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The Question of the Primary Electron Acceptor in Bacterial Photosynthesis

(*Rhodospirillum rubrum*/photoreceptor/iron-depleted particles/quantum yield/chromatophores)

P. A. LOACH AND R. L. HALL*

Department of Chemistry, Northwestern University, Evanston, Illinois 60201

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ABSTRACT **An electrophoretic purification of *Rhodospirillum rubrum* photoreceptor subunits prepared by alkaline urea–detergent disruption is described. Completely active photoreceptor subunits with less than 0.30 eq of iron (or any other transition metal) per phototrap can routinely be prepared. A new photoproduced electron paramagnetic resonance (EPR) signal has been detected in these preparations; it was shown to be due to a photoreduced species. It has a *g*-value of 2.0050 ± 0.0003, a peak-peak width of 7.0 ± 0.3 G, and a nearly Gaussian shape. The response of the new signal to microwave power is different from that of the EPR signal of the photoproduced primary electron donor of chromatophores. Quantum yield measurements of spin production show that the new signal is very efficiently formed ($\Phi = 0.6$) simultaneously with the electron donor radical. No hyperfine structure (down to 0.1 G modulation amplitude) was observed in the new signal, either at room temperature or at the temperature of liquid nitrogen. The possible identity of this molecule is discussed.**

Recent results from this laboratory (1, 2) have demonstrated that photosynthetic membrane fragments (chromatophores) of *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* can be quantitatively dissociated to yield active photoreceptor subunits. The procedure, in which chromatophores are treated with Triton X-100 and urea at high pH (AUT conditions), followed by sucrose density gradient centrifugation, causes a displacement of much of the phospholipid and a separation of about half of the protein originally present in the chromatophore membranes. The photoreceptor complex has a particle weight of about 100,000, contains most of its original complement of bacteriochlorophyll and carotenoid, and also retains high photochemical efficiency for the use of light absorbed by antenna carotenoids or bacteriochlorophyll. Iron was the only transition metal still present in the preparation at a high-enough concentration to have a direct role in the photochemical reaction. In the present report, we present data to show that iron is not required in these preparations for good photochemical activity (see also ref. 3) and, therefore, probably does not function as the primary electron acceptor in these systems.

The fact that transition metals do not play a role in the primary photochemical reaction would require that a second organic free-radical should be observable in these preparations, since the primary electron donor, a bacteriochlorophyll molecule, loses a single electron. In the case of the more intact membranous systems (e.g., chromatophores), it is well established that the only radical observed under the usual conditions of steady-state illumination is accounted for by the primary electron donor molecule (4–7). We also report here a newly observed EPR signal, which appears upon preparation of photoreceptor subunits from *R. rubrum* membrane particles. The molecule that gives rise to the signal has properties that would be expected of a primary electron acceptor.

Abbreviations: AUT particles, the phototrap-containing fraction prepared by the combined alkaline urea–Triton X-100 method for membrane dissolution; AUT-e, electrophoretically-purified AUT particles; P865, the primary electron-donor molecule, characterized by a light-induced absorbance decrease at 865 nm.

* Present address: Department of Chemistry, William Paterson College of New Jersey, Wayne, N.J. 07470.

MATERIALS AND METHODS

Triton X-100 is a product of Rohm and Haas, Philadelphia, Pa. All other chemicals were of at least reagent grade purity, and all solutions were made with deionized or deionized and distilled water.

Conditions for growth of *R. rubrum* and preparation of the chromatophore fraction from whole cells have been described (4). Details for dissolution of chromatophores by the alkaline–urea–Triton method have also been given (1, 2). The original conditions (3% Triton X-100–6 M urea, pH 11.5) were modified by the addition of 1 mM $MgCl_2$ to the sucrose gradient and dialysis buffers. This modification stabilized phototrap activity significantly, so that a pH of 12.0 was used in the initial conversion when Mg^{++} was present.

