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FLASH PHOTOLYSIS–ELECTRON SPIN RESONANCE STUDY OF THE EFFECT OF *o*-PHENANTHROLINE AND TEMPERATURE ON THE DECAY TIME OF THE ESR SIGNAL B1 IN REACTION-CENTER PREPARATIONS AND CHROMATOPHORES OF MUTANT AND WILD STRAINS OF *RHODOPSEUDOMONAS SPHEROIDES* AND *RHODOSPIRILLUM RUBRUM*

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SUMMARY

We have studied the effect of *o*-phenanthroline and temperature on the decay rate of Signal B1 in reaction-center preparations and in chromatophores from *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum*. We have shown that *o*-phenanthroline binds specifically to the reaction center protein (the binding center is probably at the iron) and when so bound inhibits the transfer of electrons from primary to secondary acceptors. We have also shown that the direct return decay time ($A^- \rightarrow P865^+$) increases with increasing temperature above approx. 150 K. This phenomenon has been interpreted within a quantum mechanical tunnelling model in which the distance of closest approach between $P865^+$ and A^- increases about 2 Å between approx. 150 and 300 K.

INTRODUCTION

Since the initial discovery of a light-induced electron spin resonance signal (hereafter called Signal B1 after Kohl [1]) in photosynthetic bacteria by Sogo et al. [2] in 1957, there has been considerable interest in this signal as a monitor of reaction center activity in these systems. It now appears certain that the free radical giving rise to Signal B1 can be identified [3–5] as the positive radical ion of the reaction center bacteriochlorophyll *a* component variously called P870 [6], P890 [7] after its absorption maximum or $P_{0.44}$ after its oxidation potential [8]. These assignments are further substantiated by the observation of Signal B1 seen in purified reaction center preparations from the R26 mutant of *Rhodopseudomonas spheroides* [3, 5] and further

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in photoreceptor subunit preparations from *Rhodospirillum rubrum* [9]. Recent work indicates that the Signal B1 free radical is a dimer cation radical of bacteriochlorophyll *a* [10, 11].

The availability of purified reaction-center protein preparations from the R26 mutant of *R. spheroides* [12, 13] has provided a clearer picture of the stoichiometry of the reaction center. By analysis it appears that this protein contains 2 bacteriochlorophyll *a* molecules with a maximum absorption at 865 nm [14] (this is the P865 component that gives rise to Signal B1 on photooxidation), 2 bacteriochlorophyll *a* molecules with a maximum absorption at 800 nm [14], 2 bacteriopheophytin *a* molecules with a maximum absorption at 760 nm [14], one iron atom and one molecule of ubiquinone-10 [15]. Apparently the iron serves as the primary acceptor* possibly in combination with ubiquinone [18–21]. That iron is involved is indicated by the effect which the potent iron chelator *o*-phenanthroline has on the system. *o*-Phenanthroline appears to block electron transfer from the primary acceptor to secondary acceptors. The inhibitory effect of *o*-phenanthroline was first discovered by Parson and Case [22] and later studied extensively by Clayton et al. [23] and Jackson et al. [34].

The kinetic behavior of the decay of Signal B1 at low temperatures is very unusual in that the 1/e decay time of approx. 30 ms is virtually temperature independent from 1.7 to 77 K [5]. This phenomenon has been explained in terms of an electron return from the primary acceptor to $P865^+$ by a quantum mechanical tunnelling process [5]. This type of electron transfer in biological systems was first suggested by Chance and co-workers [24, 25].

In this paper we present further evidence for the specific inhibitory effect of *o*-phenanthroline and for the tunneling model for electron transfer. In this study we have employed the technique of flash photolysis-electron spin resonance spectroscopy [26–28].

MATERIALS AND METHODS

All strains of bacteria [*R. spheroides* wild type (W) and a blue-green mutant (R-26) and *R. rubrum* wild type (S-1) and a blue-green mutant (BG-1)] were originally donated by Dr R. K. Clayton. All strains were grown in a modified Hutner's liquid medium [29] in a Sherer Model L24 Growth Chamber at 29 °C. Illumination was provided by a row of 40 W incandescent bulbs placed 3 inches apart and 8 inches from the bottles. All bacteria were harvested during the log phase after four days. Crude reaction centers were prepared by the method of Reed and Clayton [30]. Partially purified reaction centers were prepared according to the method of Clayton and Wang [13].

