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The photosystem of the green sulfur bacterium *Chlorobium limicola* contains two early electron acceptors similar to photosystem I

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An asymmetric EPR-line with a g factor of 2.0045 was detected after phototrapping at 196 K in membranes and in isolated photoactive P840 reaction centers of *Chlorobium limicola* f. thiosulfatophilum. The spectra resemble those of the electron acceptor A_1^- of photosystem I in chloroplasts and cyanobacteria. At 229 K a symmetric signal at $g=2.0033$, comparable to the one of the early electron acceptor A_0^- is additionally phototrapped. In contrast to membranes, the reaction center preparation does not contain appreciable amounts of photoreducible FeS-centers.

Bacterial photosynthesis; Reaction center; Phototrapping; EPR; (Green sulfur bacteria, *Chlorobium*)

1. INTRODUCTION

The similarity of plant and cyanobacterial PS II with the reaction center of purple photosynthetic bacteria on the one hand [1], and of PS I with the reaction center of green photosynthetic sulfur bacteria [2] on the other, is an interesting aspect of phylogenesis. The latter similarity is documented by the facts that green S-bacteria photoreduce NAD^+ via ferredoxin without additional energy requirement [3], that FeS centers function as early electron acceptors [4,5], and that an isolated, photoactive reaction center (RC) preparation contains the primary electron donor P840 on a 68 kDa subunit [6], as in PS I with P700. Recently light-induced optical changes have been described which probably come from bacteriopheophytin *a* [7] and might reflect the earliest electron acceptor, which is called A_0 in PS I and may be chlorophyll *a* [8].

In this study we provide evidence for the existence of another early electron acceptor in the photochemical reaction center of *Chlorobium* which resembles center A_1 of PS I [8].

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Abbreviations: DPPH, 1,1-diphenyl-2-dipicrylhydrazyl radical; PMSF, phenylmethylsulfonyl fluoride; EPR, electron paramagnetic resonance; P840, primary electron donor of the reaction center of *Chlorobium limicola;* PS, photosystem; RC, reaction center

2. MATERIALS AND METHODS

Cells of *Chlorobium limicola* f. thiosulfatophilum were grown and stored as before [6]. The P840 RC was prepared [6] with the following modifications: (i) cells were ruptured in a French press (20000 psi; see [9]) and not by sonication. The membranes were sedimented between 40000 and $130000 \times g$ and were largely free from chlorosomes already. (ii) The first extraction with octylglucoside was omitted. Membranes were resuspended to 30 μg BChl *a*/ml into the medium of the second extraction [6]. (iii) Dialysis after ammonium sulfate precipitation was against 10 mM

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phosphate plus 0.1% Triton X-100, pH 6.8, and elution from the hydroxyapatite column was with 100 mM phosphate, 0.1% Triton X-100, pH 6.8. (iv) Since photochemical activity is unstable and was reduced after sucrose density gradient centrifugation, this step was omitted. Instead, fractions from the hydroxyapatite chromatography showing a dominant concentration of the 68 kDa polypeptide on SDS-polyacrylamide gels [6] were pooled and reprecipitated with 40% ammonium sulfate. The pellet was resuspended as concentrated as possible in 20 mM Tris-HCl, pH 7.8. If not used immediately the samples were stored frozen at −70°C.

Samples were dialysed against 5 mM Tris for 2 h to reduce the buffer capacity. Further dialysis (2–3 h) against 60% glycerol resulted in about 4-fold concentration.

For EPR spectroscopy, membranes or RCs were brought to 0.1 M glycine-NaOH, pH 10. After bubbling with argon for 5 min, the samples were reduced by addition of dithionite to 10 mM and were subsequently bubbled with argon for an additional 30 min before anaerobic transfer into EPR-tubes and freezing in liquid nitrogen. For photoaccumulation [8] these EPR tubes were illuminated with a 250 W slide projector for at least 15 min in a glass dewar containing either a mixture of acetone/dry ice (196 K) or of solid/liquid ethylacetoacetate (229 K). The temperature of the bath was measured by a digital thermometer (accuracy 0.5 K). After illumination, the EPR tubes were transferred back into liquid nitrogen. EPR spectra were recorded with a Bruker ER 220 D X-band spectrometer. For the experiments at 9 K an Oxford Instruments cryosystem was used, whereas the 77 K spectra were recorded using a finger dewar filled with liquid nitrogen. For the exact determination of g factors at 77 K a double cavity with a DPPH standard was used.