For column electrophoresis, an LKB Electrofocussing apparatus (LKB Instruments, Inc., Rockville, Md.) was used, with pH 7–10 mixed ampholytes as the buffer and a stabilizing sucrose gradient. 0.2% Triton X-100 was normally present throughout the column to retard aggregation. The columns were prepared as described in the LKB Instruction Manual (I-8100-E01), with the sample applied as a band near the top of the column. The electrodes were connected so that the negatively charged components migrated toward the bottom of the column. Most columns were developed at 300 V for 18–24 hr. The temperature was maintained at 1° with a circulating-ethanol bath. For most preparations, no significant pH gradient was formed (less than 0.3 pH unit) in the region of sample separation.

Absorbance spectra and light-induced absorbance changes were measured (8, 9) with an appropriately modified Cary 14R Recording Spectrophotometer. Electron paramagnetic resonance signals were measured with a Varian E-3 Spectrometer (Varian Associates, Palo Alto, Calif.).

Iron analyses were performed with a Varian Techtron AA-5

Atomic Absorbance Spectrometer (Varian Aerograph-Varian Techtron, Park Ridge, Ill.). Standard curves for the concentration range 0.5–10 μM were obtained with fresh dilutions of standard $FeCl_3$ or $Fe(NH_4)_2(SO_4)_2$ stock solutions. The validity of analysis of unknown samples without prior digestion was checked by analysis of known concentrations of hemin, cytochrome *c*, and hemoglobin. Good agreement was found with the expected iron content. The effects on the analysis of Triton X-100, lipids, buffers, viscosity, and protein concentration were also determined.

RESULTS

When alkaline urea–Triton (AUT) particles were subjected to column electrophoresis, phototrap activity was found to correspond very closely with bacteriochlorophyll absorbance, as measured at 875 nm (Fig. 1) (3). In addition to the separation of protein and phospholipid from the photoreceptor complex, column electrophoresis also results in the separation of at least one other class of compounds: those containing iron. Detailed analysis of the protein content of these preparations will be reported elsewhere. The iron profile obtained after electrophoresis is shown in Fig. 1. The largest iron concentration is found in a fast-moving band at the bottom of the column, and there is no indication of a significant band of iron-containing material corresponding to the AUT-e bands. These data were obtained with a batch preparation (1); that is, sucrose density gradient centrifugation was omitted, and the AUT-treated chromatophores were placed directly onto the electrophoresis column. This procedure allowed us to use much higher initial concentrations of subchromatophore particles, so that the iron analyses would be quantitative. Similar results have subsequently been obtained by electrophoresis of standard alkaline urea–Triton preparations.

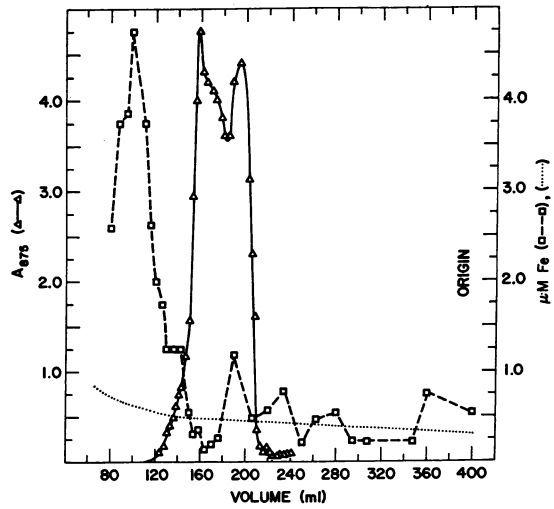

FIG. 1. Column electrophoresis of *R. rubrum* chromatophores treated with alkaline urea–Triton X-100. The batch AUT particles were prepared by incubation of chromatophores (A_{878} = 70) for 1 hr at 0° in 0.05 M phosphate (pH 12.0) containing 6 M urea, 3% Triton X-100, and 1 mM $MgCl_2$. The solution was dialyzed for 1 hr against three 1-liter changes of 0.01 M potassium phosphate buffer (pH 11.0)–1 mM $MgCl_2$, and was centrifuged for 25 min at 150,000 × *g*. The supernatant, which contained at least 90% of the pigment, was electrophoresed for 21 hr at 300 V. pH 7–10 mixed ampholytes (LKB) were used as buffer and gave a pH of 8.2. Fractions were dialyzed for 48 hr against distilled deionized water and analyzed for iron content. The *dotted baseline* represents the iron content of an identical control electrophoresis column containing no AUT material. The pigmented bands moved toward the positive electrode, which is on the *left*.

