EPR PROPERTIES OF THE ELECTRON CARRIER INTERMEDIATE BETWEEN THE REACTION CENTER BACTERIOCHLOROPHYLLS AND THE PRIMARY ACCEPTOR IN *CHROMATIUM VINOSUM*

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1. Introduction

Recent work on the photosynthetic bacterium *Rhodopseudomonas sphaeroides* has suggested the existence of an intermediary electron carrier (I) between the reaction center primary donor (a bacteriochlorophyll dimer $(BChl)_2$ absorbing at 870 nm) and the 'primary' acceptor (a quinone associated with iron, designated X). Evidence for the intermediate comes from picosecond spectroscopy, which has suggested that although the $(BChl)_2$ is oxidized within 10 ps of a flash of light [1], the primary acceptor is not reduced until some 100–200 ps later [2,3]. During this interval the electron is proposed to reside on the low redox potential I, as part of a transient $[(BChl)_2^{+\cdot} I^{-\cdot}]$ state. If normal forward photochemistry is to occur, this state reduces X in 100–200 ps [2,3] but if this is blocked by the prior chemical reduction [4] or removal [5] of X, the transient state has a lifetime of nanoseconds before decaying ($t_{1/2}$ ∿ 10 ns at 300°K; ∿ 30 ns at 80°K [4,6]). It has been suggested [7] that $I^{-\cdot}$ has some spectrophotomeric characteristics of reduced bacteriopheophytin ($BPh^{-\cdot}$).

Because $I^{-\cdot}$ has only been seen on a brief transient basis, and not uniquely, it seemed desirable to work with an experimental system in which $I^{-\cdot}$ could be trapped and stabilized for study by more conventional techniques. To this end we chose to exploit the properties of *Chromatium vinosum*, which has a similar reaction center complement to that of *Rps. sphaeroides*, but has a hydrophobically-linked cytochrome c_{553} (E_m 0 mV; pH 8) which is capable of rapid irreversible electron transfer to the light generated $(BChl)_2^{+\cdot}$ down to liquid helium temperatures [8,9] ($t_{1/2}$ 1 μs at 300°K [10,11]; 2.5 ms from 120°K down to 4.2°K [8]).

The rationale shown in fig.1, based on the above data from *Rps. sphaeroides* and *C. vinosum* was employed to trap the $I^{-\cdot}$ state in *C. vinosum* at 200°K. Starting with all components reduced except I (top), light generates the intermediary state (center) in a reversible manner, see [1–6]. Although the cytochrome c_{553} oxidation step at 200°K is 1000-fold slower than the $[(BChl^{+\cdot})_2 I^{-\cdot}]$ decay back reaction(s), its irreversibility, together with prolonged illumination at 200°K, leads to the product (bottom) containing a trapped $I^{-\cdot}$ at the expense of only cytochrome c_{553} oxidation, without *net* redox state changes of $(BChl)_2$ or X.

The results suggest that significant orbital overlap may exist between $I^{-\cdot}$ and X^{-}.

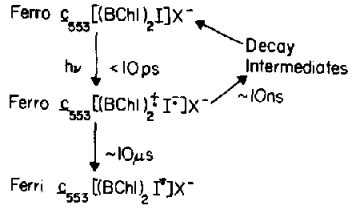

Fig.1. Experimental rationale for trapping $I^{-\cdot}$ in *C. vinosum*. The times shown are the approximate halftime at 200°K.

2. Materials and methods

A subchromatophore fraction containing the reaction center was prepared from *C. vinosum* strain D using Triton X-100 [12], and was further purified by the quantitative removal of the bulk bacteriochlorophyll pigments by treatment with acetone cooled at 200°K with solid carbon dioxide. This preparation had fully functional cytochrome oxidation reactions at low temperatures. Redox potentiometry, followed by e.p.r. analysis in a Varian E-4 spectrometer equipped with a flowing helium cryostat and temperature control, was as described previously [13]. Samples of known redox potential were stored in liquid nitrogen and kept in the dark to prevent premature irreversible photochemistry. In order to trap the reduced intermediary state, the samples were illuminated for 3 min in the 3 mm internal diameter e.p.r. tubes with a Unitron (8 V; 5 A) lamp while they were immersed in a mixture of ethanol cooled by solid carbon dioxide to ∿ 200°K; they were then transferred immediately to liquid nitrogen.

