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# Iron-Depleted Reaction Centers from *Rhodopseudomonas sphaeroides* R-26.1: Characterization and Reconstitution with $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ †

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ABSTRACT: Reaction centers (RCs) from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26.1 were depleted of Fe by a simple procedure involving reversible dissociation of the H subunit. The resulting intact Fe-depleted RCs contained 0.1–0.2 Fe per RC as determined from atomic absorption and electron paramagnetic resonance (EPR) spectroscopy. Fe-depleted RCs that have no metal ion occupying the Fe site differed from native RCs in the following respects: (1) the rate of electron transfer from $Q_A^-$ to $Q_B$ exhibited nonexponential kinetics with the majority of RCs having a rate constant slower by only a factor of ~2, (2) the efficiency of light-induced charge separation ($DQ_A \rightarrow D^+Q_A^-$) produced by a saturating flash decreased to 63%, and (3) $Q_A$ appeared readily reducible to $Q_A^{2-}$. Various divalent metal ions were subsequently incorporated into the Fe site. The electron transfer characteristics of Fe-depleted RCs reconstituted with $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ were essentially the same as those of native RCs. These results demonstrate that neither $Fe^{2+}$ nor *any* divalent metal ion is required for rapid electron transfer from $Q_A^-$ to $Q_B$. However, the presence of a metal ion in the Fe site is necessary to establish the characteristic, native, electron-transfer properties of $Q_A$. The lack of a dominant role of $Fe^{2+}$ or other divalent metals in the observed rate of electron transfer from $Q_A^-$ to $Q_B$ suggests that a rate-limiting step (for example, a protonation event or a light-induced structural change) precedes electron transfer.

The photochemical events of photosynthesis take place in a membrane-spanning complex of pigment and protein called the reaction center (RC).[1] RCs from the purple non-sulfur bacterium *Rhodopseudomonas sphaeroides* R-26.1 are composed of three 30–35-kDa polypeptides (designated L, M, and H) plus the following cofactors: four bacteriochlorophylls (BChl), two bacteriopheophytins (BPh), two ubiquinones (Q-10), and one atom of high-spin $Fe^{2+}$. A dimer of BChl serves as primary electron donor (D), and the two ubiquinones, $Q_A$ and $Q_B$, serve as primary and secondary electron acceptors, respectively. Between D and $Q_A$ is an intermediate acceptor, I, believed to be monomeric BPh interacting with BChl. Both $Q_A$ and $Q_B$ are magnetically coupled to the $Fe^{2+}$ [for reviews, see Feher & Okamura (1978, 1984), Okamura et al. (1982a,b), Parson & Ke (1982), Wraight (1982) and Crofts & Wraight (1983)].

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[1] Abbreviations: RC(s), reaction center(s); Q-10, ubiquinone-50; Q-0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; cyt, cytochrome *c*; LDAO, lauryldimethylamine *N*-oxide; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, (ethylenedinitrilo)tetraacetic acid; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; EXAFS, extended X-ray absorption fine structure; Da, dalton.

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The role of $Fe^{2+}$ has been examined by extracting it or by replacing it biosynthetically with other metal ions. Growth of bacteria in low-Fe media enriched with other metals (Feher et al., 1974) has yielded RCs containing ~0.6 Mn per RC from *Rps. sphaeroides* R-26.1 (Nam et al., 1984; Okamura et al., 1984), and ~0.9 Mn per RC from *Rps. sphaeroides* wild-type strain Y (Rutherford et al., 1985). Significant incorporation of metal ions other than $Mn^{2+}$ has not yet been obtained, however, and biosynthetic replacement is unlikely to yield RCs *without* a metal ion in the Fe site. In addition, the biosynthetic approach is complicated by the possibility that replacement of $Fe^{2+}$ may be accompanied by compensating mutations in the protein.

Extraction of $Fe^{2+}$ has involved treatment of RCs with detergents (Loach & Hall, 1972; Feher et al., 1972; Okamura et al., 1974; Prince et al., 1977) or *o*-phenanthroline plus chaotropic agents (Feher & Okamura, 1978; Dutton et al., 1978; Blankenship & Parson, 1979; Okamura et al., 1980; Tiede & Dutton, 1981). This method was also successfully employed to extract Fe from RCs of photosystem II of green plants (Klimov et al., 1980). These procedures, however, closely resemble those developed to remove the H subunit (Okamura et al., 1974; Debus et al., 1985a) and results obtained with them are, therefore, characteristic of LM rather than RCs. Indeed, several preparations have been reported to exhibit optical absorption changes characteristic of LM (Debus et al., 1985a).

In this study our approach has been to extract the $Fe^{2+}$ by a simple procedure involving reversible dissociation of the H subunit. Care was taken to remove unreassociated H and LM from the Fe-depleted samples. The resulting intact Fe-depleted RCs were subsequently reconstituted with $Fe^{2+}$ and other metal ions. Reconstitution with $Fe^{2+}$ was necessary to determine whether changes observed upon extraction of Fe resulted from its absence or from irreversible denaturation accompanying its removal. Replacement with other metal ions was necessary to determine whether changes observed in the absence of $Fe^{2+}$ could be specifically attributed to loss of the Fe or to loss of its divalent charge. To examine the role of $Fe^{2+}$ we compared the electron-transfer characteristics of Fe-deleted RCs with those of native RCs and RCs reconstituted with $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$. Preliminary accounts of this work have been presented (Debus et al., 1984, 1985b).

## Materials and Methods

*Materials.* LiSCN and $LiClO_4$ were obtained from Alfa-Ventron, horse heart cyt *c* (type VI) and Q-10 were from Sigma, Q-0 was from Biochemical Laboratories, Inc., LDAO was from Fluka Chemical Corp., and Terbutryn was from Chem Service, Inc. (West Chester, PA). Cyt $c_2$ (*Rps. sphaeroides*) was prepared following the procedures of Bartsch (1978).

The concentrations of 3–4 M stock solutions of LiSCN and $LiClO_4$ in $H_2O$ were determined by atomic absorption spectroscopy (see Metal Analysis) with a 1000 ppm standard of LiCl (Alfa-Ventron).

Solutions of 1–2 mM Q-10 in 10% sodium deoxycholate and 1 mM $cyt^{2+}$ in 10 mM Tris-HCl, pH 8, were prepared as described previously (Debus et al., 1985a). The concentrations of cyt*c* and cyt$c_2$ were determined optically with extinction coefficients of $\epsilon_{550}$(reduced) = 27.6 and 30.8 $mM^{-1}$ $cm^{-1}$, respectively (Margoliash & Frohwirt, 1959; Bartsch, 1978).

*Reaction Centers.* RCs were isolated in LDAO following the procedure of Feher & Okamura (1978) as modified by Sutton et al. (1982) and Debus et al. (1985a). The concentration of RCs was determined with the extinction coefficient $\epsilon_{802}$ = 288 $mM^{-1}$ $cm^{-1}$ (Straley et al., 1973). Typical preparations contained ~2 Q-10 per RC as determined spectrophotometrically (Butler et al., 1980; Okamura et al., 1982a). RCs depleted of $Q_B$ were prepared following the procedures of Okamura et al. (1975), except that 1 mM 2-mercaptoethanol was present throughout.

*Extraction of $Fe^{2+}$.* Our previous method of extracting $Fe^{2+}$ involved reconstitution of intact RCs from isolated H and Fe-depleted LM (Debus et al., 1984, 1985a). This procedure was tedious and limited in scale by the preparation of sufficient H. We have since developed a simple method that allows preparation of Fe-depleted RCs on a large scale. RCs ($A_{802}^{1cm}$ ~ 30 in 10 mM Tris-HCl, 0.025% LDAO, and 1 mM EDTA, pH 8) were dialyzed 2 days at 4 °C against 10 mM Tris-HCl, 0.1% sodium cholate, and 0.1 mM EDTA, pH 7.7, to remove LDAO. They were then incubated 1 h at 4 °C (with $A_{802}^{1cm}$ = 10) in 1.5 M LiSCN, 1 mM *o*-phenanthroline, 10 mM Tris-HCl, 0.03% sodium cholate, and 0.03 mM EDTA, pH 7.7. Because *o*-phenanthroline is only sparingly soluble in the presence of LiSCN, the RCs were incubated 2–3 min with *o*-phenanthroline before LiSCN was added. The LiSCN was employed to reversibly dissociate the H subunit to expose the $Fe^{2+}$ to *o*-phenanthroline. Unlike $LiClO_4$ (Feher & Okamura, 1978; Debus et al., 1985a), LiSCN does not precipitate H. Following incubation, the RCs were dialyzed 24–30 h at 4 °C against 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7, to remove the $Fe^{2+}$ and allow H to reassociate. For most preparations, a 1–2-fold molar excess of Q-10 was added from a solution of 1–2 mM Q-10 in 10% sodium deoxycholate prior to dialysis.

Following dialysis, the "crude" Fe-depleted RCs containing unreassociated H and LM were brought to a concentration of 0.1% LDAO and 0.2 M $LiClO_4$ and passed at 4 °C through a column of organomercurial agarose (Affi-Gel 501, Bio-Rad Laboratories) that had been equilibrated with 50 mM Tris-HCl, 0.1% LDAO, 0.025% sodium cholate, and 0.2 M $LiClO_4$, pH 7.7. RCs and H adsorbed to the column; LM did not. This is presumably because RCs and H possess at least one exposed sulfhydryl, whereas LM does not (Rosen, 1979). Equilibrating the column with buffers containing EDTA severely reduced its capacity for binding RCs. LDAO and $LiClO_4$ eliminated nonspecific adsorption of LM. Following removal of LM the column was washed to remove $LiClO_4$, and the RCs were eluted with 50 mM cysteine, 0.1% LDAO, 0.025% sodium cholate, and 100 mM Tris-HCl, pH 7.7. H appeared to adsorb irreversibly to the column. This may reflect strong nonspecific adsorption of H to the agarose. Following elution the purified Fe-depleted RCs were dialyzed 18–24 h at 4 °C against 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7. For most preparations, a 1–2-fold molar excess of Q-10 in sodium deoxycholate was added again prior to dialysis. The overall yield of purified Fe-depleted RCs from native RCs was ~60%.