TABLE 1. *Iron content of photoreceptor subunit preparations**

		μM		[Fe]/
		[Fe]	[P865]†	[P865]
Batch AUT-e				
Exp.	1‡	0.51	1.4§	0.35
	2	0.25	1.8	0.15
	3‡	0.70	2.1§	0.33
	4‡	0.61	3.6§	0.17
	5‡	0.62	3.2	0.19
	6‡	0.40	2.2	0.19
	7	5.5	18.0	0.30
AUT-e		0.86	2.1	0.40
		3.4	11.0	0.30

* Appropriate fractions from electrophoresis columns were dialyzed against 1 mM EDTA, followed by distilled deionized water or against distilled water only, pelleted by centrifugation, washed several times with distilled deionized water, and finally resuspended for analysis in 0.01 M Tris buffer (pH 7.5)–0.2% Triton X-100.

† Phototrap concentrations were usually measured by both ΔEPR and ΔA_{865}; an average of the two values is given. Those numbers designated by § were determined by ΔEPR only. Chromatophores were used as standard assuming $\Delta A_{865}/A_{878}$ = 0.030; $\Delta\epsilon$ for P865 = 90 mM^{-1} cm^{-1}; spins produced/P865 oxidized = 1.0. The spin to trap ratios measured for the batch AUT-e preparations were very close to those for chromatophores (1.0), and the characteristics of the EPR signals were also rather similar to those of the chromatophore signal. For regular AUT-e, the EPR signal used was that for the ubiquinone-coupled system.

‡ Analyses performed by Dr. M. Y. Okamura, Department of Chemistry, Northwestern University.

The results of analyses of several AUT-e preparations are shown in Table 1. We have not attempted to make a correction for the percent of total phototrap recovered during the experiment. Our typical overall recovery of phototrap activity in this type of experiment is 60%. The iron to phototrap ratios would be even lower if this type of correction were introduced. The data clearly show that there is less than one equivalent of iron per phototrap. Since the wavelength dependence of the absorbance photochanges of the material of low iron content in the visible and near-infrared regions is unchanged (3) as compared with the original chromatophores, and since the quantum yield for photooxidation of P865 is the same value (1.0 ± 0.1) as is observed with chromatophores (10), the low iron content of these preparations would appear to eliminate iron-containing compounds from a redox role as primary electron acceptor.

When an electrophoretically purified photoreceptor subunit preparation is examined for light-induced EPR signals, an asymmetric signal is observed (*stippled curve* in Fig. 2). After observing this asymmetric EPR signal, and after our analytical data for photoreceptor subunit preparations showed low iron contents, we began to routinely analyze each preparation for its photoproduced EPR signal relative to its photoproduced absorbance change at 865 nm. The average result