3. RESULTS AND DISCUSSION

When reduced by dithionite at high pH in the dark, membranes of *Chlorobium* show at least one FeS center at 9 K, displaying EPR signals at $g_y = 1.91$ and $g_z = 1.88$ (fig.1a). Illumination at 196 K results in the modified spectrum of fig.1b.

The appearance of two new paramagnetic centers is seen in the difference spectrum (fig.1c):

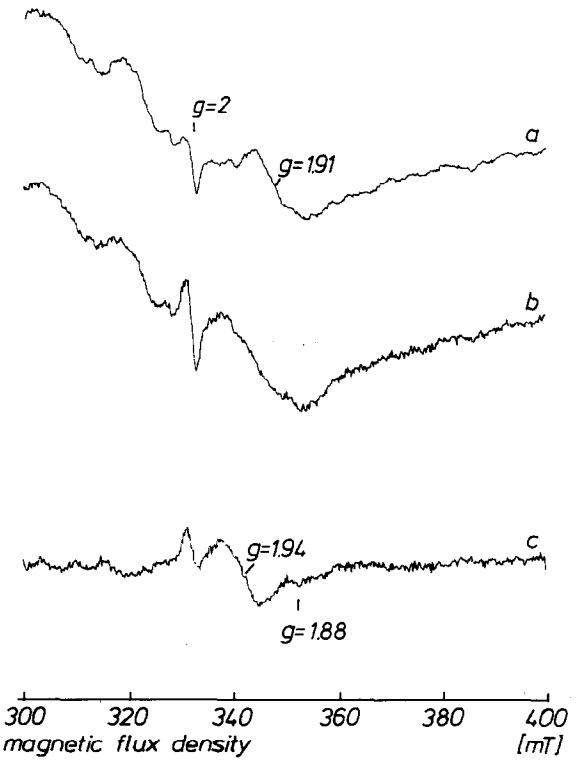

Fig.1. EPR spectra of paramagnetic centers induced in membrane samples by (a) chemical reduction with 10 mM $Na_2S_2O_4$, pH 10; (b) additional photoaccumulation at 196 K. The BChl concentration was 10 mg/ml at a BChl *a*/P840 ratio of 100. (c) The difference spectrum (b) − (a). EPR conditions: T, 9 K; microwave frequency, 9.31 GHz; microwave power, 20 mW; modulation amplitude, 2 mT; instrument gain, 2×10^5.

a singlet around $g = 2.00$ and a FeS center with $g_y = 1.94$ and $g_z = 1.88$ (the position of the g_x peak could not be determined). Thawing and keeping the sample in the dark for 5 min completely reversed the spectrum to the one in fig.1a. The presence of FeS centers in isolated RCs could not be demonstrated consistently, most likely due to variable loss during the isolation procedure. Either the polypeptides carrying FeS centers are more loosely bound or the FeS centers are more easily destroyed in comparison to PS I of chloroplasts and cyanobacteria, where harsher procedures are required to destroy these centers [10]. It should also be mentioned that there is some discrepancy concerning EPR signals associated with the RC of green sulfur bacteria. Jennings and Evans [5]

reported the photoreduction of an FeS center with $g_y = 1.90$ at cryogenic temperatures in *Chlorobium*. This finding was challenged in [11,12], showing photoreducible FeS signals at $g_y = 1.94$. In *Prosthecochloris aestuarii,* the $g_y = 1.94$ signal seems to be made up of two centers of different redox potentials, suggesting a serial function as electron acceptors [12]. These results are in line with earlier redox titration experiments in *Chlorobium* [4] stating that all low potential FeS centers have g_y values of 1.94. Thus our chemically reduced component at $g = 1.91$ could be the Rieske-center [4], whereas the photoreducible $g = 1.94$ signal corresponds to the low potential secondary electron acceptor(s). Under identical conditions of sample preparation we are able to chemically reduce centers A and B in PS I of higher plants completely. The fact that virtually no $g = 1.94$ signal is detectable under these conditions in *Chlorobium* membranes suggests a very low redox potential for this component. Thus a function comparable to the one of center X in PS I of oxygenic photosynthesis seems probable.