Fe-depleted RCs were depleted of $Q_B$ following the procedures of Okamura et al. (1975), except that 1 mM 2-mercaptoethanol was present throughout.

*Reconstitution with $Fe^{2+}$.* Fe-depleted RCs were concentrated to $A_{802}^{1cm}$ ~ 100 in a Centricon-30 microconcentrator (Amicon Corp.), diluted to $A_{802}^{1cm}$ ~ 8 with a deoxygenated solution of 1 mM $Fe(NH_4)_2(SO_4)_2$, 0.2 M LiSCN, 0.025% sodium cholate, and 10 mM Tris-HCl, pH 7.7, and incubated ~18 h at 4 °C [after Klimov et al. (1980)]. The RCs precipitated during incubation. Following incubation, the RCs were dialyzed anaerobically 1–2 days against 10 mM Tris-

Table I: Comparison of Characteristics of Native, Fe-Depleted, and Reconstituted RCs

| RC | metal content by atomic absorption (mole fraction) | EPR $g$ value (narrow peak) (±0.0001) | extent of charge separation[h] (%) (±3%) | electron transfer times $k_{AB}^{-1}$[e] ($\mu$s) (±10%) | $k_{BD}^{-1}$[f] (s) (±10%) | $k_{AD}^{-1}$[g] (ms) (±5%) | steady-state Q-0 photoreduction rate[i] ($s^{-1}$) (±5%) | $K_I$,[j] terbutryn ($\mu$M) (±30%) |
|---|---|---|---|---|---|---|---|---|
| native | 1.06 ± 0.05 Fe[a] | 2.0026 | 100 | 150 | 1.65 | 115 | 277 | 1.6 |
| Fe depleted | 0.19 ± 0.02 Fe[a] | 2.0037 | 63 | 350 | 5.0 | 200[d] | 85 | 11 |
| $+Fe^{2+}$ | 1.4 ± 0.1 Fe[a] | 2.0028 | 100 | 170 | 1.50 | 119 | 243 | 1.8 |
| $+Mn^{2+}$ | 0.83 ± 0.03 Mn[b] | 2.0027 | 103 | 160 | 1.45 | 127 | 241 | ND |
| $+Co^{2+}$ | 0.79 ± 0.05 Co[c] | 2.0028 | 100 | 170 | 1.70 | 122 | 228 | ND |
| $+Ni^{2+}$ | 0.78 ± 0.08 Ni[c] | 2.0029 | 99 | 160 | 1.70 | 120 | 200 | ND |
| $+Cu^{2+}$ | 0.86 ± 0.05 Cu[c] | 2.0027 | 101 | 150 | 2.3 | 118 | 222 | ND |
| $+Zn^{2+}$ | 0.78 ± 0.05 Zn[c] | 2.0037 | 103 | 140 | 1.20 | 130 | 230 | 1.5 |

[a] Sample also contained 0.05 ± 0.02 Mn. [b] Sample also contained 0.19 ± 0.02 Fe. [c] Sample also contained 0.19 ± 0.02 Fe plus 0.05 ± 0.02 Mn. [d] $k_{AD}^{-1}$ = 110 ± 5 ms in samples without $Q_B$ and without terbutryn present. [e] Conditions as in Figure 5. [f] Conditions as in Figure 3, but with 40 $\mu$M Q-10 and 0.2% sodium deoxycholate present. [g] Measured in the presence of terbutryn. Conditions as in Figure 3. [h] Measured 1 ms following excitation. Conditions as in Figure 3. [i] The initial steady-state rate of oxidation of cyt $c^{2+}$ by RCs illuminated in the presence of Q-0 (cyt $c^{2+}$ per RC oxidized per second). Conditions: 0.02 $\mu$M RC, 100 $\mu$M Q-0, 10 $\mu$M cyt $c^{2+}$, 0.025% LDAO, and 10 mM PIPES, pH 6.8; 21 °C; light intensity = 1.2 W/cm$^2$. [j] Measured from the kinetics of charge recombination as a function of the concentration of terbutryn. Conditions as in Figure 3.

HCl, 0.025% sodium cholate, and 1 mM EDTA, pH 7.7, resuspended, brought to 0.1% LDAO, and chromatographed on a column of DEAE-cellulose. The yield of Fe-reconstituted RCs from Fe-depleted RCs was ~50%. For some experiments the reconstituted RCs were incubated with 65 mM dithiothreitol and 1 mM *o*-phenanthroline overnight and rechromatographed on a column of DEAE-cellulose.

*Reconstitution with $Co^{2+}$.* Fe-depleted RCs were concentrated to $A_{802}^{1cm} \sim 100$, diluted to $A_{802}^{1cm} \sim 8$ with an anaerobic solution of 1 mM $CoCl_2$, 0.025% sodium cholate, and 10 mM potassium phosphate, pH 7.7, and incubated ~18 h at 4 °C. Following incubation, the reconstituted RCs were dialyzed anaerobically 2 days against 10 mM Tris-HCl, 0.025% sodium cholate, and 1 mM EDTA, pH 7.7.

*Reconstitution with $Mn^{2+}$, $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$.* Fe-depleted RCs ($A_{802}^{1cm} \sim 8$ in 10–50 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7) were incubated ~18 h at 4 °C with 10 mM $MnSO_4$, or 1 mM $NiCl_2$, $ZnCl_2$, or $CuSO_4$, and then dialyzed 2 days against 10 mM Tris-HCl, 0.025% sodium cholate, and 1 mM EDTA, pH 7.7.

*Metal Analysis.* Metal contents were determined with an atomic absorption spectrometer (Varian-Techtron AA-5) with an acetylene/air flame or a Model 61 carbon rod atomizer. Standard solutions were prepared by diluting 1000 ppm solutions of $FeCl_3$, $Mn(NO_3)_2$, $Co(NO_3)_2$, $Ni(NO_3)_2$, $Cu(NO_3)_2$, or ZnO (Fisher Scientific) into 10 mM Tris-HCl, 0.025% sodium cholate, and 1 mM EDTA, pH 8.

*Gel Electrophoresis.* SeaKem ME agarose (FMC Corp.) was dissolved in $H_2O$ at 100 °C and cooled to 65 °C. Concentrated buffer (at 65 °C) was added to final concentrations of 0.8% agarose, 50 mM Tris-HCl, 0.1% LDAO, 0.025% sodium cholate, and 0.1 mM EDTA, pH 8. (The presence of LDAO during electrophoresis was necessary to prevent adsorption of the samples onto the agarose.) The hot solution was poured onto a GelBond film (FMC Corp.) in a 9 × 13 × 0.2 cm glass mold heated to 65 °C. The gel was allowed to solidify at room temperature, cooled, and preelectrophoresed at ~10 V/cm for 30 min at 4 °C. The buffer present in the electrode reservoirs was the same as in the gel. Samples (~10 $\mu$L with $A_{802}^{1cm} \sim 8$ in 10 mM Tris-HCl, 0.1% LDAO, 0.025% sodium cholate, 0.1 mM EDTA, and 10% sucrose, pH 7.7) were electrophoresed at ~10 V/cm for 6 h at 4 °C. Following electrophoresis, the gels were fixed and stained with Coomassie Brilliant Blue R250 (Inolex) as described previously (Okamura et al., 1974) and dried in air at room temperature.

*EPR Spectra.* Spectra were recorded at 2.1 K as described previously with a 9-GHz spectrometer of local design (Butler et al., 1984). For detection of signals with light modulation (Figure 2), samples (0.1–0.2 mL) were frozen in 1-mm-thick quartz frames having one quartz cover slip attached with silicone grease. This configuration allowed the samples to be optically thin and reduced light-induced temperature modulation affects by permitting direct contact between the samples and the liquid helium bath. For detection of signals with field modulation, samples (~1.2 mL) were frozen in rexolite tubes having an inner diameter of 8 mm. The $g$ values of the narrow signals of $D^+$ or $Q_A^-$ at 9 GHz (line width ~ 10 G) were measured with constant actinic illumination and field modulation as described previously (McElroy et al., 1972).

*Optical Measurements.* The optical kinetic measurements were made as previously described (Kleinfeld et al., 1984a). The flash energy was reduced by passing the beam through solutions of $Cr(NO_3)_3$ and $Ni(NO_3)_3$. For two pulse experiments (Figure 6) a second flash was provided by a second dye laser (Phase R DL1000).

Continuous actinic illumination was provided by a Leitz-Prado 500 tungsten filament projector (500 W). The actinic illumination was filtered through $H_2O$ (1-cm path length) and a Corning CS-2-64 color filter ($\lambda$ >660 nm passed). The intensity of illumination was measured with a YSI Model 65 radiometer (Yellow Springs Instrument Co.).

## Results

*Extraction of Fe.* RCs extracted with *o*-phenanthroline and LiSCN and purified to remove unreassociated H and LM (Figure 1) contained 0.1–0.2 Fe per RC as determined by atomic absorption spectroscopy (Table I). They could be stored for several weeks at 4 °C in 0.025% sodium cholate with no loss of activity (defined as the extent of reversible bleaching of the absorption at 865 nm in response to a saturating flash of light). In the presence of 0.1% LDAO, slight losses in activity (~10%) were detected after ~12 h at 4 °C. The optical absorption spectrum of Fe-depleted RCs was identical with that of native RCs.

