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The Detection and Characterization by Electron-Paramagnetic-Resonance Spectroscopy of Iron–Sulphur Proteins and other Electron-Transport Components in Chromatophores from the Purple Bacterium *Chromatium*

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Low-temperature e.p.r. (electron-paramagnetic-resonance) spectroscopy was used to detect electron-transport components in *Chromatium* chromatophores with e.p.r. signals in the $g=2.00$ region. High-potential iron protein ($E_{m8.0}=+325$mV, where $E_{m8.0}$ is the midpoint potential at pH8) and a second component ($g=1.90$, $E_{m8.0}=+285$mV) are oxidized in illuminated chromatophores. Two iron–sulphur proteins ($g=1.94$) with $E_{m8.0}=-290$mV and $E_{m8.0}=-50$mV are present. One ($E_{m8.0}=-50$mV) is reduced on illumination. A component ($g=1.82$) with $E_{m8.0}=-135$mV is photoreduced at 10°K. The midpoint potential of this component is altered by *o*-phenanthroline and pH. The properties of this component suggest that it is the primary electron acceptor of a photochemical system. Another component ($g=1.98$) also has some of the properties of a primary electron acceptor, but its function cannot be completely defined. These results show that iron–sulphur proteins are present in the electron-transport system of *Chromatium* and indicate their role in electron transport.

Iron–sulphur proteins are widely distributed electron-transport cofactors. Soluble ferredoxins are involved as cofactors in many low-potential reactions in anaerobic bacteria and in plants (see reviews by Yoch & Valentine, 1972; Buchanan & Arnon, 1970), and membrane-bound iron–sulphur proteins are involved in mitochondrial and chloroplast electron transport. Iron–sulphur proteins are most easily detected in membrane system by low-temperature e.p.r. (electron-paramagnetic-resonance) spectrometry. Iron–sulphur proteins of the ferredoxin type have e.p.r. signals below $g=2.00$ in the reduced form, and high-potential iron protein (HIPIP) from *Chromatium* has a very characteristic spectrum centred at $g=2.04$. Although it has been known for many years that photosynthetic bacteria such as *Chromatium* contain soluble ferredoxins which are involved in carbon metabolism and nitrogen fixation, there has been no direct evidence for the involvement of these groups of proteins in photosynthetic electron transport. We have now used low-temperature e.p.r. spectroscopy to show that the photosynthetic membrane system of *Chromatium* contains both the protein HIPIP and ferredoxin-like iron–sulphur proteins, and to obtain evidence for their involvement in photosynthetic electron transport. E.p.r. spectroscopy has been extensively used to study the primary photochemical reactions of chlorophyll in plants and bacteria (see review by Weaver, 1968). It is widely accepted that the primary event in both plant and bacterial photosynthesis is the photo-oxidation of a specialized chlorophyll molecule termed the reaction-centre chlorophyll. It has been shown (Malkin & Bearden, 1971; Evans *et al.*, 1972) that in chloroplasts the primary electron acceptor can also be observed by e.p.r., and that this component, which is identified on the basis of its photoreduction at cryogenic temperatures, is probably a complex iron–sulphur centre. The primary electron acceptor in bacterial photosynthesis has proved to be more difficult to identify. Early results with absorption-spectroscopic techniques indicated that ubiquinone might be the acceptor, and Feher *et al.* (1972) have shown that in bacterial reaction centres the photoreduction of a quinone-like radical is observed by e.p.r. at liquid-helium temperatures, and Loach & Hall (1972) also observed the appearance of an unidentified radical in similar preparations. Leigh & Dutton (1972) and Dutton *et al.* (1973) have shown the reversible reduction of a component with an e.p.r. signal at $g=1.82$ at liquid-helium temperatures in *Chromatium* and *Rhodopseudomonas spheroides* reaction centres. All of these groups used reaction centres treated for different periods with detergents, removing all or most of the iron-containing electron-transport components. These treatments may disturb the sequence of electron transfer in the reaction centre. We have

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investigated the nature of the primary electron acceptor in chromatophores (membrane fragments obtained without detergent treatment) and have obtained evidence supporting the proposal that an iron protein with an e.p.r. signal at $g=1.82$ is the primary electron acceptor of a photochemical system.

Materials and Methods

Chromatium strain D was grown in a modified Pfennig's medium (Evans & Buchanan, 1965) either with succinate as carbon source or autotrophically. The nitrogen source was either NH_3 or N_2 gas. Variations in conditions for growth did not affect the results obtained. The cells were harvested with a continuous flow centrifuge and stored frozen in liquid N_2 until required.

Chromatophores were prepared by suspending the cells 1:3 (w/v) in 0.05M-Tris–HCl buffer, pH8.0, and exposing them to sonic oscillation with a Dawe Soniprobe (20kHz, 100W) for 3min. After centrifugation for 20min at 4°C at 40000*g* (MSE 18 centrifuge, 8×50ml head) to remove cell debris, the chromatophores were sedimented at 4°C at 150000*g* for 1h (MSE 50 centrifuge, 10×10ml head). The chromatophores were washed once and resuspended in the same buffer at 3.5mg of bacteriochlorophyll/ml. Bacteriochlorophyll was determined by the method of Garcia *et al.* (1968). The chromatophores catalysed cyclic photophosphorylation and a light-dependent transfer of electrons from reduced dichlorophenol-indophenol to O_2, which is coupled to ATP synthesis, similar to that described in *Rhodospivillum rubrum* chromatophores (Feldman & Gromet-Elhanan, 1972). Samples for e.p.r. spectroscopy were prepared by using 0.1ml of chromatophore suspension in 3mm internal diam. quartz tubes. Reagents were added to the suspension in the tube under a stream of N_2 gas when appropriate. Samples were either stored in the dark or illuminated (4×10^{-3} J/cm^2 per s) for 3min at room temperature (18–20°C) before freezing in liquid N_2. The samples were stored in liquid N_2.

