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PRIMARY EVENTS IN THE PHOTOSYNTHETIC REACTION CENTRE FROM *RHODOPSEUDOMONAS SPHEROIDES* STRAIN R26: TRIPLET AND OXIDIZED STATES OF BACTERIOCHLOROPHYLL AND THE IDENTIFICATION OF THE PRIMARY ELECTRON ACCEPTOR

P. LESLIE DUTTON[a], JOHN S. LEIGH[a], JR and DAN W. REED[b]

[a]*Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104 and* [b]*Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 43587* (*U.S.A.*)

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SUMMARY

ESR studies at approximately 10 °K on the reaction centre complex of the photosynthetic bacterium *Rhodopseudomonas spheroides* (strain R26), have revealed bacteriochlorophyll triplet states and a component which has an ESR absorption centred at $g=1.82$. The triplet-state bacteriochlorophyll is induced only in the light and is only detectable when the reaction-centre bacteriochlorophyll and its primary electron acceptor are reduced; the ESR triplet state signals are composed of both ESR absorption and ESR emission bands. The oxidation–reduction properties of the $g=1.82$ component and its flash-induced kinetic behavior in relation to that of P870 are those expected for the primary electron acceptor in bacterial photosynthesis.

INTRODUCTION

The problems encountered in the study of the mechanism by which photosynthetic bacteria and plants effect the transduction of electromagnetic energy into that of electrochemical energy has recently been discussed by Clayton[1]. Hiyama and Ke[2] have summarized the reported evidence for the assignation of a variety of possibilities as the primary electron acceptor.

We have recently employed the combination of oxidation–reduction potentiometry and low temperature electron spin resonance spectroscopy (ESR) to attempt to detect and measure thermodynamic properties and light-induced kinetics of the components involved in the early events of photosynthesis. We have reported direct *in situ* measurements of bacteriochlorophyll triplet states in chromatophores and in a detergent-solubilised subchromatophore preparation from *Chromatium* D (ref. 3). With the same materials an ESR signal centred at $g=1.82$ was recognised as the reduced form of an electron carrier which was closely associated with the primary photochemistry[4]. Its light-induced behaviour and chemical (dark) oxidation–reduction potential properties were shown to be consistent with the known, indirectly determined midpoint potential (approximately −135 mV at pH 7.4; see refs 5–9) of the primary electron acceptor of reaction centre bacteriochlorophyll, P883, in *Chromatium* D.

It is of some importance to extend the investigations to the much-studied reaction centre preparations from the blue-green mutant (strain R26) of *Rhodopseudomonas spheroides* (*e.g.* see refs 1 and 10–20). The solubilized reaction centre protein isolated from *Rps. spheroides* is only approx. 100000 daltons particle size and, unlike the *Chromatium* D subchromatophore preparation (approx. 500000 daltons)[21], contains no antenna bacteriochlorophyll, carotenoids or cytochromes.

MATERIALS AND METHODS

Reaction centre preparations were prepared from photosynthetically-grown *Rps. spheroides* (blue-green mutant, R26) by the method of Reed[22]. This entailed preparation of the Triton X-100-treated reaction centre complex[11], followed by further treatment with 0.1% lauryldimethylamine-*N*-oxide; a homogeneous product was obtained by DEAE-cellulose (Whatman DE-52) column chromatography. The same results were obtained with a preparation solubilised by 1% lauryldimethylamine-*N*-oxide (omitting the Triton X-100 step), followed by column chromatographic purification.

The presence of lauryldimethylamine-*N*-oxide associated with the reaction centre protein was found to interfere with a reliable use of oxidation–reduction potentiometry, so this technique was not applied for the work presented in the communication. However, manipulation of the state of oxidation–reduction of the preparation was still performed under anaerobic conditions as previously described[6]. Two principle oxidation–reduction states of the preparation were studied: (1) in which reaction centre bacteriochlorophyll (P870) was reduced. [By reduced P870 we refer to the neutral (unionized) state of P870, in contrast to the oxidized form P^+870.] and the primary electron acceptor was oxidised. This is essentially the state in which the preparation is isolated; the condition is equivalent to that described in potential region I in *Chromatium* D (ref. 3); and (2) in which both P870 and the primary electron acceptor were chemically reduced; this was effected by the addition of a fresh, dilute solution of sodium dithionite; the condition is equivalent to that described in potential region III in *Chromatium* D (ref. 3). For consistency and brevity, region I and region III will be used to describe these two states. (Potential region II is absent in *Rps. spheroides* since this potential region is identified with the specific involvement of *c*-type cytochromes in light-induced electron transfer at low temperatures.) Samples of material under these established conditions were transferred anaerobically to ESR tubes and rapidly frozen; samples were maintained in the dark at 77 °K until ready for use. ESR measurements at cryogenic temperatures were made as described previously[3].

