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IRON-SULFUR PROTEINS OF THE GREEN PHOTOSYNTHETIC BACTERIUM *CHLOROBIUM*

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SUMMARY

The iron-sulfur proteins of the green photosynthetic bacterium *Chlorobium* have been characterized by oxidation-reduction potentiometry in conjunction with low-temperature electron paramagnetic resonance spectroscopy. *Chlorobium* ferredoxin was the only iron-sulfur protein detected in the soluble fraction; no high-potential iron-sulfur protein was observed. In addition, high-potential iron-sulfur protein was not detected in the chromatophores. Four chromatophore-bound iron-sulfur proteins were detected. One is the "Rieske" type iron-sulfur protein with a g-value of 1.90 in the reduced state; the protein has a midpoint potential of +160 mV (pH 7.0), and this potential is pH dependent. Three $g = 1.94$ chromatophore-bound iron-sulfur proteins were observed, with midpoint potentials of −25, −175, and about −550 mV. A possible role for the latter iron-sulfur protein in the primary photochemical reaction in *Chlorobium* is considered.

INTRODUCTION

Membrane-bound iron-sulfur proteins function as electron carriers in such diverse energy-transducing systems as mitochondria [1], plant and algal chloroplasts [2–5], purple sulfur photosynthetic bacteria [6, 7], and purple non-sulfur photosynthetic bacteria [8, 9]. Because of our previous interest in the electron transport reactions of green photosynthetic bacteria [10, 11] we have examined chromatophores from *Chlorobium* for their content of membrane-bound iron-sulfur proteins. Our results indicate the presence in *Chlorobium* of several bound iron-sulfur proteins with properties that are significantly different from those of proteins found in other photosynthetic bacteria.

METHODS

Chromatophores from *Chlorobium limicola f. thiosulfatophilum* and *Chromatium vinosum* were prepared as previously described [11] and washed once with 50 mM potassium phosphate or Tris buffer (at the pH used for the subsequent oxidation-

reduction titrations) containing 1 mM EDTA. For the titrations performed at the pH values necessary to obtain ambient oxidation-reduction potentials of −600 mV (pH 10.0 and pH 10.5), the chromatophores were washed with 25 mM Tris buffer (pH 8.5) containing 1 mM EDTA.

For study of the soluble iron-sulfur protein fraction, the supernatant solution from centrifugation of the sonicated cells at 144 000 × *g* was passed over a 2 × 5 cm DEAE-cellulose column (equilibrated with 20 mM potassium phosphate buffer, pH 6.5). The column was washed with 50 ml of 50 mM potassium phosphate buffer (pH 6.5), and the iron-sulfur protein fraction was eluted with the same buffer containing 0.5 M NaCl. Because the soluble *Chlorobium* ferredoxin is known to be unstable, this fraction was immediately used in electron paramagnetic resonance (EPR) studies.

Oxidation-reduction titrations were performed under an Ar atmosphere as described by Dutton [12] with 0.1 M $Na_2S_2O_4$ (in 0.03 M KOH) as reductant and 0.2 M $K_3Fe(CN)_6$ as oxidant. The oxidation-reduction potential was measured with a Metrohm model 103 pH meter and a combination platinum and $Ag/AgCl_2$ electrode (Metrohm EA259) that was calibrated against a saturated quinhydrone solution at pH 7.0. The samples were transferred with a Hamilton gas-tight syringe (No. 1750) to EPR tubes made anaerobic by flushing with Ar. EPR spectra were obtained with a modified JEOL X-band spectrometer that incorporated an Airco liquid helium cooling system [13, 14].

RESULTS

Fig. 1 shows the EPR spectrum of partially purified soluble *Chlorobium* ferredoxin that had been reduced with $Na_2S_2O_4$. The spectrum had *g*-values of 2.08, 1.93, and 1.88 and a temperature dependence that was similar to that of other iron-sulfur