*Reconstitution with Metals.* Fe-depleted RCs incubated with $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, or $Zn^{2+}$ and dialyzed extensively against EDTA contained ~0.8 of the appropriate metal per RC in addition to the 0.1–0.2 Fe per RC contained in the Fe-depleted RCs (Table I).

Fe-depleted RCs incubated with $Fe^{2+}$, extensively dialyzed against EDTA, and purified by chromatography on a column of DEAE-cellulose contained 2–5 Fe per RC as determined by atomic absorption spectroscopy. Incubation with dithiothreitol and *o*-phenanthroline followed by chromatography

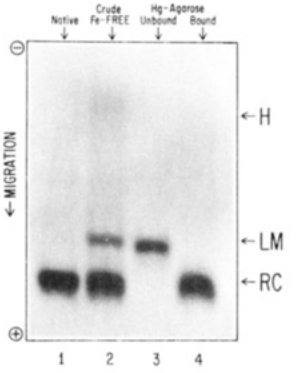



FIGURE 1: Agarose gel (stained with Coomassie Brilliant Blue R250) showing native RCs (lane 1), "crude" Fe-depleted RCs containing unreassociated H and LM (lane 2), LM removed from Fe-depleted RCs by passage through organomercurial agarose (lane 3), and purified Fe-depleted RCs eluted from organomercurial agarose with cysteine (lane 4). Note the absence of H and LM in the purified Fe-depleted RCs.

with DEAE-cellulose reduced the Fe content to ~1.4 Fe per RC (Table I). We believe the Fe in excess of 1 Fe per RC was bound nonspecifically.

*EPR Spectroscopy.* Magnetic coupling of $Q_A^-$ to a nearby paramagnetic metal ion broadens the EPR signal of $Q_A^-$, providing an assay for specific incorporation into the Fe site. EPR spectra of native, Fe-depleted, and reconstituted RCs are presented in Figure 2. The signals were detected by modulating the actinic illumination and therefore represent changes in EPR absorption ($\chi''$) produced by illumination. Consequently, the observed signals are due to the difference between the light-induced signal from $D^+$ and $Q_A^-Met^{2+}$ and the dark signal due to $Met^{2+}$. Negative EPR amplitudes reflect signals that are present in the dark (i.e., due to the divalent metal ion) but that are lost upon illumination.

Native RCs (Figure 2A) exhibited a broad signal centered at $g = 1.8$ resulting from magnetic coupling between $Q_A^-$ and $Fe^{2+}$ (Okamura et al., 1975; Butler et al., 1984; Dismukes et al., 1984). No negative signals were observed since $Fe^{2+}$ produces no EPR signal. Removal of Fe (Figure 2B) greatly diminished the broad signal. In addition, new signals attributed to the triplet state of the bacteriochlorophyll dimer (Okamura et al., 1975) were detected (arrows). The residual broad signal could be accounted for by the residual $Fe^{2+}$ and $Mn^{2+}$ in the sample (Table I). Reconstitution with $Fe^{2+}$ (Figure 2C) restored the broad signal at $g = 1.8$, demonstrating specific reincorporation of $Fe^{2+}$ into the Fe site.

Reconstitution with $Mn^{2+}$ (Figure 2D) resulted in a broad signal with negative peaks at $g = 5.2$, 2.72, 1.87, and 1.37 and positive peaks at $g = 3.6$, 2.22, 2.0, and 1.62. This broad signal is similar to the signals attributed to $Q_A^-Mn^{2+}$ in RCs containing ~0.3 Mn per RC (Feher et al., 1974). Both the derivative of the EPR absorption spectrum of Figure 2D and the EPR signal detected in darkness with field modulation (not shown) closely resemble the signals observed in RCs containing ~0.9 Mn per RC isolated from *Rps. sphaeroides* wild-type strain Y (Rutherford et al., 1985).

Reconstitution with $Zn^{2+}$ (Figure 2E) did not produce a broad signal. This was expected since $Zn^{2+}$ is diamagnetic. However, incorporation of Zn into the Fe site was indicated by the disappearance of the triplet signal. The absence of this signal was characteristic of all samples having a metal ion incorporated into the Fe site.

Reconstitution with $Cu^{2+}$ (Figure 2F) resulted in a negative signal having four hyperfine lines with $A_\parallel = 143$ G centered around $g_\parallel = 2.3$ (see insert) and a positive signal with a peak

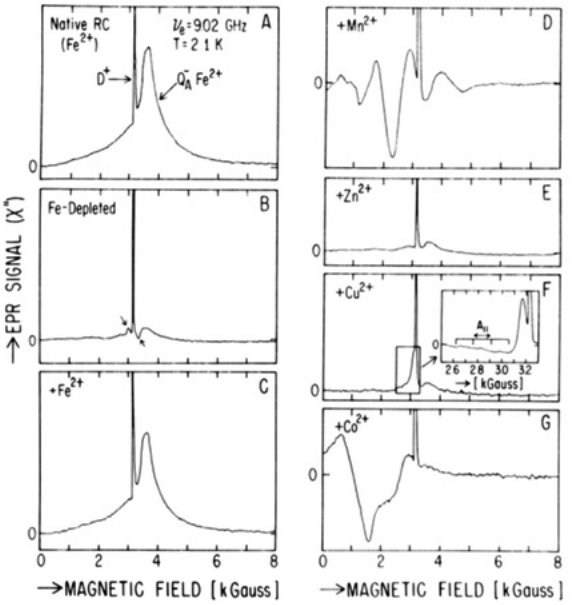



FIGURE 2: EPR spectra of native RCs (A), Fe-depleted RCs (B), and Fe-depleted RCs reconstituted with $Fe^{2+}$ (C), $Mn^{2+}$ (D), $Zn^{2+}$ (E), $Cu^{2+}$ (F), and $Co^{2+}$ (G). The signals were obtained with 3.6-Hz light modulation and are, therefore, proportional to the absorption ($\chi''$) and *not* to the usually presented derivative ($d\chi''/dH$). Samples (0.1 mL, 70 $\mu$M RC, 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7) were mixed with an equal volume of glycerol and frozen with liquid nitrogen in quartz cells (path length 1 mm). Microwave power ~$10^{-4}$ (A–C, E, F) and ~$10^{-6}$ W (D, G, and the insert to F), T = 2.1 K, $\nu_e$ = 9.02 GHz.

at $g = 2.04$. To understand these light-induced signals, the EPR spectrum in the dark was obtained by field modulation. The observed spectrum also displayed four hyperfine lines with $A_\parallel = 143 \pm 3$ G centered around $g_\parallel = 2.31 \pm 0.01$; in addition, there was a second, larger, peak at $g_\perp = 2.07 \pm 0.01$ [see Feher et al. (1986)]. The spectrum was typical for $Cu^{2+}$ in an environment with pseudoaxial symmetry (Vanngard, 1972; Peisach & Blumberg, 1974). The $g$ values observed in the light-induced spectrum (Figure 2F) could be related to those observed in darkness by a simple model (see Discussion).

Reconstitution with $Co^{2+}$ (Figure 2G) resulted in a broad signal with a negative peak at $g$ ~3.8 and a shoulder at $g$ ~2.5, and positive peaks near zero field and near $g = 2.2$. These values are consistent with the presence of high-spin $Co^{2+}$ (Bencini et al., 1981; Bertini et al., 1984).

Reconstitution with $Ni^{2+}$ (not shown) resulted in an EPR signal similar to that observed with $Zn^{2+}$. No broad EPR signal was observed, but the signals attributed to the triplet state of D disappeared.

The $g$ values of the narrow signals evident in Figure 2 are presented in Table I. In Fe-depleted RCs and in RCs reconstituted with $Zn^{2+}$ the narrow signal had a $g$ value of 2.0037. This signal represents the sum of the signals of $D^+$ and $Q_A^-$ in the absence of a nearby paramagnetic ion (Loach & Hall, 1972; Feher et al., 1972). The individual two signals of $D^+$ and $Q_A^-$ could not be resolved at 9 GHz. However, they were resolved at 35 GHz in both samples (not shown) and resemble those reported previously (Feher et al., 1972). Specific incorporation of a paramagnetic transition metal ion into the Fe site broadens the signal of $Q_A^-$, leaving only the narrow signal of $D^+$ ($g = 2.0026$), as observed in native RCs. The $g$ values of the narrow peak observed in RCs reconstituted with $Mn^{2+}$, $Co^{2+}$, and $Cu^{2+}$ (Table I) were essentially the same as that of $D^+$, indicating specific incorporation of these paramagnetic ions into the Fe site. The similarity of the $g$ value of the narrow peak observed in RCs reconstituted with