Continuous illumination of samples in the ESR cavity was provided by a Unitron lamp (8 V; 5 A; no attempts were made to use light of intensity sufficient to saturate the reactions studied). Pulsed light was from a 4-μs (full width at half height), 20-J xenon flash. Activation light was filtered through a 2-cm path of water. In measuring the xenon flash-induced ESR kinetics, it was sometimes necessary to accumulate the changes from several flashes in a Varian C-1024 computer of average transients (C.A.T.). Sample temperature was monitored with a Au/Co *versus* Cu thermocouple.

RESULTS

(*1*) *Oxidation-reduction reactions*

Reaction centres in which P870 is reduced and the primary electron acceptor is oxidised. Fig. 1A shows the ESR spectrum of the preparation under these potential region I conditions in the dark (top), under the influence of steady illumination

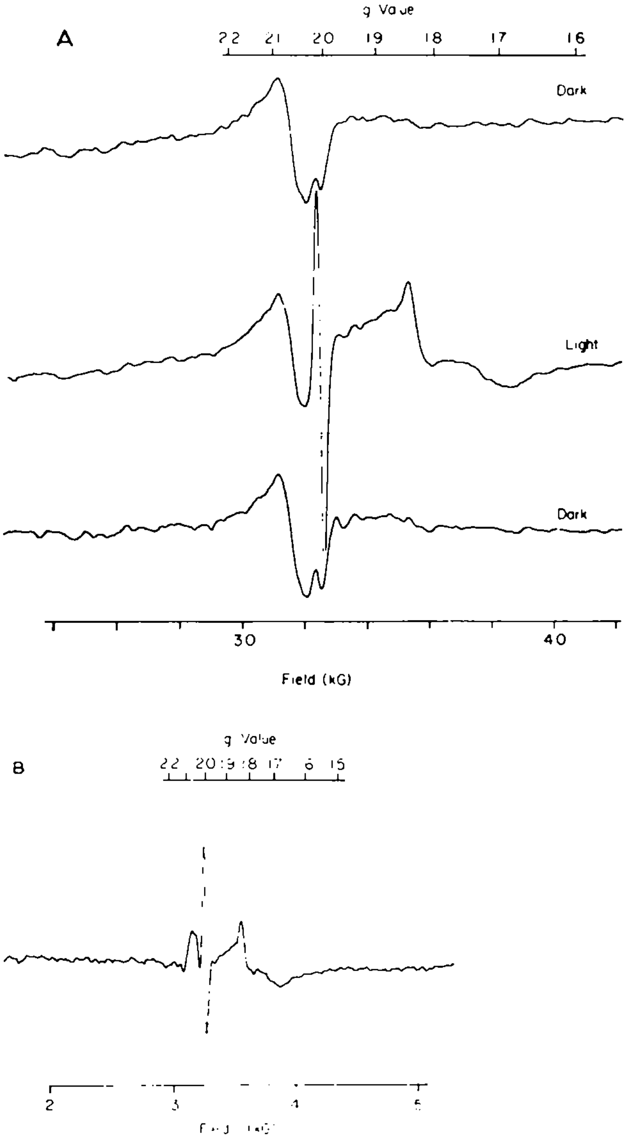

Fig. 1. (A) ESR spectra of *Rps. spheroides* (R26) reaction centre protein complex in the dark and light at a temperature of approx. 10 °K. P870 concentration, 0.5 mM. Sample conditions before freezing: anaerobic, no additions. ESR spectrometer settings: microwave power 50 mW; modulation amplitude 32 G. (B) C.A.T. light *minus* dark difference spectrum taken with a 2-fold wider magnetic field scan.