3. Results

3.1. *Cytochrome* c_{553} *oxidation*

Fig.2 shows the light induced oxidation of cytochrome c_{553} at 8°K and 200°K at different redox potentials, monitoring the EPR signal of ferricytochrome c_{553} at g 2.95 [13]. At + 200 mV, all the cytochrome is oxidized prior to illumination, and illumination has no effect upon the signal. At −50 mV where ∿ 90% of the cytochrome is reduced prior to illumination but X is oxidized, illumination at 8°K causes the expected *rapid* irreversible oxidation of cytochrome and reduction of X [9]. The size of the light induced signal is half the total cytochrome c_{553} signal consistent with previous suggestions that there are two cytochrome c_{553} hemes and a one electron capacity in X [14]. Warming the sample to 200°K and illuminating for 3 min causes only ∿ 15% further cytochrome oxidation. With material poised at −370 mV so that X is also chemically reduced (E_m ∿ −160; pH 8, see [15]), there is no light inducible cytochrome oxidation at 8°K since the familiar forward photochemical events are blocked. However, illumination at 200°K elicits the oxidation of one cytochrome c_{553} heme according to fig.1. The halftime of this oxidation is about 30 sec, and at 200°K the re-reduction has a halftime of about 20 min. At 80°K the re-reduction is undetectable even after hours.

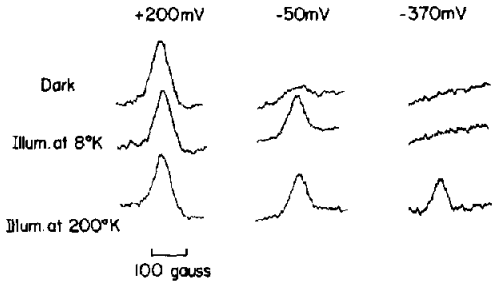

Fig.2. Light-induced cytochrome c_{553} oxidation. The reaction center–cytochrome complex (A_{883} nm = 3.0 cm^{-1}) was suspended in 50 mM Tris-Cl, pH 8.0, 1% sodium cholate with the following redox mediators; 10 μM 2,3,5,6 - tetramethylphenylenediamine for the sample poised at + 200 mV, plus 40 μM each of *N*-methyl phenazonium methosulfate, pyocyanine and 2-OH, 1,4-napthaquinone for the sample at −50 mV, plus 40 μM each of methyl and benzyl viologens for the −370 mV sample. The samples were first examined in the dark, and then after illumination at 8°K. They were then warmed to 200°K, illuminated for 3 min and cooled and re-examined at 8°K. E.p.r. spectrometer conditions: modulation amplitude 12.5 gauss, microwave power 1 mW.

3.2. *I reduction*

Fig.3 shows e.p.r. spectra in the g 2 region of the samples described in fig.2, examined in the dark after the 200°K illumination treatment. At 200 mV, where no $I^{-\cdot}$ accumulation is expected, none is obtained. In contrast at −370 mV, where the generation of $I^{-\cdot}$ is expected (see fig.1), the sample has a large signal. Some signal is also seen in the sample poised at −50 mV, but this is comparable to the amount of cytochrome oxidized during the 200°K illumination. Clearly the 'second' cytochrome heme (i.e., the 'first' one already oxidized by illumination at 8°K having been used for X reduction) is not as ready an electron source for I reduction as is the 'first' when both cytochrome c_{553} hemes are available. This is despite the fact that redox titrations using optical techniques [14] indicate that when both hemes are reduced they are kinetically and thermodynamically identical; further work is needed on this interesting observation.

The signal shown in fig.3A is in fact composed of two separate signals which can be resolved by varying temperature and microwave power (fig.3B); there is a broadened doublet with peak maxima at g 2.034

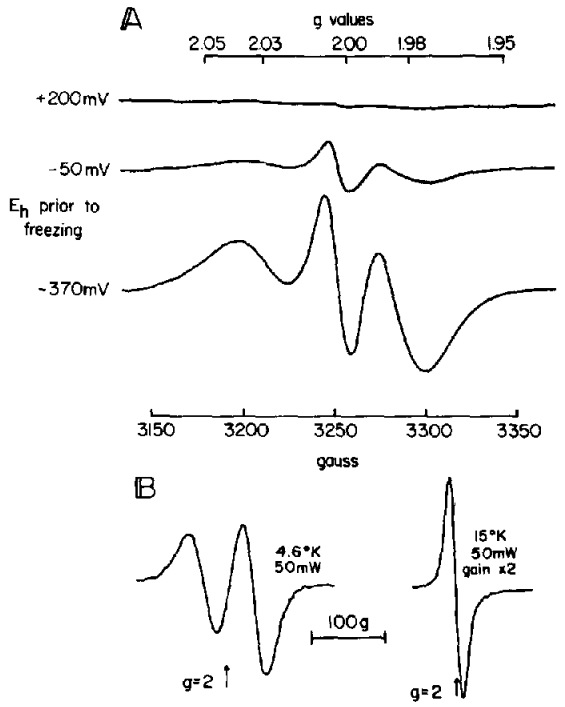

Fig.3. E.p.r. signals associated with the reduced intermediate. (A) The samples used were the same as those in fig.1. The e.p.r. signals are the difference between signals collected after the 200°K illumination *minus* the signals before this treatment; this subtraction removes the small signals (∿ 10% of −370 mV trace) due to the redox mediators. E.p.r. spectrometer conditions: modulation amplitude 5.0 gauss, microwave power 1 mW, 8°K. (B) The signal of the −370 mV sample resolved into its two components. Microwave power and temperature as shown, modulation amplitude 5.0 gauss.