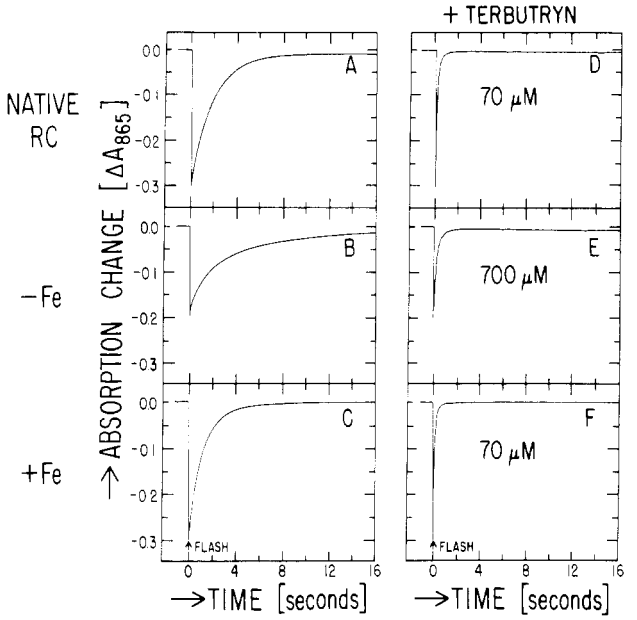
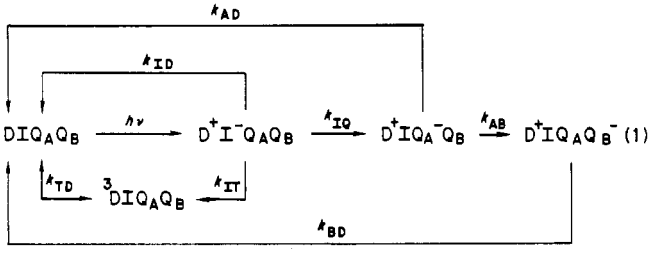



FIGURE 3: Kinetics of charge recombination at 21 °C in native RCs (A), Fe-depleted RCs (B), and Fe-depleted RCs reconstituted with $Fe^{2+}$ (C). The formation and decay of the charge-separated states $D^+Q_A^-$ or $D^+Q_B^-$ were monitored at 865 nm following a single saturating actinic flash. The effect of terbutryn on native RCs (D), Fe-depleted RCs (E), and Fe-depleted RCs reconstituted with $Fe^{2+}$ (F) is also shown. Conditions: 3 μM RC, ~0.5 μM exogenous Q-10, 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7. Terbutryn concentrations are indicated.

$Ni^{2+}$ (Table I) to that of $D^+$ indicates that $Ni^{2+}$ incorporated into the Fe site in a paramagnetic state, thereby eliminating the narrow signal of $Q_A^-$. Our inability to detect a signal from $Q_A^-$ in the presence of $Ni^{2+}$ most likely reflects an extreme broadening of this signal.

*Electron Transfer.* To determine the effect of removing Fe on electron transfer, the rates of several electron-transfer reactions were measured and compared. These reactions are schematically illustrated in eq 1. The rate $k_{BD}$ actually




represents an indirect pathway of charge recombination, involving repopulation of the state $D^+IQ_A^-Q_B$ (Wraight, 1979; Wraight & Stein, 1980; Arata & Parson, 1981; Kleinfeld et al. 1984a).

*(1) Charge Recombination.* Figure 3 compares the kinetics of charge recombination following an actinic flash exhibited by native RCs, Fe-depleted RCs, and Fe-depleted RCs reconstituted with $Fe^{2+}$. The $1/e$ time for charge recombination between $Q_B^-$ and $D^+$ ($k_{BD}^{-1}$) in native RCs at 21 °C was 1.65 ± 0.15 s (Figure 3A). The faster charge recombination time between $Q_A^-$ and $D^+$ ($k_{AD}^{-1}$ = 115 ± 5 ms) was measured in the presence of terbutryn (Figure 3D), a herbicide that inhibits electron transfer from $Q_A^-$ to $Q_B$ (Wraight, 1981; Diner et al., 1984; Okamura, 1984; Stein et al., 1984), or in RCs depleted of $Q_B$.

Charge recombination in Fe-depleted RCs in the absence of terbutryn (Figure 3B, $k^{-1} \simeq 5$ s) was slower than $k_{BD}^{-1}$ in native RCs by a factor of ~3, and the kinetics were nonexponential. In the presence of 700 μM terbutryn (Figure 3E) the observed time was 200 ± 12 ms. Terbutryn therefore appears to inhibit electron transfer in Fe-depleted RCs, but higher concentrations were required than with native RCs. In contrast to native RCs, terbutryn appeared to affect $k_{AD}$ in Fe-depleted RCs: In Fe-depleted RCs without $Q_B$ the observed recombination time was 110 ± 5 ms. Subsequent addition of 700 μM terbutryn changed the time to 200 ± 12 ms, the same value as observed in Figure 3E.

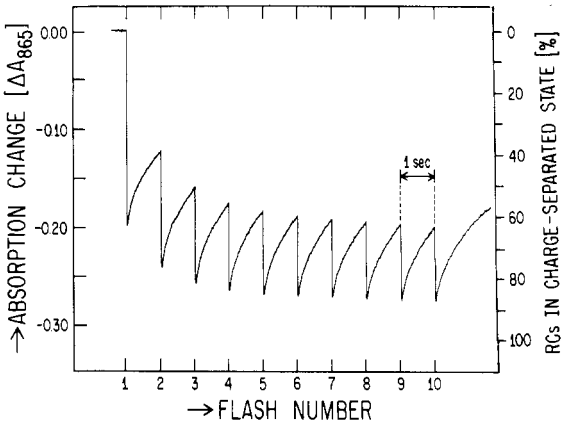



FIGURE 4: Fe-depleted RCs subjected to a series of actinic flashes spaced 1 s apart (conditions the same as in Figure 3). The $\Delta A_{865}$ produced by each flash corresponds to a charge separation ($DQ_A \rightarrow D^+Q_A^-$) in 63 ± 3% of the RCs that were not in a charge-separated state prior to the flash.

From an analysis of the kinetics of charge recombination as a function of terbutryn concentration, the dissociation constant of terbutryn ($K_I$) was estimated to have increased 7-fold in the absence of Fe (Table I). *o*-Phenanthroline also appeared to inhibit electron transfer in Fe-depleted RCs, and its $K_I$ also appeared to have increased in the absence of Fe. Complete inhibition was not achieved with *o*-phenanthroline, presumably because of the low solubility of *o*-phenanthroline in aqueous solutions. In contrast to terbutryn, *o*-phenanthroline had no apparent effect on $k_{AD}$ in Fe-depleted RCs.

In Fe-depleted RCs the extent of bleaching at 865 nm following a single actinic flash was only 63 ± 3% compared to that of native RCs (compare parts A and B of Figure 3). A 4-fold reduction in the intensity of the actinic flash had no affect, indicating that the extent of the bleaching was not limited by the flash intensity. When a series of flashes was administered (Figure 4), the amount of bleaching increased with each flash and approached a limiting value. The $\Delta A_{865}$ produced by each flash corresponded to a charge separation ($DQ_A \rightarrow D^+Q_A^-$) in 63 ± 3% of those Fe-depleted RCs that were not in a charge-separated state prior to the flash. These results indicate that the decreased (~63%) bleaching observed in the Fe-depleted RCs did not arise from a photochemically inactive fraction. Expansion of the trace in Figure 3B (not shown) revealed that the extent of bleaching was at least 95% compared to native RCs but that ~34% of the absorbance change recovered with a time constant of 30 ± 2 μs. We attribute this rapidly recovering fraction to the formation and decay of the triplet state of the bacteriochlorophyll dimer in RCs that have not undergone charge separation to $D^+Q_A^-$.

Reconstitution with $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ restored $k_{BD}$, $k_{AD}$, the extent of flash-induced bleaching at 865 nm, and the apparent dissociation constants of terbutryn and *o*-phenanthroline to values close to those of native RCs (Figure 3C,F, and Table I). From an analysis of the kinetics of charge recombination (Okamura et al., 1982a), all samples were

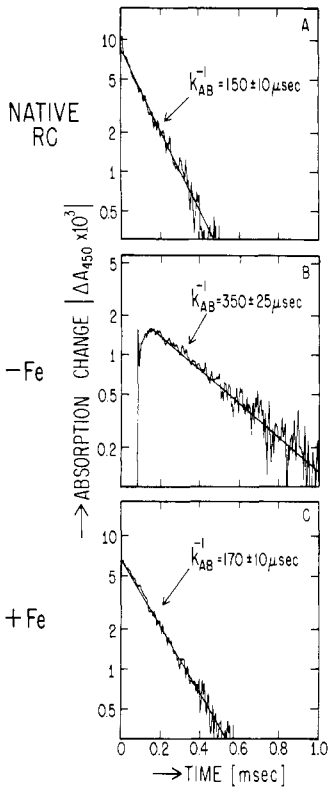



FIGURE 5: Rate of electron transfer from $Q_A^-$ to $Q_B$ measured from the difference in the extinction coefficients of $Q_A^-$ and $Q_B^-$ at 450 nm following an actinic flash. Shown are semilogarithmic plots of the data after subtraction of a constant base line corresponding to the optical absorption change, $\Delta A_{450}$, at 2 ms. Electron-transfer processes that are slower than ~1 ms do not show up in this analysis but were obtained by an alternate procedure (see Figure 6). Solid lines represent values of $k_{AB}^{-1}$, as indicated. (A) Native RC, (B) Fe-depleted RC, and (C) Fe-depleted RC reconstituted with $Fe^{2+}$. Conditions: 5 μM RC, 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7, 21 °C. Traces A and C represent the computer average of 16 traces; trace B represents the computer average of 36 traces.