(centre), and again in the dark after the illumination (bottom). Changes induced by light in the spectrum are readily observed at $g=2$, $g=1.82$ and $g=1.68$; the changes are entirely reversible. Fig. 1B shows the light *minus* dark difference spectrum of these events; no other changes were observed over the wider spectral range shown, but in the $g=2$ region two other light-induced signals, a peak at $g=2.05$ and a shoulder to the low-field side of the $g=1.82$ signal, are revealed. The peak at $g=2.05$ represents an alteration in a signal which exists in the dark and may be a heating artifact (however, this will be investigated and the results presented in future communications); unlike the shoulder (see next section) there is at present no evidence to connect it with the $g=1.82$ (1.68) signals.

Even at these very low temperatures the light-induced excited state P870 donates an electron to its primary acceptor. In the dark the electron returns from the reduced acceptor directly back to P^+870. This means that following a short flash, the kinetics of P870 photooxidation and subsequent dark rereduction should be concomitant with the reduction and reoxidation of its primary electron acceptor. Thus, if the assignations of $g=2$ signal with P^+870 and the $g=1.82$ and 1.68 signals with the reduced primary electron acceptor are correct, then the flash-induced kinetics measured at these points should be the same. This is shown in Fig. 2A. The top trace

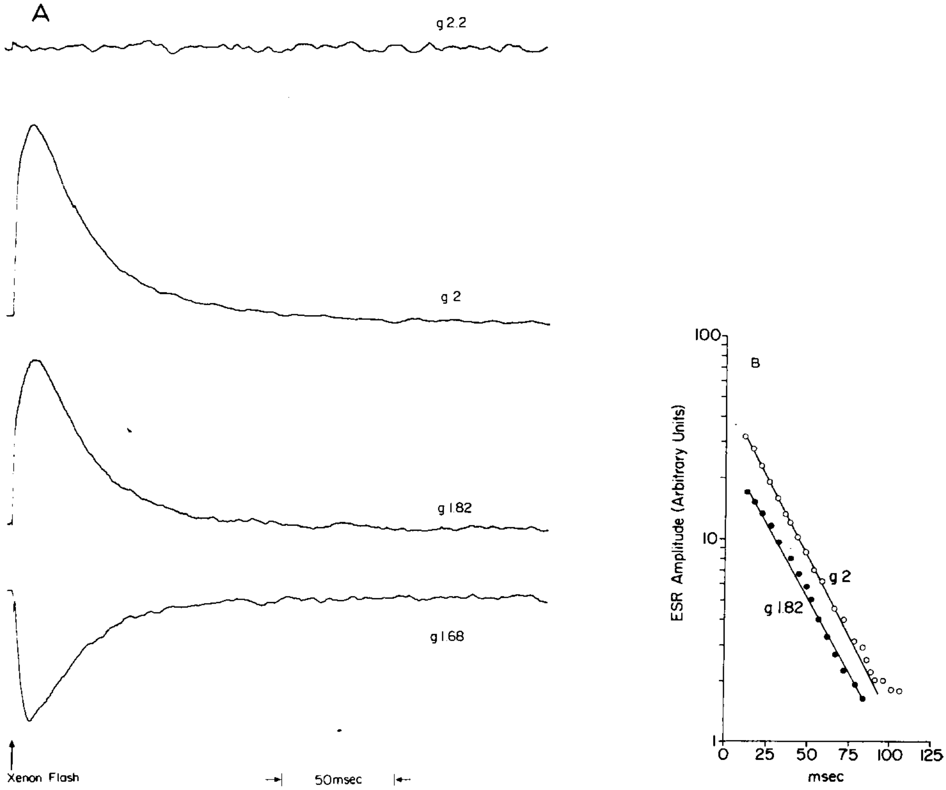

Fig. 2. (A) The kinetics of xenon flash-induced changes in *Rps. spheroides* reaction centre complex. Conditions as in Fig. 1. At $g=2.2$, $g=1.82$ and $g=1.68$ the kinetics of the change after 30 flashes, and at $g=2.0$ the change after 7 flashes were accumulated on the C.A.T. (B) Semilogarithmic plot of the dark decay kinetics of the $g=2.0$ and $g=1.82$ signals.

is a control and shows the absence of xenon flash-induced changes at $g=2.2$, a position where no ESR changes would be expected. At the maximum on the low-field side of the $g=2.0$, a signal is formed following the flash in a time which is within the instrumental rise time ($t_{1/2}$ <5 ms). Similar kinetics are seen at the maximum on the low-field side of the $g=1.82$ signal and at $g=1.68$; at $g=1.68$ the direction of the signal is appropriately inverted.