and g 1.976 which has a high temperature sensitivity (it becomes difficult to detect above 15°K) and is not easily saturated with microwave power, and a narrow signal with a line width of ∿ 15 gauss centered at g ≃ 2.003 which is less sensitive to temperature and is more readily saturated. Identical signals are generated by light in the absence of redox mediators (dithionite alone), and in similarly treated chromatophores and whole cells of the organism; indeed Evans et al. [16] have observed the signal in *C. vinosum* chromatophores treated with dithionite and illuminated while freezing. A similar spectrum can be seen in *Rps. viridis,* which also possesses a low potential cytochrome *c* which can be irreversibly photooxidized at low temperature, but which contains BChl and BPh of the *b*-type. However in this case the doublet is broader, with peak maxima at g 2.06 and g 1.96, although the g 2.003 signal is again ≃ 15 gauss wide.

3.3. *Triplet formation*

Illumination at 8°K with X reduced normally generates, with high quantum efficiency [17], a spin polarized triplet or biradical state which is probably located in the $(BChl)_2$. It has been proposed (see [1]) that this triplet is generated as the electron comes back from $I^{-\cdot}$ to the unpaired spin of the $(BChl)_2^{+\cdot}$. If this is the case, illumination at 200°K to trap the ferri-c_{553} $[(BChl)_2\ I^{-\cdot}]X^-$ state should prevent subsequent illumination at 8°K generating the triplet, because formation of the necessary $[(BChl)_2^{+\cdot}I^{-\cdot}]$ state is blocked. Fig.3 shows that after the 200°K illumination, triplet formation is only ∿ 12% of that of the untreated material. This verifies the proposal of [1], and supports the rationale outlined above that illumination at 200°K traps $I^{-\cdot}$, and that the signals (or at least one of the signals) seen in the g 2 region after treatment belong to this species.

4. Discussion

The e.p.r. analysis of the trapped state revealed (fig.2) that a maximum of one heme equivalent of cytochrome c_{553} per reaction center was oxidized

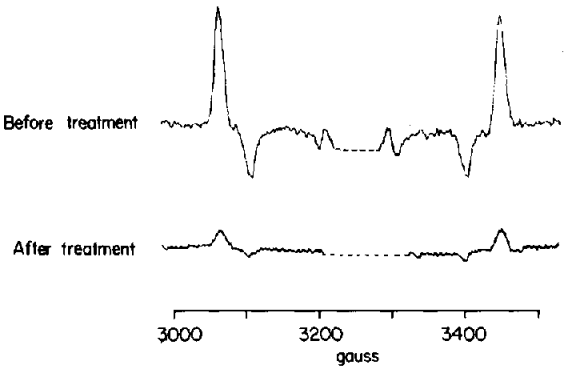

Fig.4. The spin-polarized triplet. The signals are those obtained with the −370 mV sample of fig.1 and 2 before and after the 200°K illumination. The signals are light *minus* dark spectra. E.p.r. spectrometer settings as in fig.1.

concomittantly with the generation of $I^{-\cdot}$, indicating that the I to $I^{-\cdot}$ reaction involves the addition of only one electron. Nevertheless, the $I^{-\cdot}$ of the trapped state is identified with two distinct e.p.r. signals in the g 2 region. Schepler et al. [18] have proposed (see also [21]) an exchange interaction between organic radicals and low spin $Co(II)B_{12}$ as the origin of e.p.r. 'doublet' spectra observed in enzyme reactions with coenzyme B_{12}. By analogy, the line shape of the broadened doublet of the $I^{-\cdot}$ signal corresponds to the low field component of an exchange coupling with a higher field paramagnetic center. In this respect, the reduced form of X (semiquinone-iron) has an e.p.r. signal at higher field (g_y 1.82, g_x 1.62, [13]), and this paramagnetic center could be the requisite high field partner for the lower field doublet. Indeed, the $I^{-\cdot}$ doublet exhibits relaxation behaviour similar to that of X^- [20]. Another possibility for the origin of the doublet is an interaction between $I^{-\cdot}$ and the iron of X alone (see [19] for an in vitro example).

There are two major alternatives for the observation of two distinct radical spectra for the $I^{-\cdot}$ state. First, $I^{-\cdot}$ may involve, in addition to $BPh^{-\cdot}$, another radical such as $BChl^{-\cdot}$ in a situation where only one of the radicals is sufficiently close to exchange couple with X^-. In this case, the single electron reduction indicated by cytochrome c_{553} oxidation would be distributed between $BPh^{-\cdot}$ and possibly $BChl^{-\cdot}$. The second possibility is that the electron resides solely on BPh but only part of the BPh is properly positioned to undergo exchange coupling analogous to that proposed in the B_{12} system. In this case there would be some overlap of electronic wave functions of the interacting radicals. Such an orbital overlap could provide an efficient pathway for electron transfer between $I^{-\cdot}$ and X in the normal photochemical reaction.

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