically Fe-removed RCs that have diamagnetic Zn(II) substituted specifically into the Fe site (*16*).

Transient optical measurements show that Zn(II)-binding to the RC modulates the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer. Absorbance changes at 757 nm reflect the differences in the electrochromic response of the bacteriopheophytins to the $Q_A^-Q_B$ and $Q_AQ_B^-$ quinone anion states (*6*). Optical measurements at 757 nm in isolated RCs have been primarily assigned to electron transfer, whereas electrochromic responses at other wavelengths indicate a combination of electron transfer and charge compensating events, such as proton transfer (*22*). Figure 2A shows the time course for absorbance changes associated with the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer for RCs with zero or one Zn(II) bound per RC. Zn(II) binding to the newly discovered site clearly slows down $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer. In native RCs with no Zn(II), $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron-transfer rate is heterogeneous at room temperature consisting of at least two distinct components: a major component (77%) with reaction time of 233 ± 50 μs and a minor component (23%) with reaction time of 37 ± 17 μs (*6*). In the presence of Zn(II), the electron transfer exhibits more distributed kinetics and is slowed, with the absorbance change at 757 nm continuing to build in at 10 ms. The distributed kinetics observed for Zn−RCs cannot be fit as the sum of two exponential components but requires a more dispersive kinetic model (*23*, *24*). Semiquantitative fits using three components results in lifetimes of 50 ± 10 μs (25%) and 340 ± 70 μs (47%) and an additional slow component having a reaction time of 3.2 ± 0.7 ms (28%). Thus, the slowest component of $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer of Zn−RCs is markedly longer than the 233 ± 50 μs slowest component observed in native, non-Zn containing RCs.

No significant difference in the $P^+Q_A^-$ and $P^+Q_B^-$ room-temperature recombination rates for RCs with or without Zn(II) bound was observed. Also, as shown in Figure 2B, the effect of metal binding on the room-temperature $Q_A^-Q_B \rightarrow Q_AQ_B^-$ kinetics is strikingly similar to that of the more

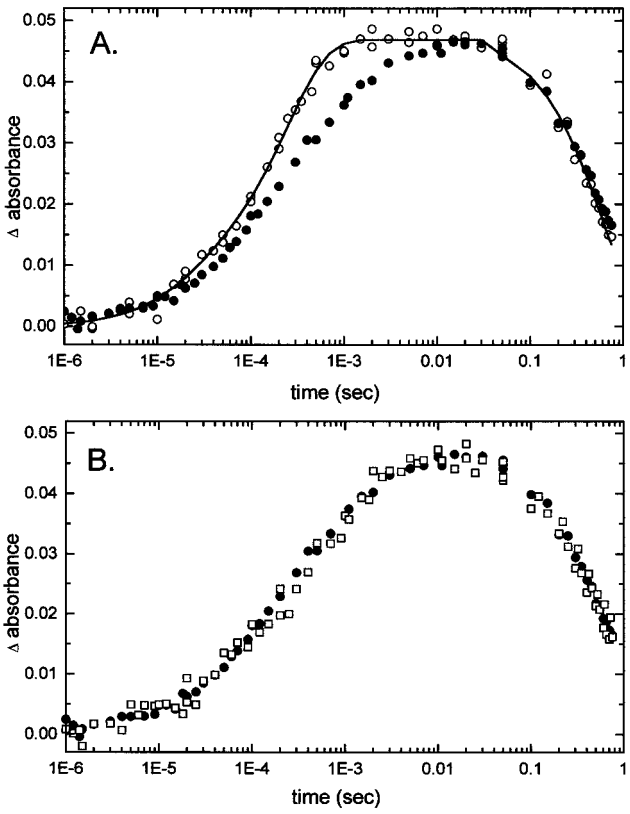

FIGURE 2: Time course for absorbance changes associated with the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron-transfer process in RCs from *Rb. sphaeroides*. (A) Optical absorption changes at 757 nm associated with quinone anions in transient P^+Q^- states were obtained for RCs with and without Zn bound. The data were collected for RCs (0.2 Zn/RC, open circles) and Zn−RCs (1.2 Zn/RC, solid circles) at 21 °C. The Zn−RCs were prepared by gel filtration chromatography. Both the RC and Zn−RC buffers contained 50 mM NaCl. The biexponential fit of RCs at 21 °C is shown (solid line). Fit parameters were $\tau_1 = 27$ μs, $A_1 = 0.21$, $\tau_2 = 258$ μs, and $A_2 = 0.79$. (B) Comparison of the effect of Zn(II) vs temperature on the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer. Data are shown for Zn−RCs (solid circles) at 21 °C and RCs at 2 °C (open squares).

global effect of cooling RCs in the absence of Zn(II) to 2 °C.

