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The redox potential of the primary quinone Q_A of bacterial photosynthesis is independent of the divalent metal ion

Susan K. Buchanan, G. Charles Dismukes and Roger C. Prince*

Department of Chemistry, Princeton University, Princeton, NJ 08544 and **Exxon Research and Engineering Company, Annandale, NJ 08801, USA*

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The function of the ferrous ion which links the primary and secondary quinone electron acceptors, Q_A and Q_B, via histidine ligands from the reaction center protein of the photosynthetic bacterium *Rhodobacter sphaeroides* (Y) has been investigated by substitution with Mn(II), Cu(II) and Zn(II), using biosynthetic incorporation of the metal ion. The midpoint potential and pH dependence for reduction of Q_A in chromatophore membranes were found to be indistinguishable for all four divalent ions, and in agreement with earlier results on the R26 and Ga strains of *R. sphaeroides* containing only iron. We therefore conclude that the divalent ion contributes no functionally significant covalent coupling to the primary acceptor and that these metals have no differential electrostatic influence. Furthermore, inhibition of photoreduction of Q_B by the herbicide ametryne was found to be the same for all four samples. Thus, these four divalent ions exhibit no differential influence on the binding properties of this herbicide in the Q_B site.

Bacterial photosynthesis; Reaction center; Ubiquinone; Electron transport; Metal substitution

1. INTRODUCTION

The initial electron transfer steps which occur during photosynthesis in plants, algae, and bacteria take place in protein complexes called reaction centers. The emergence of 3-dimensional structures, determined by X-ray crystallography, for the reaction center proteins from purple bacteria has introduced a new era in photosynthesis research [1–3]. The opportunity now exists to relate structure to function at an atomic level.

The function of the iron in reaction centers has remained unresolved, despite several studies which have succeeded in eliminating some of the possible roles. There are two quinones in the reaction center, Q_A and Q_B, which act as sequential electron carriers. EPR has shown that a ferrous ion interacts via ligand-mediated superexchange coupling with both semiquinones, Q_A^- and Q_B^-, and also with the reduced intermediary electron carrier, bacteriopheophytin$^-$ [4–9]. These interactions had earlier been considered evidence that the metal functions as an orbital bridge which is needed for efficient electron transfer [5]. An essential redox function for the ferrous ion has been eliminated by the absence of any redox chemistry [10], and the ability to replace the iron by other divalent metal ions [11–14].

In the reaction center of *Rps. viridis* the iron is coordinated to six atoms arranged in a distorted square pyramidal geometry. These are derived from five amino acid ligands from two protein subunits; four imidazole ligands from histidines and a bidentate carboxylate from a glutamic acid [15]. The NH ring proton of one of the imidazoles is H-bonded to a carbonyl oxygen atom of Q_A, which is the presumed pathway for superexchange. Located in a *trans* position on the far side of the distorted square planar array of imidazoles, one finds the binding site for Q_B and its herbicide analogs. The coordination geometry of Q_B is less well defined since atomic resolution data have not yet been published on reaction centers with Q_B bound. In *R. sphaeroides*, both Q_A and Q_B are ubiquinone molecules.

Correspondence address: S.K. Buchanan, Department of Chemistry, Princeton University, Princeton, NJ 08544, USA

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Because of the location of Q_A and Q_B in the outer coordination sphere of the metal, it had been expected that metal substitution ought to influence the kinetics of electron transfer between Q_A and Q_B in a major way. Paradoxically this is not observed, either in reaction centers in which alternate divalent metals are introduced biosynthetically [10,16], or by extraction of Fe and reconstitution with other divalent metals [12]. Extraction of the metal altogether reduces these kinetics by only a factor of two [12]. On the other hand, removing the Fe causes a 20-fold increase in the lifetime of the P^+BPh^- state and a 2-fold decrease in the formation of the subsequent state $P^+Q_A^-$ [26].

The redox properties of Q_A are also modified appreciably by metal extraction; it then functions as a 'normal' two-electron quinone, in contrast to the one-electron reduction seen in iron-containing reaction centers [17]. It is not possible to attribute this change exclusively to the removal of the metal, however, since other changes are also brought about by the extraction conditions [14]. Particularly, the protein environment around Q_A influences its redox properties [18,19].

While the midpoint potential for Q_A/Q_A^- is -45 mV and pH-independent in isolated detergent micelles of reaction centers of *R. sphaeroides* [20], in native chromatophore membranes it is -180 mV and pH-dependent below pH 9.8 [21]. The origin of this difference has never been explained, but it does point to factors outside of the reaction center itself in influencing the redox properties in vivo.

