DIRECT MEASUREMENT OF THE MIDPOINT POTENTIAL OF THE PRIMARY ELECTRON ACCEPTOR IN *RHODOPSEUDOMONAS SPHEROIDES IN SITU* AND IN THE ISOLATED STATE: SOME RELATIONSHIPS WITH pH AND *o*-PHENANTHROLINE

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1. Introduction

Recently we have revealed a redox component in both purple sulfur [1] and purple non-sulfur [2, 3] photosynthetic bacteria which in the reduced form is paramagnetic. The reduced component exhibits a striking electron spin resonance (ESR) signal centered at $g = 1.82$. The component is present in the isolated reaction center of *Rps. spheroides* [2]. In reaction centers, the $g = 1.82$ component conforms to the behavior expected for the primary electron acceptor of the reaction center bacteriochlorophyll, *P*870.

On account of the above properties of the $g = 1.82$ component and in the absence of a full chemical identification (although it is possibly an iron–sulfur protein [1, 2, 6, 7]) we have designated the $g = 1.82$ component 'photoredoxin' [8]. Thus if photoredoxin is the primary electron acceptor of *P*870, then on a redox potential dependence basis, its level of oxidation–reduction before illumination should be: a) inversely proportional to the extent of *P*870 formed in the light and b) directly proportional to the extent of light induced bacteriochlorophyll triplet formation; the relationship should be independent of whether triplet production is a process competing with the normal photochemistry or is on the main energy pathway.

In this communication, using isolated reaction centers from *Rps. spheroides*, strain R26, we prove that within experimental error these relationships between photoredoxin and light induced $^{+}P870$ and the triplet state bacteriochlorophyll are true. This strongly complements the kinetic evidence [2] that photoredoxin is the first component to receive an electron from *P*870. Further substantiation comes from the fact that in isolated reaction centers the midpoint potential (E_m) of photoredoxin and the indirectly measured E_m of the primary electron acceptor (i.e. the loss of *P*870 photooxidation with decreasing potential) show a similar relationship to pH and to added *o*-phenanthroline: both appear to be independent of pH from values 6.0–9.0 and neither are affected by *o*-phenanthroline. We confirm, however, that the E_m of the primary electron acceptor when associated with the chromatophore membrane has an apparent –60 mV/pH unit dependency and is sensitive to *o*-phenanthroline [see ref. 9].

2. Methods

Rps. spheroides (strain R26, the blue-green mutant and strain Ga, the green mutant) were grown anaerobically in the light on succinate for 1–2 days. Chromatophores were made by grinding with alumina. Bacteriochlorophyll was assayed using the *in vivo* absorption coefficients of Clayton [10]. Reaction centers from *Rps. spheroides* R26 were isolated using

the detergent lauryldimethylamine-*N*-oxide (1%) (a gift from the Onyx Chemical Co., Jersey City, New Jersey) and purified by DEAE-cellulose (Whatman DE52) column chromatography. Since this particular detergent interferes with redox potentiometry, it was exchanged after isolation for 0.1% Triton X-100. Reaction centers were also incorporated into egg lecithin phospholipid vesicles in the ratio of 1 *P*870 per 200 lecithins (egg yolk lecithin was a generous gift from Dr. V. Luzzati).

Redox potentiometry for low temperature analysis was carried out as previously described [11], samples of known potential being transferred anaerobically into 3 mm i.d. EPR tubes and rapidly frozen to liquid nitrogen temperatures in the dark using cooled isopentane for rapid heat conduction. ESR analysis was performed with a Varian E4 ESR spectrometer; the measuring temperature was approximately 10°K with spectrometer microwave power settings of 20 mW for photoredoxin and 2 mW for both the oxidized and triplet state bacteriochlorophyll signals.

3. Results

3.1. *The relationship of redox state of photoredoxin with the low temperature light induced P870 oxidation and triplet state formation*

Fig. 1 describes the redox potential dependency of the extent of light induced $^{+}P870$ and triplet state bacteriochlorophyll. The curves drawn through both sets of points define a one-electron (n = 1) redox couple with an $E_{m\,7.2}$ value of –45 mV and describe the course of oxidation–reduction of the primary electron acceptor, the redox state of which we assume governs the extent of both *P*870 photooxidation and triplet formation. The E_m is similar to other indirectly determined values for the primary electron acceptor in *Rps. spheroides* isolated reaction center preparations obtained at room temperature [12, 13].

Fig. 2 shows some typical ESR spectra of photoredoxin obtained in the dark with reaction centers poised at various redox potentials. Fig. 3 describes redox titrations of photoredoxin. Like those shown in fig. 1, the curve drawn through the points is theoretical for a one electron carrier with an $E_{m\,7.2}$ value of –45 mV. Thus the basic redox properties of photoredoxin are consistent with its identity as the primary electron acceptor of *P*870 in the reaction center protein.