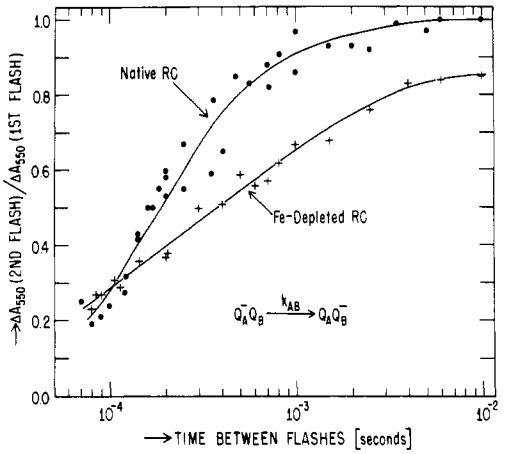



FIGURE 6: Rate of electron transfer from $Q_A^-$ to $Q_B$ in native and Fe-depleted RCs estimated from the amount of $cyt^{2+}$ oxidized following the second of two actinic flashes. The absorption change of $cyt^{2+}$ at 550 nm ($\Delta A_{550}$) following the second flash was normalized to that following the first flash and plotted as a function of the time between flashes. Conditions: 3 μM native RC or 5 μM Fe-depleted RC, 30 μM cyt $c_2$ (*Rps. sphaeroides*), 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7, 4 °C.

judged to contain at least 0.85 functional $Q_B$ per RC.

Incubation of Fe-depleted RCs with 10 mM $CaCl_2$ or $MgCl_2$ produced no change in the extent of flash-induced bleaching at 865 nm or the kinetics of charge recombination (not shown), suggesting that neither $Ca^{2+}$ nor $Mg^{2+}$ was incorporated into the Fe site.

(*2*) *Electron Transfer from $Q_A^-$ to $Q_B$.* The slow kinetics of charge recombination in Fe-depleted RCs (Figure 3B) and the apparent inhibitory effect of terbutryn (Figure 3E) suggest that functional electron transfer from $Q_A^-$ to $Q_B$ proceeds in the absence of Fe. To measure the rate of transfer, $k_{AB}$, several procedures were employed. The first made use of the differnce between the extinction coefficients of $Q_A^-$ and $Q_B^-$ in native RCs at 450 nm (Vermeglio & Clayton, 1977; Wraight, 1979; Kleinfeld et al., 1985). At this wavelength, an actinic flash induces in native RCs a transient (~150 μs) absorption *increase* corresponding to electron transfer from $Q_A^-$ to $Q_B$ ($D^+Q_A^-Q_B \rightarrow D^+Q_AQ_B^-$) superimposed on a much slower (~1.6 s) absorption *decrease* corresponding to charge recombination between $Q_B^-$ and $D^+$. Addition of terbutryn eliminates the transient increase, leaving only an absorption decrease with $k^{-1}$ ~115 ms corresponding to charge recombination between $Q_A^-$ and $D^+$. Figure 5 shows semilogarithmic plots of the transient absorption increase observed in native RCs, Fe-depleted RCs, and Fe-depleted RCs reconstituted with $Fe^{2+}$. Native RCs (Figure 5A) exhibited $k_{AB}^{-1}$ = 150 ± 10 μs. The corresponding transient absorption increase in Fe-depleted RCs (Figure 5B) yielded $k_{AB}^{-1}$ = 350 ± 25 μs and was eliminated by 700 μM terbutryn.

The magnitude of the transient increase in Fe-depleted RCs should be 63% compared to that of native RCs on the basis of the decreased (63%) yield of charge separation, assuming that the extinction coefficients of $Q_A^-$ and $Q_B^-$ are unchanged by removal of Fe. However, the observed magnitude was only ~30% (compare Figure 5A,B), suggesting that the difference between the extinction coefficients may have decreased by a factor of ~2 in the absence of Fe. An alternative explanation is that a fraction of Fe-depleted RCs have values of $k_{AB}^{-1}$ slower than ~1 ms (see later section). Such slower absorption increases are difficult to resolve from the decreases in absorption corresponding to charge recombination.

The Fe-depleted RCs also exhibited a transient absorption decay with a magnitude of $\Delta A_{450}$ = (1.4 ± 0.2) × $10^{-2}$ and $k^{-1}$ = 28 ± 2 μs (off scale on Figure 5B). This transient had approximately the same magnitue at all wavelengths 450–550 and 630–650 nm and was unaffected by 700 μM terbutryn. We attribute this transient to the formation and decay of the triplet state of the bacteriochlorophyll dimer, $^3D$, also observed at 865 nm (see previous discussion of Figure 3B).

Reconstitution of Fe-depleted RCs with $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, or $Zn^{2+}$ restored the magnitude and kinetics of the absorption change at 450 nm to values close to those of native RCs (Figure 5C and Table I) and eliminated the transient with $k^{-1}$ = 28 μs.

A second procedure for measuring $k_{AB}^{-1}$, that is more sensitive to slower values, was obtained by measuring the amount of $cyt^{2+}$ oxidized after the second of two closely spaced actinic flashes (Parson, 1969; Halsey & Parson, 1974; Debus et al., 1984, 1985a). Following the first flash, RCs rapidly oxidize 1 $cyt^{2+}$ per RC to form $DQ_A^-$. The rate of electron transfer from $Q_A^-$ to $Q_B$ was determined from the amount of $cyt^{2+}$ per RC oxidized after the second flash for varying times between flashes. The oxidation of $cyt^{2+}$ was monitored at 550 nm. The measurements were performed with cyt $c_2$ (*Rps. sphaeroides*) instead of cyt *c* (horse heart) because of the faster oxidation of $D^+$ by cyt $c_2$ (Ke et al., 1970; Overfield et al., 1979; Rosen et al., 1979). The ratio of the absorption change ($\Delta A_{550}$) resulting from the second flash to that from the first was plotted as a function of the time between flashes (Figure 6).

With Fe-depleted RCs the ratio approached a value of ~0.85 at long times. This was expected because the Fe-depleted RCs contained only ~0.85 functional $Q_B$ per RC (Figure 3B). The nonexponential shape of the curve suggests that there is a distribution of $k_{AB}^{-1}$ values in the Fe-depleted RCs. Quantitation of the data is complicated by the decreased (~63%) yield of charge separation: A fraction of the Fe-depleted RCs not undergoing charge separation after the first flash will do so after the second flash and, therefore, will contribute to the oxidation of $cyt^{2+}$ after the second flash. In addition, a fraction of the Fe-depleted RCs may form $DQ_A^{2-}$ after the second flash (see Stability of $Q_A^-$). In spite of these complications, the data show that the *slowest* components of $k_{AB}^{-1}$ in Fe-depleted RCs are at most a factor of ~10 slower than $k_{AB}^{-1}$ in native RCs. Earlier measurements indicated that a fraction of Fe-depleted RCs exhibit $k_{AB}^{-1} \sim 100$ ms (Debus et al., 1984). We believe that these very slow values can be attributed to LM that contaminated the earlier sample [$k_{AB}^{-1} \geq 100$ ms in LM (Debus et al., 1985a)]. A fraction of Fe-depleted RCs having $k_{AB}^{-1} \sim 1$–3 ms may account for the lower magnitude of the transient optical absorption increase at 450 nm observed in Fe-depleted RCs following an actinic flash (Figure 5).

A third procedure for measuring $k_{AB}$ makes use of the difference in electrochromic shifts in the near-infrared optical absorption spectrum of RCs caused by the states $D^+Q_A^-Q_B$ and $D^+Q_AQ_B^-$ (Vermeglio & Clayton, 1977; Wraight, 1979). Following an actinic flash, transient absorption decays at 774 nm corresponding to $k_{AB}$ were observed in native and reconstituted RCs and could be eliminated with terbutryn (data not shown). The observed values of $k_{AB}$ were similar to those measured at 450 nm. A transient decay with $k^{-1} = 360 \pm 30$ $\mu$s was observed in Fe-depleted RCs. Its magnitude ($\Delta A_{774}/A_{802}$) was ~60% of that observed in native and reconstituted RCs. However, this transient could not be eliminated by 700 $\mu$M terbutryn and was also evident in Fe-depleted RCs without $Q_B$. This transient at 774 nm cannot, therefore, be attributed to electron transfer from $Q_A^-$ to $Q_B$ in Fe-depleted RCs. It may reflect a conformational change in the protein induced by charge separation. The difference in behavior of terbutryn in RCs with and without divalent metal may reflect a structural change upon removal of the metal.

*(3) Electron Transfer to Exogenous Quinone.* To determine whether the flux of electrons through illuminated RCs was diminished in the absence of $Fe^{2+}$, we examined the oxidation of $cyt^{2+}$ (horse heart) by RCs illuminated in the presence of Q-0, a water-soluble analogue of Q-10 (Okamura, 1984). The initial rate of oxidation ($cyt^{2+}$ per RC oxidized per second) was 277 ± 10 $s^{-1}$ in native RCs, 85 ± 5 $s^{-1}$ in Fe-depleted RCs, and 200–240 $s^{-1}$ in Fe-depleted RCs reconstituted with $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, or $Zn^{2+}$ (Table I). A 20-fold reduction in the intensity of the actinic illumination had no effect on the relative rates of oxidation.