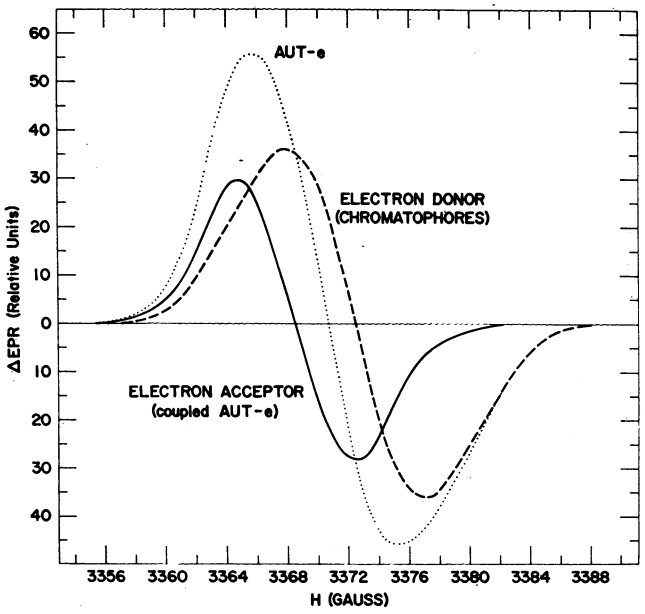

FIG. 2. Light-induced EPR signals of chromatophores (*dashed curve*), anaerobic AUT-e (*stippled curve*), and anaerobic ferrocytochrome *c*-coupled AUT-e (*solid curve*). Oxidized ubiquinone Q_2-coupled AUT-e results in a curve nearly identical with that of chromatophores. While the EPR spectra of coupled AUT-e systems are very reproducible, that of AUT-e itself varies considerably from preparation to preparation and with aerobic or anaerobic conditions. The EPR spectrum shown for AUT-e is actually the sum of the two coupled signals, but is very typical of most AUT-e signals we have observed. Modulation amplitude = 5 G, microwave power = 10 mW.

of some 25 experiments gave a spin to trap ratio of 1.7. The total range of values determined was from 1.2 to 2.2 for various AUT and AUT-e samples. The reference system in each case was a standard chromatophore preparation, for which it has been demonstrated (6) that the spin molarity, as measured by EPR, is equal to the phototrap molarity (within 5%), as measured by absorbance photochange at 865 nm.

As reported (2), we have consistently found apparently better yields of phototrap in the membrane dissolution procedure when the phototrap activity was measured by EPR photochange than when it was measured by absorbance photochange at 865 nm. The reason for this discrepancy is that an additional EPR signal appears as soon as the membranes are exposed to AUT conditions.

If the new component of the EPR photosignal is due to the one-electron reduction of some organic species, then in purified systems it may be diminished in size under steady-state and saturating-light conditions if an excess of an oxidant is added that reacts with it. Under these conditions, the primary electron-donor radical should be the predominant species observed. If oxidized ubiquinone (CoQ_6) is added to AUT-e, and a steady exciting light is applied until a steady state is reached, the shape and location of the signal closely approach that of chromatophore systems.

On the other hand, it should be possible to add reductants that react with the oxidized form of the primary electron donor and, under subsequent illumination, to reach a steady state where the photochemically-reduced species predominates. Several experiments of this type were conducted anaerobically; a typical result is also shown in Fig. 2 (*solid curve*). The light-induced absorbance changes due to P865 oxidation were also measured in these ferrocytochrome *c*-coupled systems, and were nearly completely quenched. Under these conditions, the new EPR signal is clearly observed. It appears to be nearly symmetrical, is at lower field than the primary electron-donor signal, and has a nearly Gaussian shape. It has a *g*-value of 2.0050 ± 0.003 and a peak-peak width of 7.0 ± 0.3 G. It should be noted that full reversibility is observed in all of these coupled systems. In the case of the anaerobic experiments with ferrocytochrome *c*, oxidation to ferricytochrome *c* and addition of air restore the original aerobic signal.

The decay kinetics of the newly observed radical are also consistent with its assignment to a reduced species. For example, in the presence of only ferrocytochrome *c* and in the absence of air, the signal is photoproduced, but requires hours to decay. However, if a very small amount of ferricytochrome *c* is then added to the system, complete decay of the photosignal occurs within seconds. The same type of response can also be shown with other redox couples.

The three samples whose EPR spectra are given in Fig. 2 were also measured at liquid-nitrogen (−196°) temperature. The relative shapes and locations of the signals were unchanged. No hyperfine structure could be observed in the electron-acceptor signal.