DISCUSSION

The similarity of the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ kinetics observed for Zn−RCs at room temperature to those observed for RCs [no Zn(II)] cooled to 2 °C suggests that the Zn(II) is altering important localized protein motions of the RC that are necessary for rapid $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer. A second possibility is that the Zn(II) is simply influencing the electrostatic environment near the quinones. We have several pieces of evidence which rule out a major change in electrostatic potential. First, the electrochromic spectrum of the bacteriopheophytins (BPh), which is extremely sensitive to alterations of charge near Q_A and Q_B, is identical in wild-type and Zn-bound reaction centers. Second, the $P^+Q_A^-$ and $P^+Q_B^-$ recombination rates for RCs with and without Zn(II) are indistinguishable, suggesting that the functional midpoint potential of Q_A/Q_A^- has not changed with respect to the BPh/BPh^- midpoint potential (*25*), nor has the Q_B/Q_B^- redox potential changed with respect to that of Q_A/Q_A^- (*26*, *27*). Third, $Q_A^-Q_B$ to $Q_AQ_B^-$ electron-transfer rates do not become distributed in response to pH changes above the p*K* of a

titratable group in the Q_B pocket (*26*), but rather change monotonically. Therefore, these three pieces of evidence strongly demonstrate that Zn(II) binding is not simply an electrostatic effect.

Zn(II) binding is most likely altering the dynamics of conformational changes in the RC thereby influencing electron transfer. Binding of Zn(II) to a specific coordination site potentially could alter the mobility of the metal ligands and surrounding region of polypeptide as well as change essential localized protein conformations important for electron and proton transfer. The observed distributed kinetics support this structural role for Zn(II). The need for a dispersive kinetic model suggests that the RC exists in a distribution of slowly interconverting conformations that are observed as differences in the rates of the absorbance changes associated with the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer (*6*). Upon Zn(II) binding, a wider distribution is observed than in the absence of Zn(II) at room temperature, indicating that the interconversion between substates is slowed. Thus, we have found a local protein environment that apparently controls protein dynamics important for electron transfer in the RC and that, remarkably, mimics the global temperature effect. These experimental results support the view (*9*, *10*) that $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer is an example of gated electron transfer, i.e., electron transfer is controlled by protein conformational change (*11*, *12*). Zn(II) has been found to be a key structural component in a variety of biological systems, stabilizing conformations of polypeptide domains to facilitate interactions between proteins and other macromolecules (*28*). Our results indicate a possible new role for Zn(II): influencing electron transport by enforcing the conformation of a specific protein domain.

A second metal ion is not observed in the *Rb. sphaeroides* RC X-ray crystal structures (*1*−*3*). Most likely Zn(II) was not bound to the RCs used for crystallization as typical crystallization conditions employ high concentrations of anions, which would be expected to precipitate surface bound Zn(II). Interrogation of the crystal structure for typical Zn(II) ligands, such as histidine and cysteine, reveals a potential metal-binding site that includes a grouping of histidine residues on the surface of the H-subunit (H68, H126, and H128) and the L-subunit (L211). Interestingly, these histidines are positioned beneath the Q_B-binding pocket and surround a water channel that is proposed to be a proton pathway to Q_B (*1*, *29*, *30*).

We are currently investigating the location and coordination environment of the Zn site, as well as the specific mechanism for Zn(II) regulation of electron and proton transport both in isolated and native membrane bound RCs from several species of purple photosynthetic bacteria. Further studies of this Zn site will provide us with important information about the kind of protein dynamics and localized structure that are important for $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer. Potentially, these results could lead to the first experimental determination of sites of protein motions coupled to photosynthetic electron transfer and provide important insight into how local protein environments control energetics and dynamics of nuclear reorientations that are important for efficient electron transfer.

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