The decay of the signals in the dark appear to follow first-order kinetics (Fig. 2B). The measured half-times of the signal decay are 19.3 ms ($g=2.0$), 20.0 ms ($g=1.82$) and 17.4 ms ($g=1.68$). The half-times given are the average of 5, 7 and 2 determinations done at $g=2.0$, 1.82 and 1.68, respectively. At $g=2$ none of the individual values varied more than 1 ms from the mean value, while at $g=1.82$ the values varied up to 3 ms of the mean value.

Reaction centres in which both P870 and the primary electron acceptor are reduced. If the component responsible for the $g=1.82$ and $g=1.68$ signals is the reduced primary electron acceptor, these signals should be produced on chemical reduction (*i.e.*, in region III); in addition, when the primary electron acceptor is in the reduced state, P870 photooxidation should be prevented and so no $g=2$ signal should be formed on illumination. Fig. 3 reveals that upon chemical reduction the $g=1.82$ and 1.68 signals are already present in the dark (top). The shoulder on the

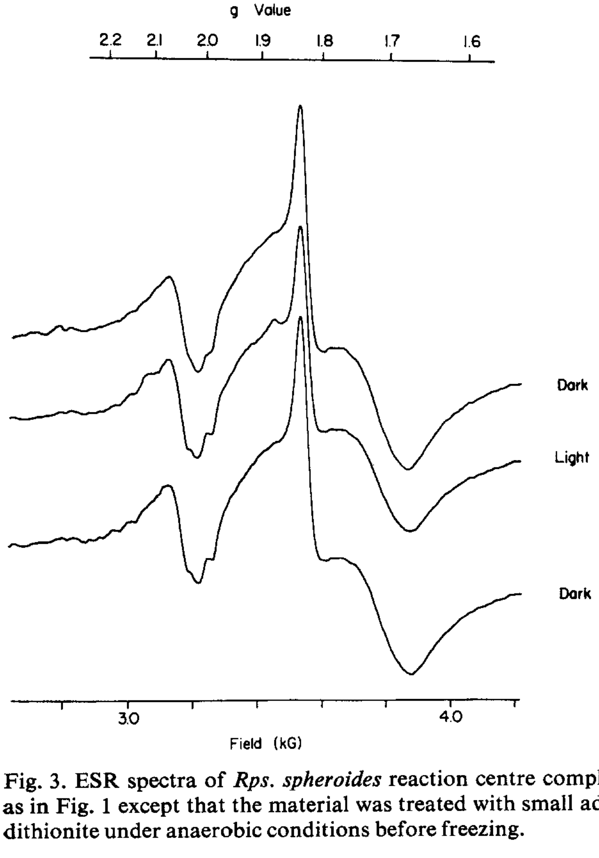

Fig. 3. ESR spectra of *Rps. spheroides* reaction centre complex in the dark and light. Conditions as in Fig. 1 except that the material was treated with small additions of a fresh solution of sodium dithionite under anaerobic conditions before freezing.

low-field side of the $g = 1.82$ signal is also apparent; thus, since the shoulder is observed on illumination and on chemical reduction it is not unreasonable at this time to regard it as the low-field component $g = 1.88$–1.94) of a "rhombic" ESR absorbance of which the $g = 1.82$ signal is the centre band (g_y) and the $g = 1.68$ signal is the high-field band (g_x). Illumination (centre) induces no further increase in these signals and as predicted, no $g = 2$ signal is developed upon illumination.

The temperature dependence of the g = 1.82 signal. The temperature sensitivity of the amplitude of the $g = 1.82$ signal is described in Fig. 4. The signal is almost undetectable at 19 °K with no apparent general broadening of the $g = 1.82$ signal. The experiment was performed at a microwave power of 50 mW which is not sufficient to saturate the signals (the microwave power dependency showed that the signals are not saturated even at 100 mW).