As was discussed above, two organic radicals are present in photoexcited photoreceptor subunit preparations, and

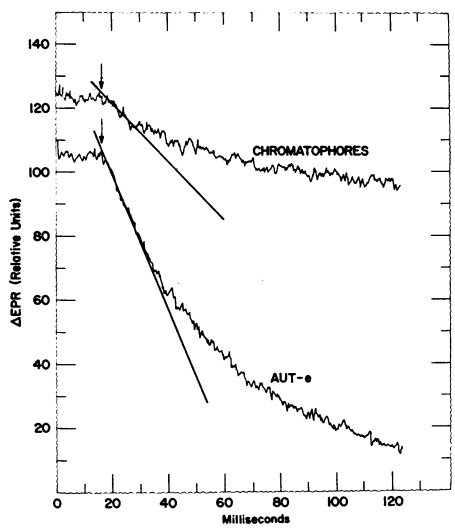

Fig. 3. Relative rate of radical formation in chromatophores and AUT-e. Continuous illumination is allowed to fall on the sample (*arrow*). A change in the negative direction represents radical formation. The excitation wavelength (880 nm) was selected by use of a Baird Atomic B-9 narrow band pass filter. Repetitive data was accumulated (6) by use of a Nuclear Data Enhancetron, together with a mechanical chopper. The field setting for this experiment was 3376.0 G. A power of 10 mW and a modulation amplitude of 5 G were used. Repetitive light and dark periods of 2 sec and 22 sec, respectively, were used for the chromatophore sample, and 0.2 sec and 2 sec, respectively, were used for the AUT-e sample. Although not recorded, the results of single initial light periods were in qualitative agreement with the results shown. Similar rate measurements were also made at field settings of 3377.5, 3364.5, and 3366.0 G.

conditions may be used where only one radical predominates. The response of each of these signals to microwave power at room temperature was determined by use of these conditions. The new radical, which appears to be a one-electron reduced species, was more readily saturated than the primary electron-donor radical.

Perhaps the most crucial test of whether the additional EPR signal arises from the primary electron-acceptor species is to measure the quantum efficiency for its spin production. Again using the *R. rubrum* chromatophore system as a standard whose quantum efficiency for spin production is known (6), we directly compared this reference system to the purified photoreceptor preparations. Typical kinetic data are shown in Fig. 3. A greater initial slope is evident for the photoreceptor subunit preparation, which indicates that more EPR signal per quantum is being produced in the AUT-e preparation than in chromatophores. Similar rate comparisons were made at three other locations in the field. At each field setting, the rate of change due to oxidation of the primary electron donor was subtracted from the total by conducting an identical measurement with a sample of chromatophores. In both cases, the samples absorbed all the light (880 ± 10 nm) incident upon them. The shape and location data of the reduced radical, as shown in Fig. 2, together with its double-integrated area,† allow the rate of spin production to be calculated for each experiment.

In measurement of spin concentrations, a correction of the data is desirable because the rate measurements, as well as the earlier spin comparison measurements, were made at high power (10 mW) in order to have maximum signal to noise ratio. However, the measurements are best compared at low power, where the amount of each radical increases linearly with the square root of the power (6, 11). From the relative "power saturation" profiles, a correction can be applied to convert the ratio (reduced signal to chromatophore signal) at 10 mW to what the ratio would have been at 1.25 mW (the factor used was 1.17). By this treatment of the data, the average quantum yield (four determinations) for production of the reduced radical was 0.6 ± 0.2.

DISCUSSION

The absence of all transition metals except iron in purified reaction center (12) and photoreceptor subunit (2, 3) preparations has been clearly demonstrated. The data on preparations of *R. rubrum* photoreceptor subunits reported here show that iron is not present in sufficient concentration to play a stoichiometric role in phototrap function. It is with these latter systems that we can demonstrate a new, one-electron, photoreduced organic species.

The newly discovered photoproduced EPR signal in photoreceptor subunit preparations has a g-value of 2.0050 ± 0.0003 and has a peak-peak width of 7.0 ± 0.3 G. Among biomolecules known to exhibit similar properties, most porphyrin radicals would seem to be ruled out by their g-value, which is near 2.0027 for the oxidized species (13) and 2.0021 for the reduced species (14). Semiquinones of the ubiquinone and plastoquinone type are known to exhibit EPR signals near g = 2.0045 (15, 16). Such molecules were among the first suggested to play a possible role in the primary photochemical event in photosynthesis (17), and model systems involving quinones and chlorophyll have been extensively studied (18–20). Although the narrowness and lack of observable hyperfine structure in the reduced radical in our samples is not consistent with any known quinone radicals, an immobility imposed by protein might prevent the observation of hyperfine structure and give rise to a narrower signal (16). Other molecules, such as pteridines (21, 22) and flavins (23, 24), are also known to exhibit semiquinone forms that have EPR signals centered near g = 2.0045, but they also have broader signals.