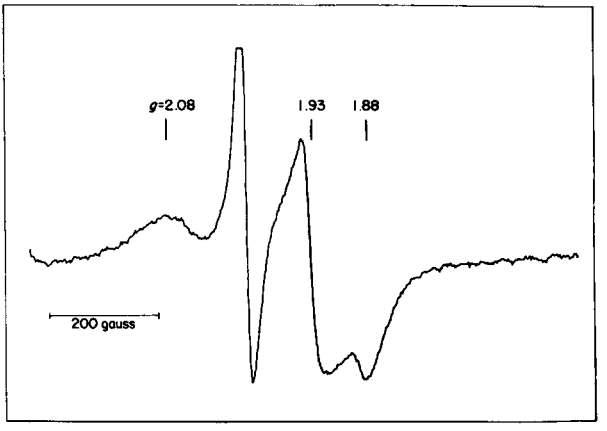

Fig. 1. EPR spectrum of dithionite-reduced soluble ferredoxin from *Chlorobium*. The soluble protein was isolated as described in Methods and was reduced with 5 μmol of $Na_2S_2O_4$ (prepared anaerobically in 0.01 M NaOH) prior to freezing to 77 °K. EPR conditions: frequency, 9.20 GHz; modulation amplitude, 10 G; microwave power, 10 mW; amplifier gain, 200; temperature, 15 °K.

proteins (see ref. 15). The residual signal in the $g = 2.0$ region probably originates from a small amount of oxidized ferredoxin. The EPR spectrum of soluble *Chlorobium* ferredoxin is similar to that recently reported for a ferredoxin from *Rhodospirillum rubrum* [16] that has g-values of 2.07, 1.94, and 1.89. No EPR signal of the high-potential iron-sulfur protein type [6, 7, 17] was observed when ferricyanide was added to the partially purified soluble extract. (In contrast to other iron-sulfur proteins, the high-potential iron-sulfur protein exhibits an EPR signal in the oxidized state; see ref. 17.) There was also no EPR signal that could be attributed to the high-potential iron-sulfur protein in *Chlorobium* chromatophores poised at oxidation-reduction potentials above +400 mV, a potential sufficiently high to oxidize the membrane-bound high-potential iron-sulfur protein in the purple sulfur bacterium *Chromatium* [6, 7]; it therefore appears that *Chlorobium* does not contain this electron carrier.

Fig. 2 shows the EPR spectra of *Chlorobium* chromatophores poised at oxidation-reduction potentials of +155 and +15 mV (pH 8.5). A membrane-bound component that exhibits an EPR signal in the reduced state with g-values of 1.90 and 1.79 is apparent at +15 mV but absent at the more positive potential. This EPR spectrum is similar to that of the "Rieske" $g = 1.90$ iron-sulfur protein first found in mitochondrial Complex III [9, 18] and subsequently found in numerous photosynthetic systems [6–9, 19].

Fig. 3 shows oxidation-reduction titrations of the $g = 1.90$ iron-sulfur protein in *Chlorobium* chromatophores at two pH values. The experimental points can be fit to $n = 1.0$ titrations with midpoint oxidation-reduction potentials of +165 and +60 mV at pH 6.8 and pH 8.4, respectively. The titrations were reversible at both pH

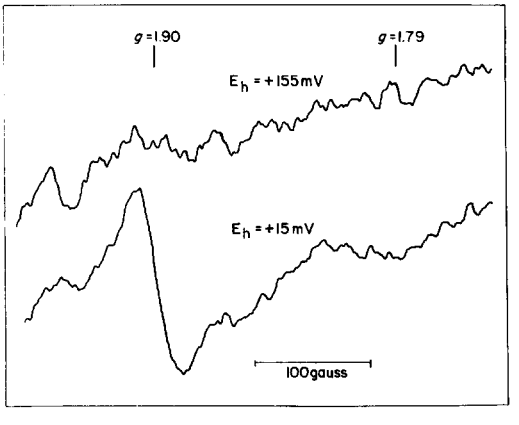

Fig. 2. EPR spectrum of the membrane-bound $g = 1.90$ iron-sulfur protein in *Chlorobium* chromatophores. The reaction mixture contained 100 mM Tris buffer (pH 8.5), *Chlorobium* chromatophores (at a *Chlorobium* chlorophyll concentration of 7 mM) and the following oxidation-reduction mediators: 2,6-dichlorophenolindophenol (60 μM), 2,3,5,6-tetramethyl-*p*-phenylenediamine (50 μM), 1,2-naphthoquinone (50 μM), phenazine methosulfate (50 μM), phenazine ethosulfate (50 μM), duroquinone (50 μM), and pyocyanine (50 μM). Samples were withdrawn at the indicated oxidation-reduction potentials and spectra were recorded with the following EPR conditions: frequency, 9.21 GHz; modulation amplitude, 10 G; microwave power, 10 mW; amplifier gain, 710; temperature, 15 °K.