*Stability of $Q_A^-$. (1) With Respect to Reduction.* Generation of $Q_A^-$ either by reduction with sodium dithionite or by illumination in the presence of excess $cyt^{2+}$ produced weak semiquinone EPR and ENDOR signals in Fe-depleted RCs compared to RCs reconstituted with $Zn^{2+}$ (data not shown). This suggests that $Q_A^-$ is unstable with respect to reduction to $Q_A^{2-}$ when no metal ion occupies the Fe site. To test this hypothesis, we monitored the absorption of $Q_A^-$ at 450 nm in response to a series of actinic flashes in samples depleted of $Q_B$. Diaminodurene and sodium ascorbate were present to reduce $D^+$. (The $1/e$ reduction time was 9.7 ± 0.3 ms under the conditions used.) Figure 7 shows the effect of 14 successive

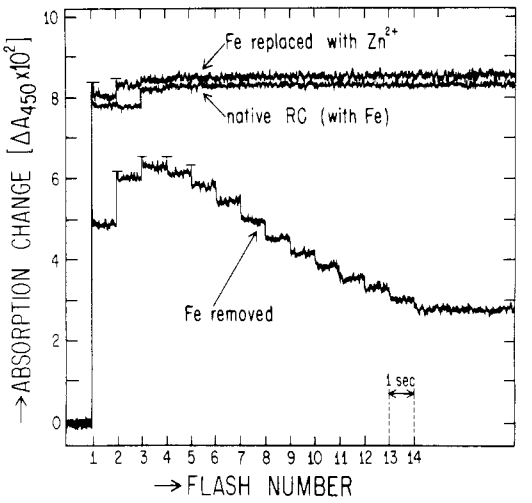



FIGURE 7: Stability of the semiquinone anion $Q_A^-$ in response to a series of actinic flashes spaced 1 s apart as measured optically at 450 nm. The native and Fe-depleted RCs contained 1.03 ± 0.03 and 1.00 ± 0.03 Q-10 per RC, respectively. The RCs containing $Zn^{2+}$ were reconstituted from these Fe-depleted RCs. Conditions: 17 $\mu$M RC, 0.5 mM diaminodurene, 10 mM sodium ascorbate, 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7, 21 °C. The diaminodurene was added from a fresh solution of 50 mM diaminodurene in ethanol.

actinic flashes on native RCs containing 1.03 ± 0.03 Q-10 per RC, Fe-depleted RCs containing 1.00 ± 0.03 Q-10 per RC, and RCs containing $Zn^{2+}$ reconstituted from these Fe-depleted RCs. Following a single actinic flash, native RCs exhibited an absorption change, $\Delta A_{450}$, corresponding to formation of $DQ_A^-$ (Figure 7). Some increase in $\Delta A_{450}$ on the second and third flashes was expected because the rate of reduction of $D^+$ by diaminodurene is only ~10-fold greater than $k_{AD}$. [The lack of increase in native RCs following the second flash is presumably because a small fraction (~3%) contained $Q_B$.] Subsequent flashes had no effect. Fe-depleted RCs exhibited a change in $\Delta A_{450}$ following every flash. Because only 63% undergo charge separation on each flash (Figure 4), a significant increase in $\Delta A_{450}$ on the second and thrid flashes was expected. On the fourth and subsequent flashes, however, the $\Delta A_{450}$ decreased by 7.5 ± 1.5% following each flash, indicating loss of $Q_A^-$, presumably because of formation of $Q_A^{2-}$. Reconstitution with $Zn^{2+}$ (Figure 7) or $Fe^{2+}$ (not shown) restored the apparent stability of $Q_A^-$ to repeated actinic flashes.

*(2) With Respect to Oxidation or Protonation.* From the persistence of the optical absorption change, $\Delta A_{450}$, in the dark following a *single* actinic flash in samples with and without $Q_B$, neither $Q_A^-$ nor $Q_B^-$ appeared significantly less stable with respect to loss of their electron in Fe-depleted RCs compared to native RCs (data not shown). The observed optical absorption changes at 450 nm were characteristic of unprotonated ubisemiquinone (Land et al., 1971). Thus, neither $Q_A^-$ nor $Q_B^-$ became directly protonated in the absence of a transition metal ion.

## Summary and Discussion

In this study we examined the electron-transfer characteristics of RCs from *Rhodopseudomonas sphaeroides* R-26.1 depleted of Fe and reconstituted with $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$. Extraction of Fe involved incubation of RCs with *o*-phenanthroline in the presence of the chaotropic agent LiSCN, followed by extensive dialysis to remove the Fe and purification with organomercurial agarose to remove unreassociated H and LM. The resulting RCs (Figure 1) contained 0.1–0.2 Fe per RC as determined by atomic absorption

and EPR spectroscopy (Table I and Figure 2). Subsequent reconstitution with $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ (Table I) resulted in incorporation of these metal ions into the native Fe site as determined by EPR spectroscopy (Figure 2).

*EPR Spectroscopy.* The light-induced EPR signals of $Q_A^-Fe^{2+}$ (Figure 2A) has been accounted for by a magnetic exchange interaction between $Q_A^-$ and $Fe^{2+}$ (Butler et al., 1984; Dismukes et al., 1984). Exchange interactions can also account for the light-induced EPR signal of $Q_A^-$ complexed to $Mn^{2+}$, $Cu^{2+}$, and $Co^{2+}$ (Figure 2D,F,G). For two spins coupled by an exchange interaction, the observed $g$ values will be the average of the $g$ values of the two isolated spins if the coupling energy is greater than the difference in the Zeeman energies of the two individual spins, and the magnetic dipolar interaction is small (Kokoszka & Gordon, 1969). If these conditions are satisfied, the observed light-induced EPR signals (positive peaks in Figure 2D,F,G) are related to the narrow signal of $Q_A^-$ ($g = 2.0045$) and the signals of $Mn^{2+}$, $Cu^{2+}$, and $Co^{2+}$ in the dark (negative peaks in Figure 2D,F,G) by the relationship:

$$g_{light} = \tfrac{1}{2}(g_{dark} + 2.0045) \quad (2)$$

This relationship accounts for the signals of $Q_A^-$ complexed to $Cu^{2+}$ (Figure 2F) and for most of the signals of $Q_A^-$ complexed to $Mn^{2+}$ and $Co^{2+}$ (Figure 2D,G) (with the exception of the peaks near zero field, which are perturbed by the zero field splittings). For example, the Mn sample (Figure 2D) exhibits negative peaks corresponding to $g = 5.2$, 2.72, 1.87, and 1.37. Equation 2 predicts light-induced positive peaks at $g = 3.6$, 2.36, 1.94, and 1.69. This is in good agreement with the observed peaks at $g = 3.6$, 2.22, 2.0, and 1.62. A more complete description of the EPR signals of $Q_A^-$ complexed to $Mn^{2+}$, $Cu^{2+}$, and $Co^{2+}$ will require an analysis of the spin Hamiltonians of the different systems.

*Electron Transfer from $Q_A^-$ to $Q_B$.* The slow kinetics of charge recombination in Fe-depleted RCs (Figure 3B) and the apparent inhibitory effect of terbutryn (Figure 3E) suggest that functional electron transfer from $Q_A^-$ to $Q_B$ proceeds in the absence of $Fe^{2+}$. "Functional" electron transfer requires that $k_{AB} \gg k_{AD}$ (see Eq 1). Since $k_{AD}^{-1} \sim 110$ ms in Fe-depleted RCs (without terbutryn present), $k_{AB}^{-1}$ must be faster than ~10 ms in the absence of $Fe^{2+}$. This conjecture was borne out by the measurements of Figures 5 and 6, which show that a majority of the Fe-depleted RCs exhibited values of $k_{AB}^{-1}$ that were slower by only a factor of ~2 compared to native RCs and that the *slowest* components of $k_{AB}^{-1}$ were slower by only a factor of ~10. These results demonstrate that the $Fe^{2+}$ does not play an obligatory role in electron transfer from $Q_A^-$ to $Q_B$. The similarity of $k_{AB}$ in RCs reconstituted with $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ (Table I) confirms this conclusion: If the valence orbitals of the metal ion played an obligatory role in this electron-transfer reaction, substitution of $Fe^{2+}$ by other metal ions would be expected to affect the rate (Nam et al., 1984). The previously reported loss of electron transfer in RCs depleted of Fe (Blankenship & Parson, 1979) presumably resulted from a concomitant loss of the H subunit during removal of Fe (Debus et al., 1985a). The lack of an obligatory role for $Fe^{2+}$ in electron transfer from $Q_A^-$ to $Q_B$ confirms conclusions drawn previously from studies involving biosynthetic replacement of $Fe^{2+}$ by $Mn^{2+}$ (Nam et al., 1984; Okamura et al., 1984).

Quinone extraction and reconstitution studies led Okamura et al. (1975) to propose the "iron wire" hypothesis in which the $Fe^{2+}$ plays a dominant role in the electron transfer from $Q_A^-$ to $Q_B$. The observation of magnetic interactions between $Fe^{2+}$ and both $Q_A^-$ and $Q_B^-$ (Wraight, 1977, 1978; Okamura, et al., 1978; Rutherford & Evans, 1979, 1980; Butler et al., 1984; Dismukes et al., 1984) gave support to this hypothesis. Suggestive evidence for this hypothesis also comes from the recently determined crystal structure of the RC from *Rps. viridis* (Deisenhofer et al., 1984, 1985a,b), which shows the $Fe^{2+}$ to be located between $Q_A$ and the presumed site for $Q_B$ and to be connected to $Q_A$ via one histidine ligand and to $Q_B$ via another. [The structure of the RC from *Rps. viridis* is believed to be homologous to that of the RC from *Rps. sphaeroides* (Allen et al., 1986)]. A similar structure was proposed on the basis of EXAFS measurements by Bunker et al. (1982). Furthermore, recent ENDOR measurements on RCs reconstituted with $Zn^{2+}$ suggest that both $Q_A$ and $Q_B$ are connected to these histidines via hydrogen bonds (Lubitz et al., 1985; Feher et al., 1985). Hence, it seems likely that the mechanism of electron transfer from $Q_A^-$ to $Q_B$ should involve the $Fe^{2+}$. However, our present results show that the *observed* rate is changed only slightly when the $Fe^{2+}$ is removed (Figures 5 and 6) and is essentially unchanged when the $Fe^{2+}$ is replaced by other divalent metal ions (Table I). To reconcile these seemingly contradictory results, we postulate the existence of a rate-limiting step that precedes electron transfer. Such a rate-limiting step may, for example, be a protonation event and/or a light-induced conformational change. Evidence for such a mechanism comes from a comparison of $k_{AB}$ in RCs cooled to cryogenic temperatures under illumination (e.g., in the charge-separated state $D^+Q_AQ_B^-$) and RCs cooled in darkness (Kleinfeld et al., 1984b). Our observation of an optical absorption decay at 774 nm with $k^{-1} = 360 \pm 30$ μs in Fe-depleted RCs without $Q_B$ or in the presence of 700 μM terbutryn may reflect such a rate-limiting step. Furthermore, native RCs without $Q_B$ or in the presence of *o*-phenanthroline exhibit an electrogenic step with a time constant of ~100 μs (Y. Blatt, A. Gopher, D. Kleinfeld, M. Montal, and G. Feher, unpublished results), which may also reflect such a mechanism. Replacement of $Fe^{2+}$ by divalent metal ions of similar ionic radii, as in our present study, would not be expected to appreciably change the rate of protonation or of a structural change, but *removing* the $Fe^{2+}$ *may*. The slower $k_{AB}^{-1}$ values observed in Fe-depleted RCs (Figures 5 and 6) may reflect either a change in the rate-limiting step or a significant change in the electron tunneling rate from $Q_A^-$ to $Q_B$, which may become the rate-limiting step in the absence of a metal ion.