We would summarize experimental results that have a bearing on the primary electron acceptor molecule as follows: our first interpretation of the reversible reductive quenching of the absorbance and EPR photochanges, which gave a midpoint potential of −0.02 V (4, 8, 25–31), is still compatible with all existing data, that is, that the phototrap is shut-off because the primary electron acceptor molecule is reduced by the added titrant. If we assume that this redox dependency does reflect the reduction of the primary electron acceptor molecule, then because no EPR signal is observable at low potential in light or dark (8, 10), it may be concluded that: (*a*) the dark reduction is a two-electron reduction of the primary electron acceptor or (*b*) the dark reduction is a one-electron reduction of the primary electron acceptor, which interacts so strongly with a paramagnetic transition metal that its EPR signal is greatly broadened.

Similar preparations from other photosynthetic materials should be examined for the new EPR signal reported here. We find that our photoreceptor subunit preparation from *Rhodopseudomonas spheroides* does indeed show an increased EPR spin to phototrap ratio upon exposing the membranous material to AUT conditions. A complete analogy to the *R. rubrum* system seems to exist from the limited measurements we have made with the *R. spheroides* system (unpublished experiments). Very recently, a confirmation of the new radical was reported by Feher *et al.* (32). They found that it could be observed in their reaction-center preparations from the R-26 mutant of *R. spheroides* after addition of sodium dodecyl sulfate, and presented evidence for iron separation under these conditions.

We want to underscore the fact that, of the methods used to purify reaction-center or phototrap systems, the AUT procedure is the only one that: (*a*) completely dissociates all wild-type, mutant, green-plant, and algae systems tested; (*b*) results in sufficient dissociation to allow separation of nearly all iron-containing material; (*c*) allows observation of a reduced radical that may be from the primary electron-acceptor molecule; and (*d*) gives a low particle weight preparation in which the antenna bacteriochlorophyll and carotenoids are still a functioning part of the complex (1, 2).

Trace amounts of the new radical reported herein may have been observed previously by Kohl *et al.* (33) in whole cells of *R. rubrum*. They observed very small amounts of a dark signal, whose g-value was 2.0054 and whose half-width was about 11 G. A tentative identification of the signal with the flavoprotein succinic dehydrogenase was suggested. They showed that upon illumination the signal decreased, whereas the normal oxidized electron-donor radical increased. Depending on the internal redox balance, the integrity of the whole cell suspension, and the nature of the parameters con-

† We are grateful to Professor Brain Hoffman of the Department of Chemistry at Northwestern University for the use of his computer program for double integration of EPR signals.

tributing to the dark and light steady-state conditions, it may be that the electron acceptor radical described herein could be observed in trace amounts in the intact organism. However, we have not been able to observe the new signal with our chromatophore preparations, even when we attempt to couple the system by adding various redox components.

The properties of the newly observed free radical do not appear to be consistent with the usual properties of some of those molecules most often suggested to serve as the primary electron acceptor (bacteriochlorophyll, bacteriopheophytin, pteridines, and flavins). It is, of course, possible that the reduced radical we have observed is that of a secondary electron acceptor molecule still very tightly coupled to the phototrap (note that the quantum yield for its photoreduction is 0.6). Rather than suggest an identity for the newly observed radical, we would like to recall that it took 12–15 years after the discovery of the primary electron-donor free radical by EPR (34, 35) before the identity of the molecule from which it came was well documented and generally accepted. Perhaps with the purified reaction center and photoreceptor subunit preparations now available, the identity of this newly observed radical may not take as long to unequivocally establish.

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