To test the influence of the divalent ion's charge density and orbital configuration on the properties of Q_A, we sought to measure the midpoint reduction potential and pH dependence for Q_A/Q_A^- in chromatophore membranes for which the reaction center Fe was replaced with Mn, Cu, and Zn using biosynthetic substitution to eliminate possible artifacts due to extraction.

2. MATERIALS AND METHODS

R. sphaeroides wild-type cells (Y strain) were grown phototropically as described by Reed and Clayton [22]. Cultures were the kind gift of Dr F. Reiss-Husson, CNRS, France. The method of biosynthetic substitution of the non-heme Fe(II) ion has been described [13,14]. Metal-substituted cultures were grown on Hutner's medium (containing no yeast extract) with 7.2×10^{-8} M $FeSO_4$, plus 2.7×10^{-4} M $CuSO_4$, 1.8×10^{-3} M $MnSO_4$ or 3.0×10^{-3} M $ZnSO_4$. All chemicals were reagent grade except where analytical grades were available. All solutions were prepared using deionized water having undetectable amounts of Fe, Cu, Mn, and Zn as determined by atomic absorption. Chromatophores were prepared with a French pressure cell, and reaction centers were isolated from the same batch of chromatophores used for the redox titrations and examined by atomic absorption analysis according to the procedure of Jolchine and Reiss-Husson [23]. Metal analysis for Fe, Cu, Mn and Zn was performed by flameless atomic absorption spectroscopy as described in [10].

Flash-induced optical absorbance changes were measured with the double-beam spectrophotometer described in [24]. This involved filtered xenon flash excitation (10 μs pulse width at half-height, $\lambda > 700$ nm spectral range) with the absorbance amplitude recorded at 1 ms following the flash peak. Redox potentiometry followed the method of Dutton [25]. The midpoint of the primary quinone acceptor was monitored indirectly by following the amplitude of the flash-induced charge separation as a function of the redox potential measured before the flash. The potential was set using sodium dithionite and potassium ferricyanide as the titrants; 10 μM 2,3,5,6-tetramethylphenylenediamine, *N*-methylphenazonium methosulfate, 2-hydroxy-1,4-naphthoquinone, and pyocyanin were used as mediators. The solution also contained 5 μM valinomycin as a mobile ionophore. Photooxidation of the bacteriochlorophyll special pair was monitored at 605–540 nm. Cytochrome *c* was measured optically as the ascorbate-reduced minus ferricyanide-oxidized spectrum (table 2). All reaction mixtures contained 100 mM KCl plus an appropriate pH buffer at 20 mM.

Table 1

Atomic absorption data for *R. sphaeroides* grown in normal and metal-enriched media

Metal enrichment	Resonance line (A; element)	Relative sensitivity[a] (μg/ml)	Fe/RC	Cu/RC	Mn/RC	Zn/RC
Fe(II)	2483(Fe)	0.045	1.00 + 0.02	<0.02	<0.02	<0.02
Cu(II)	3247(Cu)	0.063	0.25 + 0.02	0.85 + 0.02	<0.02	<0.02
Mn(II)	2795(Mn)	0.018	0.39 + 0.02	<0.02	0.71 + 0.02	<0.02
Zn(II)	2139(Zn)	0.0057	0.50 + 0.02	<0.02	<0.02	0.60 + 0.02

[a] Concentration giving 1% absorption

3. RESULTS

Analysis of the metal content of reaction centers prepared from chromatophore membranes used in the redox titrations is given in table 1. Cell growth in the appropriate medium achieves 85% replacement of Fe by Cu, 71% replacement by Mn, and 60% replacement by Zn. The remaining transition metal content is made up largely of Fe(II). For all metal substituted reaction centers, the total metal content is about 1.1 per reaction center on the basis of an extinction coefficient at 802 nm of 2.88×10^5 M^{-1} cm^{-1} [5]. Table 2 shows the amounts of cytochrome *c* and reaction centers in chromatophores from cells grown on different divalent cations (from which the reaction centers used for the metal analysis of table 1 were made). Inducing growth with other metals in the reaction center does not seem to interfere with other iron containing proteins (at least as assayed by the cytochrome content), nor does it appear to alter the reaction center concentration in the cells.