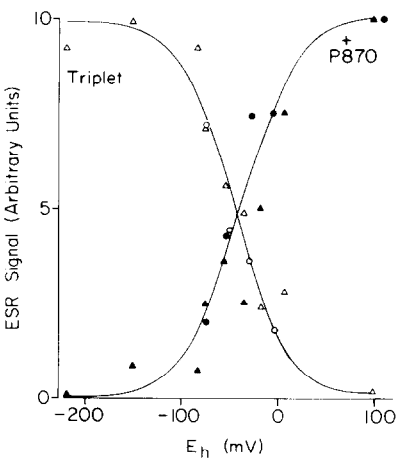

Fig. 1. Redox potential dependence of the extent of light-induced oxidized and triplet state reaction center bacteriochlorophyll in *Rps. spheroides* reaction center protein (*P*870 concentration 80 μM) in 50 mM morpholinopropane sulphonate buffer at pH 7.0. The following mediating dyes were present: 50 μM phenazine ethosulfate; 100 μM pyocyanine and 50 μM 2-hydroxy-1,4-napthaquinone. Oxidized *P*870 ($^{+}P870$) was assayed at g 2.00 (peak to peak amplitude); triplet state was assayed by the low field band at g 2.13 [see ref. 2]. Titrations were performed with reaction center protein in 0.1% Triton X-100 (○ for triplet, ● for $^{+}P870$) and combined with egg yolk lecithin at a *P*870:phospholipid ratio of 1:200 (△ for triplet, ▲ for $^{+}P870$). The titration was performed over a 30–45 minute period, samples being taken in reductive and oxidative phases. The line drawn through the points is theoretical for a n = Nernst curve. Further details are given in the Methods.

3.2. *The relationship of the E_m of the primary electron acceptor with pH*

If identification of photoredoxin as the primary electron acceptor is correct we should further expect that the pH dependence of the E_m of photoredoxin is the same as that for the primary acceptor obtained indirectly *via* assay of light induced $^{+}P870$ as a function of potential. This is shown to be the case in fig. 4A. From pH 5.7–9.1 the E_m values of photoredoxin, and the indirectly determined primary electron acceptor are the same within experimental error. Reed et al. [12] found a similar result in reaction centers measuring the E_m of the primary electron acceptor by a fluorescence technique.

A very different pH dependency, however, is encountered when the reaction centers are associated with the chromatophore membrane. Fig. 4B shows

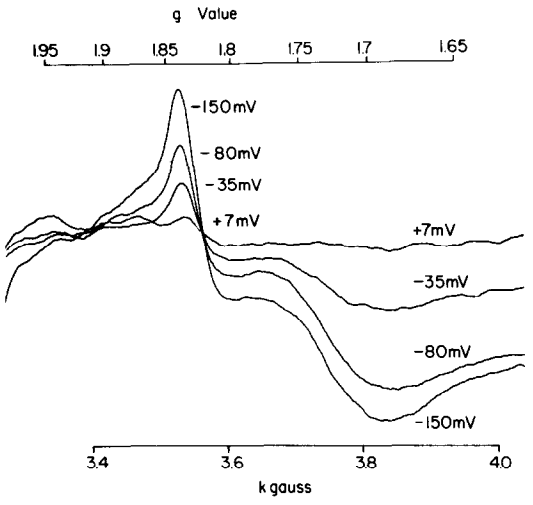

Fig. 2. Dark ESR spectra of photoredoxin as a function of redox potential in the *Rps. spheroides* reaction center protein. The conditions were as in fig. 1; the reaction centers were suspended in 0.1% Triton X-100.

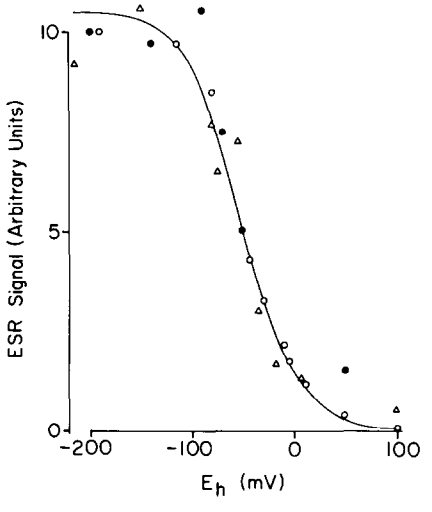

Fig. 3. Redox potential titration of photoredoxin in *Rps. spheroides* reaction centers. The conditions of the titration were as given in fig. 1. The state of reduction of photoredoxin was assayed by the amplitude of the g 1.82 band (low field peak to high field trough). The titrations were done in 0.1% Triton X-100 (●, ○: two different preparations) and combined with egg yolk lecithin at a ratio of 200:1 phospholipid:*P*870 (Δ). The line through the points is a theoretical n = 1 Nernst curve.