The free energy difference, $\Delta G$, between the semiquinone states $Q_A^-Q_B$ and $Q_AQ_B^-$ can be related to $k_{AD}$ and $k_{BD}$ by the relationship (Kleinfeld et al., 1984a):

$$\Delta G = -kT \ln\left(\frac{k_{AD} - k_{BD}}{k_{BD}}\right) \quad (3)$$

where $k_{BD}$ represents the indirect pathway of charge recombination involving repopulation of $D^+IQ_A^-Q_B$. The decrease of $k_{BD}$ in Fe-depleted RCs (a factor of ~3; see Figure 3B and Table I) suggests that $\Delta G$ increased from 67 to ~100 meV in the absence of a metal ion in the Fe site (calculated with $k_{AD}^{-1} = 110$ ms in Fe-depleted RCs, i.e., without terbutryn). This apparent increase in $\Delta G$ suggests a change in the environment of one or both quinones when no metal ion occupies the Fe site. A change in the environment of $Q_A$ is suggested by ENDOR studies with Fe-depleted RCs and RCs reconstituted with $Zn^{2+}$. The environment of $Q_A^-$ in RCs containing $Zn^{2+}$ appears to be asymmetric with respect to the quinone oxygens (Lubitz et al., 1985; Feher et al., 1985). This asymmetry appears to be less in Fe-depleted RCs (W. Lubitz, R. J. Debus, E. C. Abresch, R. A. Isaacson, M. Y. Okamura, and G. Feher, unpublished results), suggesting that the asymmetry

results from an electrostatic interaction with the metal ion. The loss of this interaction may account for the possible change in the relative extinction coefficients of $Q_A^-$ and $Q_B^-$ at 450 nm in Fe-depleted RCs (Figure 5B).

*Electron Transfer from $I^-$ to $Q_A$.* The decreased (63%) quantum yield of flash-induced charge separation in Fe-depleted RCs (Figures 3 and 4), together with the observations of a transient optical absorption decay with $k^{-1} = 28–30$ μs (Figures 3 and 5) and a triplet EPR signal (Figure 2B), suggests that formation of the triplet state, $^3D$, competes with electron transfer to $Q_A$ in the absence of a metal ion. Such competition can be accounted for by the simple model of eq 1. The state $D^+I^-Q_AQ_B$ can decay by three pathways: (i) charge recombination with rate constant $k_{ID}$, (ii) decay to the triplet state with rate constant $k_{IT}$, and (iii) electron transfer to $Q_A$ with rate constant $k_{IQ}$. Since the lifetime of the actinic flash (~400 ns) is much longer than $k_{ID}^{-1}$ (10–20 ns), RCs undergoing charge recombination from $D^+I^-Q_AQ_B$ to the ground state will be reexcited during the lifetime of the flash. At the termination of the flash, the RCs will be partitioned between the three states $^3DIQ_AQ_B$, $D^+IQ_A^-Q_B$, and $D^+IQ_AQ_B^-$, whose lifetimes (~10 μs, ~0.1 s, and ~1 s, respectively) are all longer than the lifetime of the flash. The fraction, $F$, of RCs in the charge-separated states $D^+Q_A^-$ and $D^+Q_B^-$ is given by

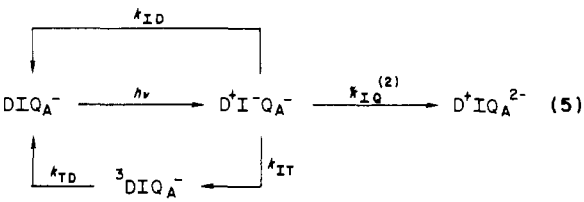
$$F = \frac{k_{IQ}}{k_{IT} + k_{IQ}} \tag{4}$$

In Fe-depleted RCs this fraction appears to be 0.63 ± 0.03 compared to 1.0 in native RCs (Figures 3 and 4). The lower value of $F$ can be accouted for by either a decrease in $k_{IQ}$ or an increase in $k_{IT}$. To estimate $k_{IQ}$ the rate of decay of $I^-$ ($k_{decay}$) was measured optically following short (~30 ps) actinic flashes by C. Kirmaier and D. Holten. In the simple model of eq 1, $k_{decay} = k_{IQ} + k_{IT} + k_{ID}$. Preliminary measurements indicate that $k_{IQ}$ decreased by a factor of 20–50 in Fe-depleted RCs compared to native RCs (Holton et al., 1986). Reconstitution with $Zn^{2+}$ restored the rate.

A possible role of the metal ion may be to facilitate electron transfer from $I^-$ to $Q_A$. A mechanism to accomplish this is provided by the electrostatic interaction between $Q_A^-$ and the divalent metal ion, which would change the redox potential of $Q_A^-$ (Dutton et al., 1978; Morrison et al., 1982; Wraight, 1982; Crofts & Wraight, 1983). The electrostatic interaction can influence the electron transfer rate in several ways. One is via a *static* change in the redox potential. Current electron transfer theories predict that the proper redox potential is required to maximize the rate of electron transfer (Hopfield, 1974; Jortner, 1976). A detailed investigation of the influence of redox energy on $k_{IQ}$ in RCs has been performed by Gunner et al. (1981, 1982) and Liang et al. (1981).

Another possibility is that the electron-transfer rate is influenced by *dynamic* effects of the electrostatic interaction between $Fe^{2+}$ and $Q_A^-$. Current theories of electron transfer emphasize mechanisms for coupling the electron-transfer reaction to lattice vibrations. Mechanisms for doing this involve reorganization of the structure of the reactants and products, e.g., changes in solvation (Marcus, 1956; Levich & Dogonadze, 1961; Kakitani & Mataga, 1985) or differences in bond length (Hopfield, 1974; Jortner, 1976). A possible specific mechanism for electron transfer to the $Q_A$–$Fe^{2+}$ complex involves changes in the $Q_A$–$Fe^{2+}$ distance (De Vault, 1986). Fluctuations in this coordinate due to vibrations modulate the electrostatic energy of the product state, $IQ_A^-Fe^{2+}$. This modulation may facilitate electron transfer from $I^-$ to $Q_AFe^{2+}$.

*Reduction of $Q_A^-$ to $Q_A^{2-}$.* The loss of $Q_A^-$ in Fe-depleted RCs subjected to a series of actinic flashes in the presence of exogenous electron donors (Figure 7) suggests that photochemical reduction of $Q_A^-$ to $Q_A^{2-}$ occurs readily in the absence of a metal ion. This also accounts for the weak EPR and ENDOR signals of $Q_A^-$ observed in Fe-depleted RCs illuminated in the presence of $cyt^{2+}$. The rate of photochemical reduction of $Q_A^-$ can be estimated with a simple model schematically illustrated by eq 5. In analogy with eq 4, the



(5)

fraction, $F^{(2)}$, of RCs with $Q_A^{2-}$ at the termination of a saturating ~400-ns actinic flash is given by

$$F^{(2)} = \frac{k_{IQ}^{(2)}}{k_{IT} + k_{IQ}^{(2)}} \tag{6}$$

The decrease in $\Delta A_{450}$ following the fourth and subsequent flashes in Fe-depleted RCs (Figure 7) suggests that this fraction is ~0.075 in the absence of a metal ion. From the quantum yield for formation of the triplet state $^3DIQ_A^-$ [0.1–0.2 at 20 °C (Parson et al., 1975; Parson & Monger, 1977)] and the value of $k_{ID}^{-1}$ (10–20 ns), one infers for native RCs a value of $k_{IT}^{-1}$ ~100 ns [for more detailed discussions of triplet formation in RCs see Norris et al. (1982), Ogrodnik et al. (1982), Schenck et al. (1982), and Chidsey et al. (1984)]. If $k_{IT}$ is unaffected by removal of the Fe, this relationship implies that $[k_{IQ}^{(2)}]^{-1} \sim 1$ μs in Fe-depleted RCs. This represents a $10^4$-fold increase in rate over that measured in native RCs [$[k_{IQ}^{(2)}]^{-1}$ ~10 ms (Okamura et al., 1979)].