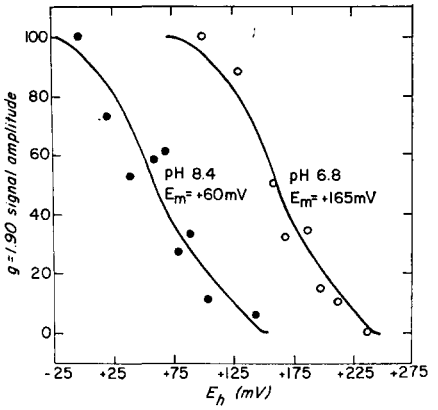

Fig. 3. Oxidation-reduction titrations of the $g = 1.90$ iron-sulfur protein in *Chlorobium* chromatophores. Reaction conditions as in Fig. 2 with 100 mM potassium phosphate buffer (pH 6.8) added where indicated.

values. Fig. 4 shows the pH dependence of the midpoint potentials of the *Chlorobium* $g = 1.90$ iron-sulfur protein over the pH range from 6.8 to 8.4. These potentials fit a -60 mV per pH unit dependence expected for a component that takes up one H^+ per electron.

In addition to the component that shows a signal at $g = 1.90$, at oxidation-reduction potentials lower than $+75$ mV *Chlorobium* chromatophores contain components that exhibit an EPR signal at $g = 1.94$. Fig. 5 shows EPR spectra of *Chlorobium* chromatophores at three different oxidation-reduction potentials ($+100$, -100, -200 mV). The signals at $g = 1.94$ indicate the presence of additional iron-sulfur proteins in *Chlorobium* chromatophores. The dependence of the magnitude of the

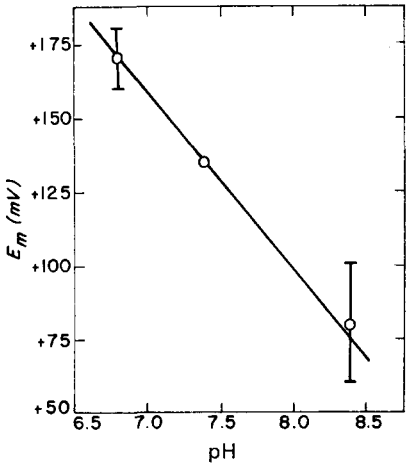

Fig. 4. Effect of pH on the midpoint oxidation-reduction potential of the *Chlorobium* $g = 1.90$ iron-sulfur protein. Reaction conditions as in Fig. 2 with 100 mM potassium phosphate buffer used at pH 7.4.

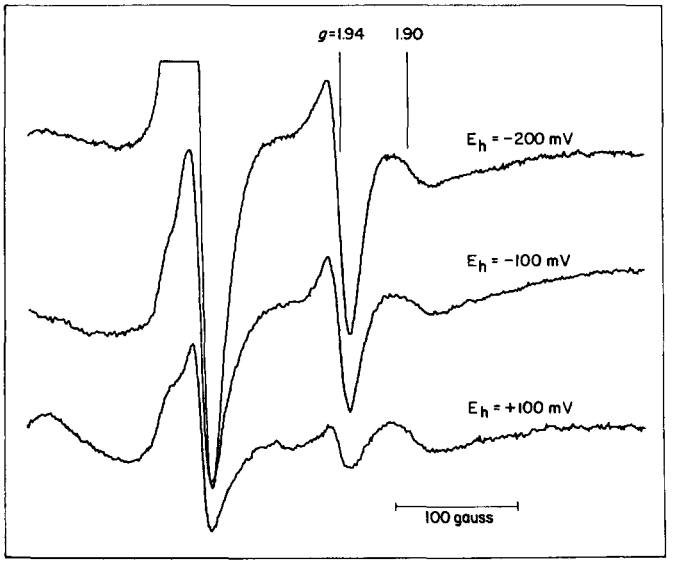

Fig. 5. EPR spectra of membrane-bound $g = 1.94$ iron-sulfur proteins in *Chlorobium* chromatophores. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), *Chlorobium* chromatophores (at a *Chlorobium* chlorophyll concentration of 3 mM) and the following oxidation-reduction mediators (at a concentration of 50 μM): phenazine methosulfate, phenazine ethosulfate, 5-hydroxy-1,4-naphthoquinone, duroquinone, pyocyanine, 2-hydroxy-1,4-naphthoquinone, anthraquinone-1,7-disulfonate, anthraquinone-2-sulfonate, and benzyl viologen. Samples were withdrawn at the indicated oxidation-reduction potentials and spectra were recorded with the following EPR conditions: frequency, 9.21 GHz; modulation amplitude, 6.0 G; microwave power, 10 mW; amplifier gain, 200; temperature, 40 °K.