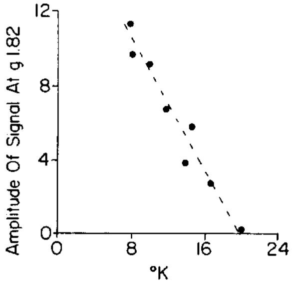

Fig. 4. Temperature dependence of the $g = 1.82$ signal. Experimental conditions were the same as in Fig. 3.

With this extreme temperature dependence of the $g = 1.82$ signal we were aware of the possibility of heating artifacts. With regard to the $g = 1.82$ signal in region III (Fig. 3) it can be noticed, in fact, that the signal amplitude decreased slightly on illumination. This is due to light-induced sample heating; removal of the water filter caused the signal to decrease further. Some sample heating was also observed following a xenon flash as shown by a flash-induced signal decrease at $g = 1.82$ in region III. The kinetics of heating were faster than the instrumental rise time, and under our experimental conditions (*e.g.* helium flow rate, *etc.*) the cooling rate was approximately first order up to 100 ms with a $t_{1/2}$ of 98 ms. Thus, concerning the measured reduction–oxidation kinetics of the $g = 1.82$ signal in region I (Fig. 2) the slowness of the course of sample recooling with respect to the rate of reoxidation of the $g = 1.82$ component will lead to small errors in the measured rates; however, even for a 6 °C temperature rise, the error introduced on the measured half-time of $g = 1.82$ component dark reoxidation would be less than 10%. Work geared toward a full quantitation of the extent of the heating effect is underway so that we can assess the relationship of the amplitude of the $g = 1.82$ signal formation following a single flash in region I with that chemically induced in region III.

(2) *Energy transfer reactions*

Under conditions where the light-induced excited singlet state P870 can undergo rapid oxidation by reducing its primary electron acceptor (potential region I) the familiar $g = 2$ signal (P^+870) is evident as we have already seen in Fig. 1. Fig. 5A

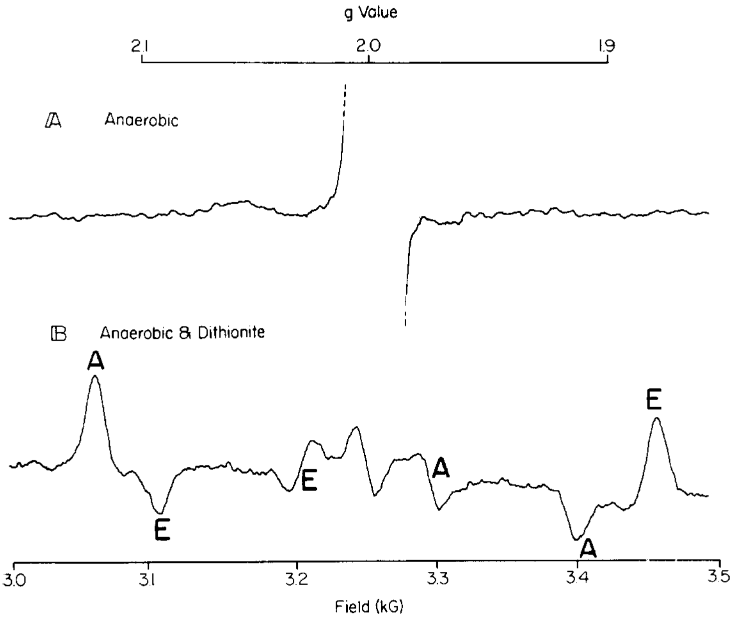

Fig. 5. Light *minus* dark difference spectra of (A) the oxidised bacteriochlorophyll and (B) the triplet-state bacteriochlorophyll. In A the sample conditions were as in Fig. 1 and in B the conditions were as in Fig. 3. ESR spectrometer settings: microwave power 1.2 mW; modulation amplitude 10 G.

shows again the light *minus* dark difference spectrum under such conditions but using a lower microwave power to maximise the bacteriochlorophyll signals. Under region III conditions (Fig. 5B) the light-induced $g=2$ signal is insignificant because P870 photooxidation is prevented by the prior state of reduction of the primary electron acceptor. Instead, a new set of signals is now apparent; the signals are rapidly and reversibly formed in the light. These signals cannot be induced in the dark under any oxidation–reduction states. The signals are comprised of two rhombic ESR transitions, one resulting in microwave absorption (signals labelled A) and the other resulting in microwave emission (signals labelled E). The overall properties of the signals suggest they represent bacteriochlorophyll in the triplet state. Similar signals have been reported and discussed previously with respect to chromatophores and subchromatophore preparations from *Chromatium* D, *Rhodospirillum rubrum* (blue-green mutant) and *Rps. spheroides* (strain Ga) (ref. 3). Identification of the signals in the reaction centre protein from *Rps. spheroides* (R26), which has no carotenoids or antenna bacteriochlorophylls, makes it more likely that the triplet state resides in the reaction centre bacteriochlorophyll molecules.