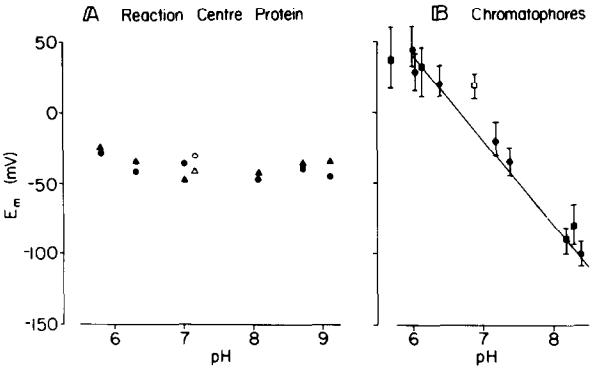

Fig. 4. The pH dependency of the measured E_m of the primary electron acceptor in A) the reaction center protein and B) the chromatophore membrane of *Rps. spheroides*: A) The reaction centers were at a *P*870 concentration of 70–80 μM in 0.1% Triton X-100. The E_m of the primary acceptor was assayed by the extent of the light induced g 2.00 signal (●) as in fig. 1 or by the extent of the g 1.82 signal in the dark (▲) as in fig. 3. The E_m of the primary electron acceptor measured in the presence of 4 mM *o*-phenanthroline (4 hr preincubation) is given by the open symbols. (○ for light induced g 2.00 and △ for dark g 1.82 signals). Each E_m determination was obtained by assay of six samples taken both reductively and oxidatively over a 30 min period. The limits of observable experimental error of the E_m values were typically ±15 mV by measuring photoredoxin and ±20 mV for measuring the light induced g = 2 signal; B) The chromatophores of *Rps. spheroides*, both the Ga (●) and the R26 (■) mutants were used at bacteriochlorophyll concentrations in the range of 70–100 μM. The E_m of the primary electron acceptor was assayed by the light induced g 2.00 signals as in fig. 1, the general procedure being the same as for the isolated reaction center protein. The E_m of the primary electron acceptor in the presence of 2 mM *o*-phenanthroline was performed with *Rps. spheroides* R26 chromatophores (□) is the average of two closely agreeing determinations. The limits shown are the maximum and minimum values possible for the fitting of a Nernst theoretical curve to the experimental points.

The pH buffers used were: 50 mM morpholine ethane sulphonate, 50 mM KCl, from pH 5–6.5; 50 mM Morpholinopropane sulphonate, 50 mM KCl, from pH 6.5–7.5; 100 mM Tris–HCl or glycyl–glycine buffer, from pH 7.5–9.0. Redox mediators were 50 μM amounts of phenazine methosulphate, phenazine ethosulphate and 2-hydroxy-1,4-napthaquinone and 120 μM pyocyanine.

that the E_m of the primary acceptor exhibits a −60 mV per pH unit dependency from pH 6–8.5 for both *Rps. spheroides* R26 and Ga mutants. Jackson et al. [9] have previously reported a similar dependence for the room temperature determination using *Rps. spheroides* Ga chromatophores.

3.3. *The effect of o-phenanthroline on the E_m of the primary electron acceptor*

Another difference between reaction centers in the isolated state and chromatophore membrane bound state is in the effect of the electron transfer inhibitor *o*-phenanthroline [14, 15] on the measured E_m of the primary electron acceptor. For reaction centers in the isolated state, *o*-phenanthroline has no significant effect ($\leqslant$ 10 mV) as shown in fig. 4A. However, in the chromatophore an approx. 30 mV increase in the measured E_m is encountered (fig. 4B); this is comparable with the 40 mV increase reported by Jackson et al. [9].

4. Discussion

4.1. *The identification of photoredoxin as the primary electron acceptor*

Photoredoxin in the isolated reaction center protein has an E_m value of −45 mV and an *n*-value of unity. The E_m of photoredoxin appears to be essentially independent of pH from 5.7–9.1; neither is it significantly affected by *o*-phenanthroline. The same properties exist for the primary electron acceptor in the isolated reaction center when determined indirectly by measurement of light induced extents of reaction center bacteriochlorophyll oxidation and triplet formation, or by other indirect methods of Clayton et al. [12, 13]. The close correlations of these chemical and thermodynamic data firmly complement the previously reported [2] closely matching flash induced kinetics at 8°K of photoredoxin reduction and subsequent dark reoxidation and *P*870 oxidation and dark rereduction and therefore strongly support the view that photoredoxin is the first electron transfer component to receive an electron from light activated *P*870.