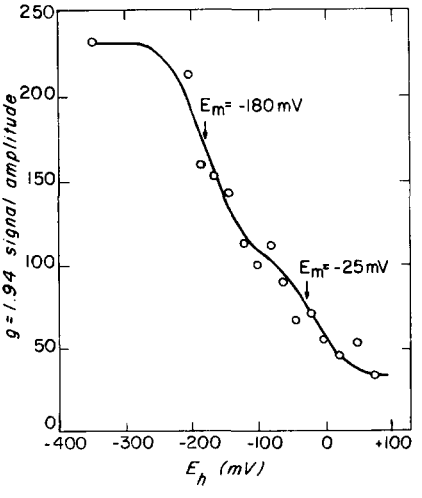

Fig. 6. Oxidation-reduction titrations of $g = 1.94$ iron-sulfur proteins in *Chlorobium* chromatophores. Reaction conditions as in Fig. 5.

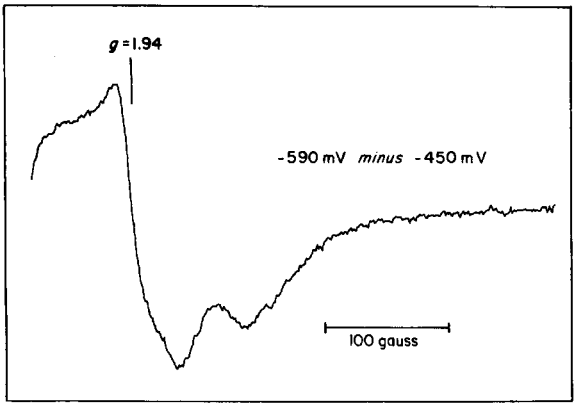

Fig. 7. EPR spectrum of a low-potential, membrane-bound iron-sulfur protein in *Chlorobium* chromatophores. The reaction mixture contained 150 mM glycine buffer (pH 10.0), *Chlorobium* chromatophores (at a *Chlorobium* chlorophyll concentration of 7 mM) and the following oxidation-reduction mediators (at a concentration of 100 μM): phenazine methosulfate, pyocyanine, 2-hydroxy-1,4-naphthoquinone, anthraquinone-2-sulfonate, benzyl viologen, methyl viologen, and triquat (1,1′-trimethylene-2,2′-dipyridylium dibromide). EPR spectra were recorded at oxidation-reduction potentials from −400 to −590 mV and the difference between the spectra at −590 and −450 mV was obtained by computer subtraction. EPR conditions: frequency, 9.20 GHz; modulation amplitude, 10 G; microwave power, 5 mW; amplifier gain, 110; temperature, 10 °K.

$g = 1.94$ signals on oxidation-reduction potential is shown in Fig. 6. The data fit the theoretical curve drawn for two components with midpoint potentials of −25 and −180 mV, respectively. Several titrations gave values of −25 (±20) and −175 mV (±20 mV), with identical values being obtained at pH 7.0 and pH 8.0. Some preparations exhibited a small residual signal at $g = 1.94$ that persisted even at potentials above +100 mV, but this signal was variable and did not appear to represent a significant component.

No increase in the magnitude of the $g = 1.94$ signal of *Chlorobium* chromatophores was observed as the potential was lowered from −250 to −475 mV. However, if the potential was lowered further, an additional signal was observed. Fig. 7 shows an EPR difference spectrum of *Chlorobium* chromatophores poised at −590 mV compared to chromatophores poised at −450 mV. The signal at $g = 1.94$, which originates from the reduced form of a very low potential iron-sulfur protein, is temperature sensitive and could be observed at temperatures near 10 °K but not at temperatures above 20 °K. It was not possible to obtain a well-defined titration endpoint for this low-potential iron-sulfur protein. Based on the observation that no signal appeared at potentials more positive than −500 mV, the midpoint potential is estimated to be near −550 mV. By contrast, in titrations of *Chromatium* chromatophores in the potential range from −400 to −610 mV no iron-sulfur proteins with midpoint potentials more negative than −350 mV were detected.