DISCUSSION

The concept that the photosynthetic unit exists as a discrete entity in photosynthesis was proposed 40 years ago[24]. Efforts principally from Clayton's laboratory over the past 10 years (see ref. 23) have brought the reaction centre into an experimental reality and has provided an invaluable vehicle for the elucidation of the mechanism of light energy transfer and its transduction into electrochemical potential energy. The establishment in this paper of the direct measurement of bacteriochlorophyll in the triplet excited state and the recognition of the $g = 1.82$ component in the reaction centre protein complex enhances their importance and will simplify future investigations.

The position of bacteriochlorophyll triplet state in energy transfer

The triplet excited state of the reaction centre bacteriochlorophyll is only apparent when both the P870 and its primary electron acceptor are chemically reduced prior to illumination (region III). In chromatophores of *Rps. spheroides*, *Rsp. rubrum* and *Chromatium* D exact correlations exist between the redox-potential dependence of the appearance of the light-induced triplet signal and the disappearance of the light-induced oxidation of reaction centre bacteriochlorophyll (given by the EPR signal at $g = 2.0$) as the primary electron acceptor is progressively chemical ly reduced prior to activation. Fluorescence behavior resulting from the excited singlet decay of P870 in *Rps. spheroides* reaction centres follows the same pattern[1,8,16,17]. Thus as with the enhanced bacteriochlorophyll fluorescence yield, the formation of significant steady-state levels of triplet-state bacteriochlorophyll occurs when photochemistry in the reaction centre bacteriochlorophyll is prevented. This information cannot lead to any decision between the triplet excited state being (a) part of the primary energy transfer pathway between the excited singlet bacteriochlorophyll and the oxidised end product, or (b) a possible means of excited singlet decay, since its role in either alternative could lead to the same effect. The answer must come from kinetic studies. So far in this direction with *Rps. spheroides* chromatophores poised in potential region III, we have measured the kinetics of the triplet following a 20-ns ruby laser pulse. The triplet is formed in less than $t_{1/2}$ of approx. 500 ns (instrumental rise time) and decays with a $t_{1/2}$ of approx. 5 μs. In *Chromatium* D the triplet kinetics in potential regions I, II and III have been presented[3] but the kinetics were entirely instrument limited (instrumental rise time approx. 50 μs); however, an amount of flash-induced signal which was apparent in region I of *Chromatium* D (it was about 15% of that detected in region III, or region II on the second flash) may prove important but its exact nature has not yet been clarified. If the signal detectable in region I following a flash is bacteriochlorophyll in the triplet state then its kinetics of formation obviously would have to be rapid enough to compete with the other competing events; its rate of decay, however, could help decide its role in the events of photosynthetic energy transfer. The expected kinetics of the triplet in region I if on the primary energy transfer pathway would rise concomitant with the decay of the singlet, and decay concomitant with the transfer of the electron to the primary acceptor. If not primary its decay may well be as long as 5 μs, the same as that measured in region III.

The position of the $g = 1.82$ components in primary photochemistry

The criteria we have used as a basis for our evaluation of primacy of electron acceptors in photochemistry are: (a) at cryogenic temperatures the acceptor should exhibit kinetics of flash-induced photoreduction and subsequent dark reoxidation which are identical to the course of reaction centre bacteriochlorophyll photooxidation and subsequent dark rereduction; (b) the acceptor should be oxidisable and reducible by chemical agents in the dark; its oxidation–reduction potential properties should be the same as the values obtained by independent methods of approach; (c) the acceptor should be present in isolated subchromatophore and reaction centre protein preparations; however, the thermodynamic and kinetic parameters of oxidation and reduction encountered in the isolated preparations should be the same as those exhibited by the intact organism; (d) the behavior of the acceptor component should be consistent with other known reactions, such as low-temperature cytochrome oxidation, which are closely associated with the light reaction.