The signal around $g = 2.00$ was further examined at 77 K and low microwave power. Under these conditions FeS centers are not detected and no EPR feature in the region of $g = 2$ was observed with samples frozen in the dark. After illumination at 196 K the signal shown in fig.2a was obtained with membranes as well as with isolated reaction centers. The line position and shape closely resembles the A_1^- spectrum of higher plants [8]. The signal is asymmetric with a g factor of 2.0045 ± 0.0003 and a linewidth of 1.05 mT as compared to 2.0043 and 1.036 mT determined for A_1^- in PS I of chloroplasts [8]. Further illumination at 229 K changed the spectrum (fig.2b). The difference spectrum is shown in fig.2c. It shows a symmetric line at $g = 2.0033 \pm 0.0002$ with a peak-to-peak with ΔH_{pp} of 1.46 mT, comparable to the signal attributed to the electron acceptor A_0 in PS I. The latter values are slightly different from the ones published by Swarthoff et al. [13] for *Prosteco-chloris aestuarii,* another green S-bacterium ($g =$ 2.0038 and linewidth 1.296 mT). However, their spectrum was obtained by photoaccumulation at 273 K, followed by rapid cooling to 250 K, which could lead to a rather undefined state of reduction.

Our results are very much reminiscent of the situation in PS I of plants, where phototrapping at

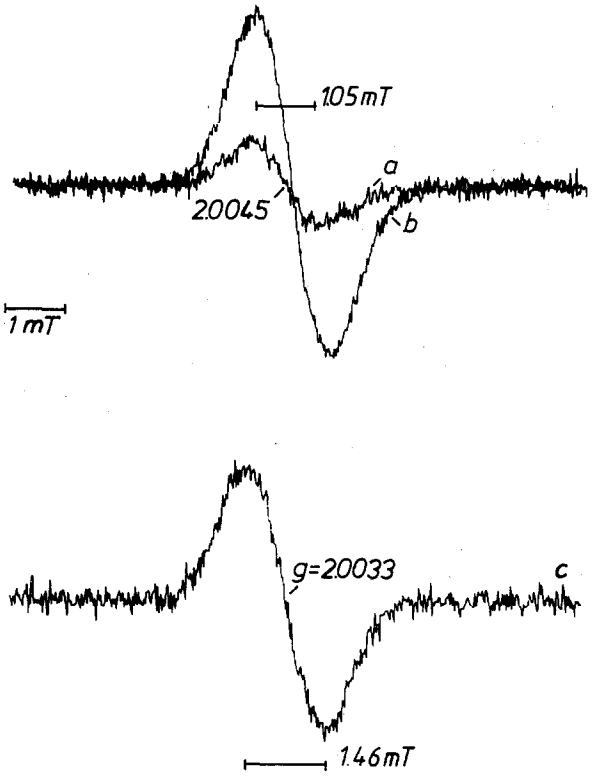

Fig.2. EPR spectra of paramagnetic centers accumulated in isolated RCs by illumination at 196 K (a) and 229 K (b). The BChl concentration was 100 μg/ml at a BChl *a*/P840 ratio of 40. (c) The difference spectrum (b) – (a). EPR conditions: *T*, 77 K; microwave frequency, 9.46 GHz; microwave power, 63 μW; modulation amplitude, 0.2 mT; instrument gain, 1×10^6.

the two temperatures (196 and 229 K) leads to the accumulation of two paramagnetic species attributed to the two early electron acceptors A_0 and A_1 [8].

It has been suggested that the EPR signal of A_1^- of chloroplasts represents a semiquinone [8,14]. This idea has gained further support by the observation that the optical difference spectrum of A_1^-/A_1 resembles the UV-difference spectrum of phyllosemiquinone-phylloquinone [15,16]. Moreover, the core of PS I from chloroplasts and cyanobacteria, the P700-reaction center, contains two bound phylloquinone molecules [17]. However there is evidence arguing against the identification of A_1 with phylloquinone [18].

A quinone as early electron acceptor was also suggested for the green S-bacterium *Heliobac-*

terium chlorum [19]. There, a signal at $g = 2.0038$ with a ΔH_{pp} of 1.5 mT (X-band) was photoinduced in membrane samples frozen in the light at low redox potentials. Line broadening to 1.8 mT under Q-band conditions together with the power saturation characteristics of the signal were taken as indications that the signal may be due to a semiquinone. Either the different spectral parameters of the signal described in [19] (compared to our signals) are due to the specific species investigated, or, again, the method of photoaccumulation used accounts for the different spectra. To check the quinone hypothesis we are presently examining the quinone content of our RC samples. Whatever the nature of the redox centers, the results presented here suggest the presence of two distinct early electron acceptors in *Chlorobium*, which seem to have rather close similarity to the respective centers in the PS I of algae and higher plants.

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