Confidence that the $g_y = 1.82$ and $g_x = 1.68$ signals observed in the isolated protein, which we associate with reduced photoredoxin, do represent an intact component which exists in the parent bacterium is gained from fig. 5: the ESR spectrum of dithionine-reduced whole cells exhibits signals resembling the line-shape, microwave saturation and temperature dependence of the g_y and g_x bands of reduced photoredoxin; slight positional differences, however, are noted in fig. 5.

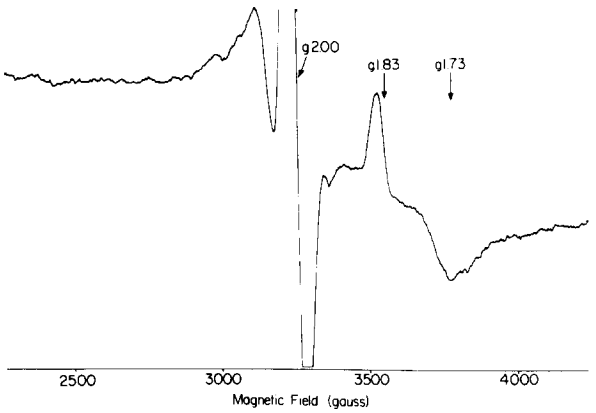

Fig. 5. The presence of photoredoxin in whole cells of *Rps. spheroides* (R26). The cells (800 μM bacteriochlorophyll) were reduced by sodium dithionite before freezing. ESR conditions were the same for the assay of photoredoxin in the isolated reaction center protein.

4.2. *The properties of the primary electron acceptor in the isolated protein and in the chromatophore membrane*

Why, in the isolated state, does the E_m of the primary electron acceptor (photoredoxin) exhibit a pH dependency nor any significant affect by *o*-phenanthroline, yet when associated with the chromatophore display a −60 mV pH unit dependency (indicating the uptake of a single proton after a one electron reduction) and a small but definite positive shift in the presence of *o*-phenanthroline (indicating its preferential binding to the reduced primary acceptor)? A simple reason could be that in the detergent-isolated state, the approach to equilibrium of reduced photoredoxin with *o*-phenanthroline or the hydrogen ion activity of the various buffers used has not been significant during the 20–30 min period of each titration. This would be surprising, however, in view of the high concentration of *o*-phenanthroline used (4 mM) and the 3000-fold range of hydrogen ion activity examined. If this is the case, then presumably when the primary electron acceptor is in the chromatophore, the supporting membrane facilitates a more rapid equilibrium. Even in the chromatophore, however, evidence from studies [16, 17] of hydrogen ion binding following flash induced reduction of the primary electron acceptor, suggests that hydrogen ion

binding does not rapidly follow (i.e., on the millisecond time scale) the reduction of the primary electron acceptor.

An alternative explanation could be that the effects of pH and *o*-phenanthroline are not direct [cf. ref. 9], there being a requirement for an endogenous component of the chromatophore membrane to elicit the E_m shifts.

A point of importance to pH/E_m relationships in general, rests on the time-scale of the hydrogen ion binding or release following the respective reduction or oxidation of a redox couple. Most determinations of this type are performed over a time period long enough for the redox couple in question to maintain equilibrium with the hydrogen ion activity. Under operating conditions however, in which electron transfer is perhaps much more rapid than the hydrogen ion binding-release, the E_m of the redox couple could be far removed from that indicated from measurements in which the system is closer to equilibrium with hydrogen ion activity. In the case of the primary electron acceptor in the chromatophore we have the situation in which it becomes rapidly reduced; in the time interval before the hydrogen ion binding, the E_m of the redox couple (unprotonated reduced form) could be as electronegative as that dictated by the pK of the reduced form. For such a pK of 12 for example, the E_m of the pulse-reduced primary electron acceptor at pH 7.0 would be approximately −320 mV (in contrast to the −25 mV at pH 7.0 with the reduced form protonated). In this state the primary acceptor would have an E_m similar to that of pyridine nucleotide; this contrasts with a 99.999% level of reduction required to achieve the same potential (i.e. −320 mV) if the primary electron acceptor is protonated immediate to its reduction. The possibility exists that the redox span of the light reaction (defined by the $E_{m\,7}$ values of *P*870 (440 mV) and its acceptor (−25 mV) [18, 19] determined in equilibrium with hydrogen ions; the span possibility expands to a total of 585 mV if *P*870 and the acceptor are operating at 90% oxidized and reduced respectively) is in fact much wider potential range than anticipated.

Note added in proof

M.C.W. Evans and S.G. Reeves have shown in *Chromatium* D (personal communication) that *o*-phenanthroline induces an approximate 140 mV positive shift in the E_m of photoredoxin measured directly in the chromatophore; the shift is commensurate with that expected for the primary acceptor from indirect measurements [9]. This is further direct chemical evidence that photoredoxin is the first electron acceptor for the photoactivated reaction center bacteriochlorophyll complex

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