Redox titrations of chromatophores were conducted at pH values from 6 to 10.5. A representative pair of titration curves of the primary acceptor is shown in fig.1 (here, for Mn-enriched reaction centers). It is seen that the titrations are fully reversible and fit a one-electron titration shown by the solid curves. The E_m/pH relationship is plotted in fig.2, which also incorporates the earlier data of Prince and Dutton [21] for *R.*

Table 2

Amounts of cytochrome *c* and reaction centers in chromatophores from *R. sphaeroides* Y grown on different divalent cations

Metal	Cytochrome *c*	Reaction center
	(μM/100 μM bacteriochlorophyll)	
Fe	0.67	0.91
Cu	0.85	1.04
Mn	0.65	0.91
Zn	0.80	0.97

Cytochrome *c*: determined from ascorbate (+ 5 μM *N*-methylphenazonium methosulfate) minus ferricyanide difference spectra, and therefore includes both cytochromes c_1 and c_2. An E_{mM} of 20 cm^{-1} was used [27]. Reaction center: measured from the total change at 605–540 nm after 8 saturating flashes in the presence of ascorbate, valinomycin and 3-undecyl-2-hydroxy-1,4-naphthoquinone. An E_{mM} of 29.8 cm^{-1} was used [27]

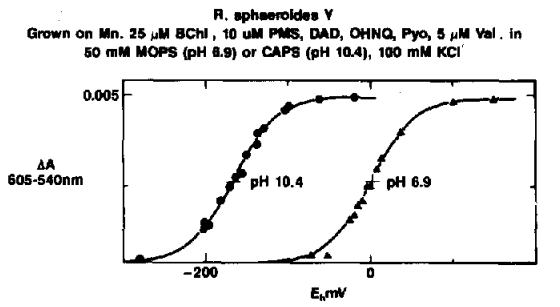

Fig.1. Redox titrations of Q_A in reaction centers containing Mn (71% replacement of Fe). Chromatophores of *R. sphaeroides* Y (25 μM bacteriochlorophyll) were suspended in 20 mM *N*-morpholinopropanesulfonate (pH 6.9) or cyclohexylaminopropanesulfonate (pH 10.4), 100 mM KCl with the redox dyes listed in the text, and 5 μM valinomycin. The extent of reaction center photo-oxidation was assayed at 605–540 nm, 1 ms after a saturating single-turnover flash. The titrations show data obtained in both reductive and oxidative titrations.

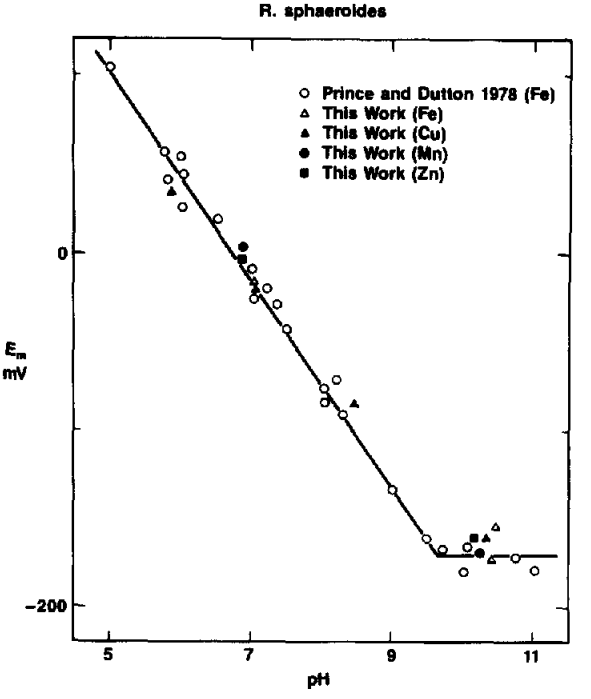

Fig.2. The pH dependence of the midpoint potential (E_m) of Q_A/Q_A^- in *R. sphaeroides*. The figure shows earlier data from strains Ga and R26, collected in [19], together with data reported here from strain Y. These experiments used the buffers of fig.1, or 3-(dimethyl(hydroxymethyl)methylamino)-2-hydroxypropanesulfonate at pH 8.5.

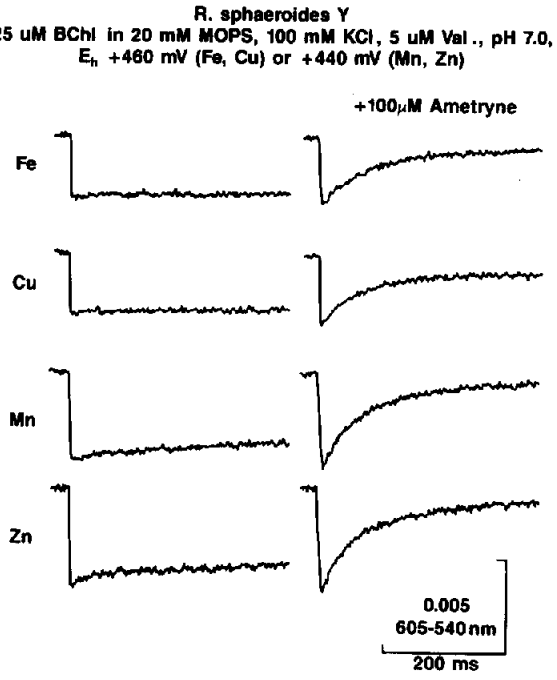

Fig.3. The effect of ametryne in reaction centers containing different metals. Chromatophores (25 μM bacteriochlorophyll) were suspended in the buffer of fig.1, without the redox dyes, and the ambient potential (E_h) poised at 460 mV (Fe, Cu) or 440 mV (Mn, Zn). Ametryne was added to 100 μM as indicated.