Reconstitution with $Fe^{2+}$ or $Zn^{2+}$ restored the stability of $Q_A^-$ against photochemical reduction to $Q_A^{2-}$ (Figure 7). The ability of $Zn^{2+}$ to substitute for $Fe^{2+}$ shows that the apparent increase in $k_{IQ}^{(2)}$ observed in Fe-depleted RCs reflects the loss of an electrostatic interaction between $Q_A^-$ and the metal ion. The data of Figure 7 suggest that $Q_A$ becomes a two-electron acceptor in the absence of a metal ion in the Fe site. This explains the weak EPR and ENDOR signals of $Q_A^-$ observed in Fe-depleted RCs treated with sodium dithionite, as was also reported by Dutton et al. (1978). In protic environments Q-10 is a two-electron acceptor, whereas in aprotic solvents it behaves like a one-electron acceptor (Chambers, 1974). This suggests that the environment of $Q_A$ in native RCs is not readily accessible to solvent protons. This conclusion can be reconciled with the suggestion that $Q_A^-$ resides in a protic environment (Morrison et al., 1982) by assuming that $Q_A^-$ hydrogen bonds with nearby residues on the protein. Evidence for hydrogen bonds to the carbonyl oxygens of $Q_A^-$ comes from EPR studies (Hales & Case, 1981) and ENDOR studies (Lubitz et al., 1985; Feher et al., 1985). In the absence of a metal ion, $Q_A^-$ seems to be more accessible to solvent protons, as evidenced from the fact that $Q_A$ acts as a two-electron acceptor. Protons may enter the vacant Fe site and stabilize $Q_A^{2-}$ either by protonating it directly or by protonating the distal nitrogen of the histidine (the nitrogen that had been coordinated to the $Fe^{2+}$), causing the release of a proton from the proximal nitrogen (the nitrogen that is hydrogen bonded to the quinone).

*Physiological Role of Fe.* What are the possible physiological consequences of the changes observed in RCs without

$Fe^{2+}$ or any other transition metal ion? The small changes in $k_{AB}^{-1}$ in Fe-depleted RCs are unlikely to be significant since $k_{AB} \gg k_{AD}$ even with a 10-fold decrease in $k_{AB}$; consequently, the efficiency of electron transfer from $Q_A^-$ to $Q_B$ would essentially be unaffected (see eq 1). However, the apparent 50-fold decrease in $k_{IQ}$ would substantially decrease the quantum efficiency of light-induced charge separation. Furthermore, the concomitant formation of triplet states may initiate photochemical reations that are deleterious to protein function. The apparent $10^4$-fold increase in $k_{IQ}$[2] suggests that $Q_A$ could become photochemically reduced to $Q_A^{2-}$, especially under conditions of high light intensity and particularly since the $Q_B$ site may not be fully occupied in vivo (Robinson & Crofts, 1983). The consequences of the formation of $Q_A^{2-}$ are not clear. $Q_A^{2-}$ is readily protonated; the resulting ubihydroquinone may bind differently to the $Q_A$ site, or its redox potential may be higher than that of $Q_B$. In either case, efficient electron transfer to $Q_B$ may be impaired.

The flux of electrons from $cyt^{2+}$ to exogenous Q-0 in illuminated RCs was diminished by only a factor of ~3 in Fe-depleted RCs (Table I), suggesting that neither the reduced quantum efficiency nor the formation of triplet states has serious consequences with respect to electron transfer. However, these experiments were performed in the presence of a large excess of Q-0. Under these conditions $Q_A^-$ may lose its electron rapidly, thereby preventing the formation of $Q_A^{2-}$.

The ubiquitous presence of $Fe^{2+}$ (or in some cases $Mn^{2+}$) in association with quinones in RCs from photosynthetic bacteria and green plants suggests that it serves an important physiological function. One possibility is that the unfilled valence orbitals of $Fe^{2+}$ (or $Mn^{2+}$) protect the RC from photochemical damage by quenching excited states of the quinones. Another possibility is that $Fe^{2+}$ regulates the assembly of intact RCs by switching off assembly under oxidizing conditions when $Fe^{2+}$ becomes oxidized to $Fe^{3+}$. Under oxidizing conditions RC levels are known to be depressed (Kaplan, 1978; Ohad & Drews, 1982). The $Fe^{2+}$ may also play an important structural role; it is known to be ligated to four transmembrane helices of the L and M subunits (Deisenhofer et al., 1985a,b). The changes observed in Fe-depleted RCs may reflect reversible conformational changes. The nonexponential nature of the kinetics of charge recombination between $Q_B^-$ and $D^+$ (Figure 3B) and the distribution of $k_{AB}^{-1}$ values (Figure 6) in Fe-depleted RCs suggest that the protein is less rigid in the absence of a metal ion.

RCs reconstituted with $Mn^{2+}$, $Co^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ are well suited for magnetic resonance studies designed to address questions about the relationships between electron transfer processes, proton binding, dynamic conformational changes, and the electronic structure of the primary reactants. ENDOR experiments with RCs reconstituted with the diamagnetic metal ion $Zn^{2+}$ have already yielded information about the electronic structure and the environments of $Q_A^-$ and $Q_B^-$ (Lubitz et al., 1985; Feher et al., 1985). Experiments to determine the ligation of the metal ion to nitrogens by analyzing the superhyperfine structure of RCs reconstituted with $Cu^{2+}$ (see Figure 2F, insert) are in progress (Feher et al., 1986). The ease of reconstituting Fe-depleted RCs with $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ suggests that other metal ions may also be incorporated into the Fe site. Studies with RCs reconstituted with mono- and trivalent metals, or metals with different ionic radii, should yield further insight into the importance of charge or metal size on the electron-transfer processes in bacterial RCs. These studies can be extended to RCs from photosystem II of green plants. Klimov et al. (1980) have shown that the $Fe^{2+}$ of RCs of photosystem II can be extracted and reincorporated. Reconstitution of these RCs with $Mn^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Zn^{2+}$, and other metal ions, together with spectroscopic measurements, should provide structural information not presently available since no crystals of these RCs have yet been obtained.

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**Registry No.** Fe, 7439-89-6; Fe(2+), 15438-31-0; Mn(2+), 16397-91-4; Co(2+), 22541-53-3; Ni(2+), 14701-22-5; Cu(2+), 15158-11-9; Zn(2+), 23713-49-7.

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# Detection, Characterization, and Quenching of the Intrinsic Fluorescence of Bovine Heart Cytochrome *c* Oxidase†

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ABSTRACT: The intrinsic fluorescence of lauryl maltoside solubilized bovine heart cytochrome *c* oxidase has been determined to arise from tryptophan residues of the oxidase complex. The magnitude of the fluorescence is approximately 34% of that from *n*-acetyltryptophanamide (NATA). This level of fluorescence is consistent with an average heme to tryptophan distance of 30 Å. The majority of the fluorescent tryptophan residues are in a hydrophobic environment as indicated by (1) the fluorescence emission maximum at 328 nm and (2) the differing effectiveness of the quenching agents: $Cs^+$, $I^-$, and acrylamide. Cesium was ineffective up to a concentration of 0.7 M, whereas quenching by the other surface quenching agent, iodide, was compiex. Below 0.2 M, KI was ineffective whereas between 0.2 and 0.7 M 15% of the tryptophan fluorescence was found to be accessible to iodide. This pattern indicates that protein structural changes were induced by iodide and may be related to the chaotropic character of KI. Acrylamide was moderately effective as a quenching agent of the oxidase fluorescence with a Stern–Volmer constant of 2 $M^{-1}$ compared with acrylamide quenching of NATA and the water-soluble enzyme aldolase having Stern–Volmer constants of 12 $M^{-1}$ and 0.3 $M^{-1}$, respectively. There was no effect of cytochrome *c* on the tryptophan emission intensity from cytochrome *c* oxidase under conditions where the two proteins form a tight, 1:1 complex, implying that the tryptophan residues near the cytochrome *c* binding site are already quenched by energy transfer to the hemes of the oxidase. The lauryl maltoside concentration used to solubilize the enzyme did not affect the fluorescence of NATA. In contrast, the fluorescence spectral maximum of indole was shifted to a shorter wavelength near the critical micelle concentration of lauryl maltoside, indicating that indole had partitioned into the detergent micelle. The fluorescence spectrum of indole in a lauryl maltoside micelle resembles the tryptophan fluorescence of cytochrome *c* oxidase. These data support the idea that in a membrane protein there are two possible environments that may give rise to the fluorescence properties of tryptophan seen with cytochrome *c* oxidase: the interior of the protein and the protein/lipid or protein/detergent interface. The ability of $Cs^+$ and $I^-$ to quench the fluorescence of indole in lauryl maltoside micelles is much greater than the ability of these agents as quenchers of the oxidase fluorescence. These quenching data suggest that in cytochrome *c* oxidase the fluorescent tryptophan residues are buried in the protein. Thus, quenching studies represent a method for distinguishing between the two possible types of hydrophobic environments in integral membrane proteins. It is concluded that the fluorescent tryptophans in cytochrome *c* oxidase are asymmetrically located in the complex, removed from the cytochrome *c* binding region, and buried in the interior of the protein rather than at the protein/lipid interface.

Fluorescence spectroscopy has been an important tool in the study of the dynamics of protein conformation. Intrinsic fluorescence of protein molecules most commonly arises from the aromatic amino acid tryptophan (Lakowicz, 1983). Tryptophan fluorescence is highly sensitive to environment and is thus a useful structural probe (Burstein et al., 1973).

Cytochrome *c* oxidase (EC 1.9.3.1; cytochrome *c*:oxygen oxidoreductase) is an integral protein of the inner mitochondrial membrane. This protein consists of 10–13 nonidentical subunits with a total molecular weight of 200 000 and contains

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