The flash photolysis-electron spin resonance apparatus and techniques are described elsewhere [26–28, 31]. All experiments were carried out under light-saturating conditions. All light was passed through a dark red filter, Corning CS2-64. The flash lamp (Xenon Corp. Novatron 185) had a rise time of approx. 10 μs and a decay time of approx. 50 μs. The horizontal light output of the lamp is about 0.5 $J \cdot rad^{-1}$.

* Although Feher [12] was the first to suggest that iron is involved in the primary acceptor, Feher et al. [16] later favored relegating iron to a secondary acceptor. However, later work by Dutton and Leigh [17] shows that iron is almost certainly part of the primary acceptor.

Low temperatures were achieved by use of a Varian E257 variable temperature accessory.

Samples for flash photolysis-ESR investigation were diluted so that the OD in the aqueous solution flat cell did not exceed 1.0 at 800 nm.

RESULTS AND DISCUSSION

Effect of o-phenanthroline

We used reaction centers obtained by treating chromatophores of the R26 mutant of *R. spheroides* with Triton X-100 [30]. These were partially purified by $(NH_4)_2SO_4$ fractionation. Since *o*-phenanthroline is only slightly soluble in water, *o*-phenanthroline was first dissolved in 95% ethanol and then added to the reaction centers in a 1 M solution so as to give the desired *o*-phenanthroline concentration. A control with addition of pure 95% ethanol showed no effect on the decay of Signal B1.

At room temperature the flash photolysis-ESR kinetics for reaction centers with no *o*-phenanthroline added exhibited a single first-order decay with a 1/e decay time τ of approx. 1.2 s (see Fig. 1a). Addition of *o*-phenanthroline caused the kinetics to change from monophasic to biphasic. Fig. 1b shows a typical biphasic decay. Analysis of the biphasic decay demonstrated that the slow phase was first order with $\tau = 1.2$–1.5 s. (This time was, within experimental error, the same as τ without addition of *o*-phenanthroline). The fast phase was also first order with $\tau \approx 100$ ms.

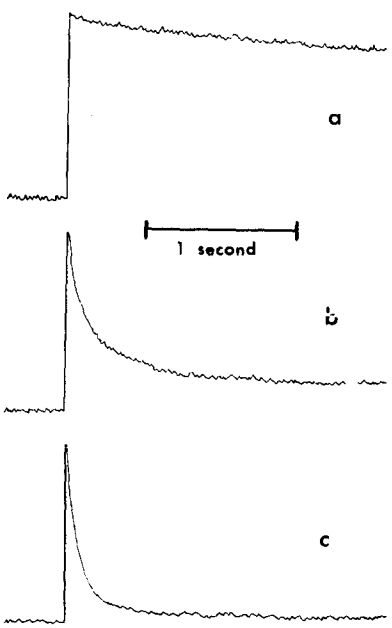

Fig. 1. Decay kinetics of Signal B1 in a Triton reaction center preparation from the R-26 mutant of *R. spheroides* following a saturating light flash (a) [*o*-phenanthroline] = 0, (b) [*o*-phenanthroline] $= 2.0 \cdot 10^{-3}$ M; (c) [*o*-phenanthroline] $= 9.1 \cdot 10^{-3}$ M. ESR conditions: microwave power, 20 mW; modulation amplitude, 10 G.

TABLE I

SLOW AND FAST-DECAY COMPONENTS OF SIGNAL B1 IN TRITON REACTION CENTERS FROM THE R-26 MUTANT OF *R. SPHEROIDES* IN THE PRESENCE OF *o*-PHENANTHROLINE

[*o*-Phenanthroline] (mM)	τ_{slow}(s)	τ_{fast}(ms)	(%) A_{slow}	A_{fast}/A_{slow}
0	1.2±0.1	–	100	0
0.10	1.3±0.1	*	100	≈ 0
0.50	1.2±0.1	132±30	63	0.59±0.08
0.99	1.0±0.2	144±40	53	0.88±0.11
1.64	1.4±0.2	100±15	29	2.4±0.4
1.96	1.5±0.3	85±15	31	2.2±0.3
2.9	1.4±0.3	97±10	26	2.9±0.5
3.2	1.5±0.3	111±10	25	3.0±0.5
3.8	1.4±0.3	96±10	18	4.7±1.0
4.8	*	121±10	14	6.0±1.5
9.1	*	113± 7	5	20±13

* Signal-to-noise did not permit a time constant measurement.