sphaeroides Ga and R26 strains. There is an apparent pK for the reduced form of the primary acceptor at pH 9.8. Above this pH, the E_m appears to be pH-independent for all membrane preparations and has a value of −180 mV. Below pH 9.8 the E_m increases by 60 mV per unit drop in pH for all samples, as illustrated by the slope of the straight line plot.

Fig.3 shows a comparison of the rates of $P^+Q_A^-$ recombination in the absence and presence of the herbicide ametryne (a terbutryn analog), which is thought to inhibit electron transfer between Q_A and Q_B by displacement of Q_B [1]. In this experiment the solution potential was kept high (450 ± 10 mV) in order to insure chemical oxidation of cytochrome c_2, which in its reduced form would compete with recombination for reduction of P^+. In the absence of the herbicide, the re-reduction of P^+ is slow because the electron comes from Q_B^-. When the latter is displaced by the herbicide, the electron returns from Q_A^-, in a faster reaction. For native Fe-containing and for metal-substituted chromatophore preparations, the acceleration of the recombination by the herbicide is identical and corresponds to the expected kinetics for recombination.

4. DISCUSSION

The observed dependence of −60 mV/pH for the midpoint potential of the Q_A/Q_A^- couple in chromatophores of strain Y cells (fig.2) indicates that the Mn-, Cu- and Zn-enriched preparations follow the same behavior as Fe-containing samples from this same strain as well as the R26 and Ga strains. Below pH 9.8 the transient reduction of Q_A involves one electron and one proton, while above this pH only an electron is involved. The E_m above the pK is −180 mV for all chromatophores studied.

These data show that there is no differential influence of the divalent metals on the E_m/pH profile for reduction of the primary acceptor in the chromatophore membrane. Therefore, the different valence electron configurations of the divalent metal ions, which are $4s^03d^x$ (x = 5, 6, 9, 10: Mn, Fe, Cu, Zn), do not affect the reduction potential of the primary acceptor. This means that the weak electron-exchange coupling of 0.1–0.5 cm^{-1} seen between Fe(II) and Q_A^- [27,28] cannot be appreciably larger for these other metals. Even the redox-inactive metal, Zn, functions as well as the potentially redox-active Mn, Cu, and Fe. We conclude that the metal plays no direct redox role in the function of the primary acceptor. Furthermore, there is no appreciable covalent mixing of metal orbitals with the semiquinone orbitals, since this would surely result in different potentials for the primary acceptor. It should be pointed out that while oxidation of Fe(II) to Fe(III) is possible in green plant photosystem II [31], this occurs under non-physiological conditions and has not been seen in bacterial reaction centers or chromatophores.

Likewise, there is no differential electrostatic effect on the E_m or the pK because of the different relative charge densities for these ions, estimated at 0.8, 1.0, 1.1 and 1.0 (for Mn, Fe, Cu and Zn, respectively [28], although this small predicted difference may not necessarily be reliably estimated using this approximation). This does not exclude a direct electrostatic influence of the metal ion on

the E_m or pK, since reaction centers having no divalent transition metal ion in the site have not been found. Attempts to grow cells on media enriched with magnesium or calcium were unsuccessful, perhaps because of size restrictions (relative charge densities 1.4 and 0.4, respectively); nor has success been reported in introducing them by extraction and reconstitution [12].

The various metal ions also exert no large differential influence on the binding properties of ametryne to the Q_B site as shown in fig.3. This result could perhaps have been anticipated from herbicide binding studies in *Rps. viridis* crystals, showing that the Fe(II) is not directly involved in the binding of herbicides located in the Q_B site [1,15].

Semi-empirical electronic structure calculations of the influence of a divalent charge on the pK_a for ionization of the ring NH proton of a coordinated imidazole have shown that the charge is capable of stabilizing the imidazolate anion by a large amount (−60 kcal/mol in vacuum) [16]. A model in which this stabilization is put forward as a part of the possible function of the divalent charge has appeared [32]. This essentially electrostatic role for the metal ion is consistent with the present results.

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