DISCUSSION

The iron-sulfur protein compliment of *Chlorobium* is quite different from that of the other types of photosynthetic bacteria. Unlike the purple sulfur bacterium

Chromatium [6, 7, 17], *Chlorobium* has neither soluble nor bound high-potential iron-sulfur protein. The content of bound iron-sulfur proteins with EPR signals at $g = 1.94$ (in the reduced form) and midpoint oxidation-reduction potentials between +50 and −350 mV is different in *Chlorobium* from either the purple sulfur or purple non-sulfur bacteria. Whereas *Chlorobium* has two such iron-sulfur proteins in this oxidation-reduction potential range, with midpoint potentials of −25 and −175 mV, the proteins of the purple sulfur bacterium *Chromatium* have midpoint potentials near −75 [6, 7] and −290 mV [7] and the purple non-sulfur bacteria (*Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata*) have three iron-sulfur proteins, with midpoint potentials near +30, −200, and −350 mV [8, 9]. However, the most striking differences between the iron-sulfur proteins from *Chlorobium* and from other photosynthetic species are the properties of the $g = 1.90$ protein and the presence of an iron-sulfur protein with a midpoint potential more negative than −500 mV.

The $g = 1.90$ iron-sulfur protein in *Chlorobium* chromatophores has a midpoint potential of +160 mV (at pH 7.0). This value is approx. 125 mV more negative than the values reported for the $g = 1.90$ protein in purple sulfur [6, 7] and purple non-sulfur [8, 9] photosynthetic bacteria. This midpoint potential is also considerably more negative than the potential of the protein in mitochondria [9] and in chloroplasts [19]. The midpoint potential of the $g = 1.90$ protein in *Chlorobium* is consistent with the protein functioning as an electron carrier that donates electrons to the reaction center bacteriochlorophyll through cytochrome *c*. The midpoint potential for the photooxidizable cytochrome *c* chromatophores from *Chlorobium* is +220 mV [12], slightly more positive than the midpoint potential of the $g = 1.90$ protein. A similar relationship exists in other photosynthetic bacteria where the photooxidizable *c*-type cytochromes have midpoint potentials between +285 and +340 mV [20, 21]. Similarly, the reaction center bacteriochlorophyll in *Chlorobium* chromatophores has a midpoint potential of +330 mV [12] and the reaction center bacteriochlorophylls in the purple sulfur and purple non-sulfur photosynthetic bacteria have considerably more positive midpoint potentials of nearly +450 mV (see ref. 22 for a recent summary of these values). Because the $g = 1.90$ iron-sulfur protein in *Chlorobium* can take up a proton on reduction in the pH range from 6.8 to 8.4, it is possible that it functions as a proton carrier as well as an electron carrier. In this regard, the *Chlorobium* $g = 1.90$ iron-sulfur protein differs from those in other organisms where no protons are involved in reduction [8, 9, 19].

Perhaps the most noteworthy observation reported above is the detection of an iron-sulfur protein ($g = 1.94$ EPR signal in the reduced form) with a midpoint potential near −550 mV in *Chlorobium* chromatophores. Recent work by Prince and Olson [23] on the effect of oxidation-reduction potential on cytochrome *c* photooxidation in a partially purified reaction center complex from *Chlorobium* suggests that the primary electron acceptor of *Chlorobium* has a midpoint potential between −500 and −550 mV. The results of Prince and Olson [23] and the known involvement of an iron-sulfur protein with a midpoint potential near −550 mV at the primary electron acceptor site of plant Photosystem I [3–5, 24] suggest such a role for the low-potential iron-sulfur protein in *Chlorobium*. We have not been able to detect photoreduction of this component at cryogenic temperatures (a usual criterion for a primary electron acceptor, see refs. 22 and 25 for reviews), perhaps because

of poor illumination of the entire sample as a result of the extremely high sample absorbance.

Further evidence for a possible role of the −550 mV iron-sulfur protein in *Chlorobium* as the primary electron acceptor comes from the observation that representative species of both the purple sulfur bacteria (*Chromatium vinosum*, see above) and purple non-sulfur bacteria (*Rhodospirillum rubrum*, D. C. Yoch of this laboratory, unpublished observations) contain no iron-sulfur proteins with mid-point potentials between −400 and −625 mV. These two types of photosynthetic bacteria appear to have primary electron acceptors that function near −180 mV [22, 26], rather than the −550 mV value reported for *Chlorobium* [23].

The presence of a low-potential iron-sulfur protein ($E_m \approx -550$ mV) in *Chlorobium* chromatophores which could function as the primary electron acceptor would be consistent with the previous demonstration of a direct ferredoxin-dependent, uncoupler-insensitive, reduction of NAD by this organism [27–29]. Such a ferredoxin-dependent reduction has not been demonstrated in other photosynthetic bacteria. The similarities between the reaction in *Chlorobium* chromatophores and the reaction in chloroplast Photosystem I merit detailed consideration in subsequent investigations.

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