The work presented here with the $g = 1.82$ component goes some way to satisfy these criteria. The kinetics of flash-induced formation (instrument limited) and dark decay of the $g = 1.82$ signal are within experimental error concomitant with flash-induced formation and dark decay of P^+870. The readily measurable rates of decay of these signals in these isolated reaction centre proteins are similar to those reported for P870 in chromatophores[4]. The $g = 1.82$ signal is clearly induced by chemical reduction in the dark. We have not yet measured its midpoint potential directly. However, in chromatophores from *Rps. spheroides* (strain Ga) we have established that the signal is fully formed at a redox potential of -220 mV; this potential is appropriate if the midpoint potential of the $g = 1.82$ component has the expected value of approximately -50 mV (values for the primary acceptor measured indirectly range from -20 to -80 mV; see refs 8,16 and 25–27).

A recent determination by Clayton *et al.*[18] indicates the electron capacity of the primary electron acceptor molecule in *Rps. spheroides* is unity; the fact that most determinations of the course of oxidation–reduction of the primary electron acceptor yield n values of 1, supports this finding (see refs 8 and 16; Leigh, J. S. and Dutton, P. L., unpublished result; Lindsay, J. G. and Dutton, P. L., unpublished result, but *cf.* ref. 26). With *Chromatium* D there is clear evidence that the electron capacity of the primary electron acceptor is only one; this comes from the flash-induced reactions at 77 °K; in potential region II where ferrocytochrome c_{553} irreversibly reduces photooxidised P883 in 2 ms, leaving the primary electron acceptor permanently reduced, a second flash induces no further oxidation of cytochrome c_{553} (ref. 28) or P883 (ref. 3 and 28). This would not be the case if the electron capacity of the primary electron acceptor were more than one.

How do these results relate to other reported paramagnetic species which are currently under consideration as primary electron acceptors? McElroy *et al.*[29] have described a very broad signal which was induced on illumination of a reaction centre preparation from *Rps. spheroides*; it was considered possibly to be an iron compound. Loach and Hall[30] reported in isolated *Rps. rubrum* subchromatophore preparations containing less than 0.3 iron atoms per P870 a narrow signal at $g = 2.005$; Feher *et al.*[13] subsequently revealed this signal on illumination of a *Rsp. spheroides* reaction centre preparation but only after treatment with sodium dodecylsulphate which released most of the iron. They presented evidence that the signal was a ubi-semiquinone[13].

Of importance to the consideration of primacy of these signals is the question of whether photooxidation of P870 itself is sufficient to decide that the primary electron acceptor is still attached after various increasingly vigorous fractionation procedures. At this time we feel that unless the basic criteria outlined at the beginning of this section are satisfied, it is not sufficient. In this regard, Feher *et al.*[13]

have reported that the kinetics of P870 oxidation–reduction in their sodium dodecyl sulphate-treated preparations are considerably altered with respect to those encountered in the chromatophore or their reaction centre preparation before sodium dodecylsulphate treatment. It is of some relevance in this regard, that chlorophyll even in solution is capable of catalyzing a variety of coupled oxidations and reductions which are driven in the light against the electrochemical potential (see ref. 31).

In our preparations we have avoided any steps which would expose the preparation to high detergent concentrations (*e.g.* that encountered during ammonium sulphate flotation) and have employed only sucrose density gradients and column chromatography to effect isolation and purification of the reaction-centre complex. We believe that iron in the reaction-centre system is an obligate part of the primary acceptor molecule which under physiological conditions operates to the virtual exclusion of other molecules which could, in its absence, serve as alternate acceptors. We have proposed that the $g = 1.82$ signal is a reduced iron–sulphur protein. An iron–sulphur protein as the primary acceptor is attractive from the known physicochemical properties of these proteins; they are small, highly structured molecules, and the majority of those known have n values of 1 and oxidation–reduction midpoint potentials below 0 mV. Computer analysis of the lineshape of the $g = 1.82$ signal (Blumberg, W. E., personal communication) reveals that it is not inconsistent with its possible identity as an iron–sulphur protein with an iron content per molecule as little as unity. However, the necessary work is underway to isolate and characterize the $g = 1.82$ component and to further describe the properties of the reaction centre with and without this molecule.

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