The ratio of the fast-phase amplitude (A_{fast}) to the slow-phase amplitude (A_{slow}) increased with increasing *o*-phenanthroline concentration. Fig. 1c shows that at $9.1 \cdot 10^{-3}$ M *o*-phenanthroline the decay is almost all fast. The results are summarized in Table I.

Use of reaction centers prepared by another method [13] gave essentially the same results. Dialysis of *o*-phenanthroline-treated reaction centers in buffer (0.01 M Tris, pH 7.5) abolished the fast decay induced by *o*-phenanthroline indicating that the *o*-phenanthroline is not permanently bound. Glycerol addition had no effect on the fast-decay time constant. This fact is important to the temperature dependence studies described below. The linewidth and saturation behavior of Signal B1 were unchanged by the *o*-phenanthroline addition.

The effect of *o*-phenanthroline addition on chromatophores prepared from wild-type *R. spheroides*, *R. rubrum* and a blue-green mutant BG-1 of *R. rubrum* was qualitatively the same as for reaction center preparations. There was, however, some variation in the magnitude of the effect for a given concentration of *o*-phenanthroline (see later discussion).

The type of biphasic kinetic behavior which we observed for Signal B1 on addition of *o*-phenanthroline has been found for at least two other components interacting with reaction centers (RC): (1) mammalian cytochrome *c* [32] and (2) phenazine methosulfate [33]. In both of these cases the behavior is interpreted in terms of a specific equilibrium binding mode. Our system is similar except that we assume that *o*-phenanthroline binds specifically to the iron which is assumed to be at least part of the primary acceptor moiety. For equilibrium binding the following equilibrium should prevail

$$RC + o\text{-phenanthroline} \rightleftharpoons RC \cdot o\text{-phenanthroline}$$

where RC · *o*-phenanthroline represents a reaction center protein with a molecule of *o*-phenanthroline complexed to the iron. The corresponding equilibrium constant is

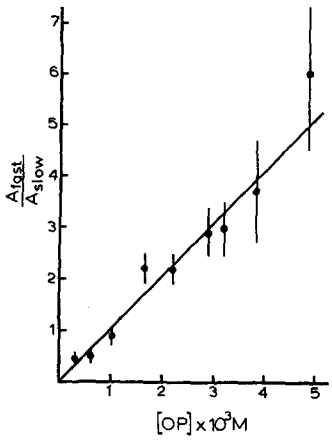

Fig. 2. Plot of A_{fast}/A_{slow} versus the *o*-phenanthroline concentration for the concentration data in Table I.

$$K = \frac{[\text{RC} \cdot o\text{-phenanthroline}]}{[\text{RC}][o\text{-phenanthroline}]}$$

We will assume that the fast-decay component is due to direct return of the electron from A^- (the primary acceptor) to $P865^+$; this process is assumed to be the only one allowed when *o*-phenanthroline is complexed to the iron*. The slow decay component then represents those reaction centers which do not have *o*-phenanthroline complexed. Hence, the amplitude of the fast component (A_{fast}) immediately after the flash should be proportional to the RC · *o*-phenanthroline concentration, and the amplitude of the slow component (A_{slow}) should be proportional to the reaction center concentration. Rearrangement of the equilibrium expression then gives

$$\frac{[\text{RC} \cdot o\text{-phenanthroline}]}{[\text{RC}]} = K[o\text{-phenanthroline}] = \frac{A_{fast}}{A_{slow}}$$

Thus a plot of A_{fast}/A_{slow} vs the *o*-phenanthroline concentration should be a straight line passing through the origin with a slope equal to K. The data are presented in Table I and plotted in Fig. 2. It is clear that the predicted linearity is found. Similar graphs were obtained for the various chromatophore preparations [31]. Table II lists the values of K obtained. Although there is some variation from one system to another, the K values are remarkably similar. From this information we conclude that the interaction of *o*-phenanthroline with the reaction center protein is not much affected by whether or not the protein is free or membrane bound.

Temperature dependence of the decay of Signal B1

McElroy et al. [5] found that between 1.7 and 77 K the decay of the light-induced Signal B1 is temperature independent and first order with a 1/e time constant

* At first sight it may seem strange that an inhibiting reaction (complexation of *o*-phenanthroline) causes an increase in the decay rate of Signal B1. The reason is that in the absence of *o*-phenanthroline electron transfer from the primary to the secondary acceptor is much faster than the direct return route. Once past the primary acceptor, the decay is relatively slow [23].

TABLE II

EQUILIBRIUM BINDING CONSTANTS FOR *o*-PHENANTHROLINE

System	$K \times 10^{-3}$ (l · mole^{-1})
Reaction center protein preparations from the R-26 mutant of *R. spheroides*	1.2±0.1
Chromatophores from wild type *R. spheroides*	3.0±0.2
Chromatophores from the BG-1 mutant of *R. rubrum*	1.2±0.2
Chromatophores from S-1 (*R. rubrum*)*	6.2±0.8

* S-1 chromatophore had a 30-ms fast decay (normal electron transfer) that interfered with determination of direct electron return 30-ms component. The data was collected later from aged S-1 chromatophores.

of approx. 30 ms. At these temperatures the only decay process possible is direct electron return from A^- to $P865^+$. Now that we are confident that *o*-phenanthroline can act as an inhibitor of electron transfer to secondary acceptors, we have been able to extend the temperature range for the decay of Signal B1 via direct return to approx. 300 K. The results are plotted in Fig. 3.

As noted in the introduction McElroy et al. [5] interpreted the temperature independence of the decay time between 1.7 and 77 K as evidence for a quantum mechanical tunnelling mechanism and indeed this does seem to be a reasonable explanation based on simple model calculations. However, when the temperature range is extended to higher temperatures, we find that the decay time τ increases with increasing temperature, contrary to the behavior of almost all chemical reactions.

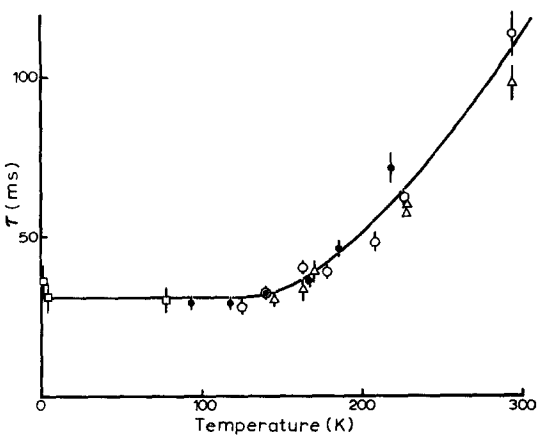

Fig. 3. Plot of the 1/e decay time (τ) of Signal B1 versus temperature for a variety of preparations: ●, untreated reaction centers from the R-26 mutant of *R. spheroides*; ○, reaction centers with $3.2 \cdot 10^{-3}$ M *o*-phenanthroline; △, chromatophores from the wild strain of *R. spheroides* with $3.0 \cdot 10^{-3}$ M *o*-phenanthroline; □, data from ref. 5 for untreated reaction centers from the R-26 mutant of *R. spheroides*.

As an explanation we propose that at temperatures above approx. 150 K the reaction center protein is able to "breathe" or expand in such a way that the distance of closest approach between $P865^+$ and A^- (the primary acceptor) increases slightly. Since the tunnelling time is very sensitive to the thickness of the barrier, a small change in distance will lead to a large change in τ. Indeed a simple calculation shows that the distance between $P865^+$ and A^- needs to increase by only approx. 2 Å to change τ from 30 to 100 ms. This small change in structure should be possible for a molecule such as the reaction center protein (mol. wt approx. 60 000). Our interpretation is therefore not unreasonable and provides further support for a tunnelling mechanism for the direct electron return in bacterial photosynthesis.

One final comment concerns the question of whether or not the phenomena observed with reaction center preparations accurately reflect the structure and behavior of the total organism. The fact that both in the case of the *o*-phenanthroline effect and in the temperature dependence of τ the chromatophore preparations behaved essentially the same as the reaction center preparations speaks strongly that indeed one can in this case probably extrapolate the reaction center observations to at least the level of an intact chromatophore membrane.

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