E.p.r. spectra were obtained with a Varian E4 spectrometer. Samples were cooled to between 6° and 30°K by a stream of helium gas passing through a quartz dewar inside the e.p.r. cavity. The temperature was monitored by a thermocouple placed upstream of the sample.

Oxidation–reduction potentiometry was carried out, essentially as described by Dutton (1971), in an anaerobic vessel continuously flushed with O_2-free N_2. The oxidation–reduction potential was measured with a platinum electrode (Radiometer, Copenhagen, Denmark; type P101) and a standard calomel electrode (Radiometer; type K401). Samples were transferred to pre-gassed e.p.r. tubes through a stainless-steel transfer tube and frozen under a stream of N_2. The following compounds were used as appropriate as mediators between the electrode and the electron carriers in the chromatophores: Methyl Viologen ($E'_0=-440$mV), 200μM; Benzyl Viologen ($E'_0=-311$mV), 200μM; phenosafranine (3,7-diamino-5-phenylphenazinium chloride) ($E'_0=-239$ mV), 100μM; 2-hydroxy-1,4-naphthaquinone ($E'_0=-149$mV), 100μM; Methylene Blue ($E'_0=+11$mV), 100μM; 5-hydroxy-1,4-naphthaquinone ($E'_0=+30$ mV), 100μM; phenazine methosulphate ($E'_0=+80$mV), 100μM; 1,2-naphthaquinone ($E'_0=+143$mV), 100μM; *NNN'N'*-tetramethyl-*p*-phenylenediamine ($E'_0=+260$mV), 10μM. The potential of the reaction mixture was adjusted with 0.1M-potassium ferricyanide or 1% (w/v) sodium dithionite in 0.1M-Tris–HCl buffer, pH9.0.

The naphthaquinone derivatives were obtained from Koch–Light Ltd. (Colnbrook, Bucks., U.K.). Other mediators and chemicals used in the experiments were from British Drug Houses Ltd. (Poole, Dorset, U.K.).

Results

We have examined the e.p.r. spectra of chromatophore preparations under different conditions of oxidation or reduction at temperatures between 6° and 30°K. In the present paper we describe the components that we have detected with e.p.r. signals in the $g=2.00$ region. We have observed components with signals in the $g=3.00$ and $g=6.00$ regions, which may be attributed to cytochromes, but we have not studied these in detail except to determine that they are due to different components from those giving rise to signals around $g=2.00$.

Fig. 1 shows the e.p.r. spectrum of protein HIPIP in chromatophores together with the spectrum of the purified protein HIPIP. The protein HIPIP has an e.p.r. signal in the oxidized form, whereas all the other components discussed in this paper have e.p.r. signals in the reduced form. When the chromatophores are kept in the dark the protein HIPIP is reduced (Fig. 1, trace 1); however, on illumination at room temperature the protein HIPIP becomes oxidized (Fig. 1, trace 2). Comparison of the signal in the illuminated chromatophores with that of purified protein HIPIP (Fig. 1, trace 3) confirms that the signal at $g=2.04$ and the associated minor peaks are due to this protein HIPIP. In the presence of electron donors such as sodium dithionite or reduced dichlorophenol–indophenol the protein HIPIP is reduced even after illumination. Potentiometric titration indicates that the oxidation–reduction potential of the protein HIPIP in the chromatophore membrane is $E_{m8.0}=+325$mV where $E_{m8.0}$ is the midpoint potential measured at pH8. This is close to that of the pure protein ($E'_0=+350$mV). Because the

signal of the protein HIPIP is very sharp, the gain setting of the spectrometer used to obtain the spectra in Fig. 1 is one-tenth of that used in the other spectra. In Fig. 1 (trace 2) a light-induced free-radical signal (g=2.0) is observed. This signal is probably due to the reaction-centre bacteriochlorophyll, which becomes oxidized on illumination.

If the spectrum of chromatophores kept in the dark or in the presence of an electron donor is examined below $g = 2.00$ at a higher gain a signal at $g = 1.90$ is also observed (Fig. 2, trace 1). This signal disappears on illumination (Fig. 2, trace 2) in the absence of an electron donor. In the presence of ascorbate and dichlorophenol-indophenol or dithionite the signal is unaffected by illumination (Fig. 3). Oxidation–reduction titration shows that the signal disappears on oxidation. The titration curve for this signal fits the theoretical curve for a one-electron-accepting centre with a potential of +285mV.

Fig. 3 shows the spectrum of the chromatophores in the presence of electron donors at 25°K. These spectra show a signal at $g = 1.94$ characteristic of iron–sulphur proteins of the ferredoxin type. In the presence of dithionite (Fig. 3) this signal is large and it is unaffected by illumination. When the electron donor is ascorbate or dichlorophenol–indophenol (Fig. 3, traces 1 and 2 respectively), the signal is only present if the chromatophores are illuminated before they are frozen, and this light-induced signal is smaller than the dithionite-induced signal. Oxidation–reduction-potential titration (Fig. 4) shows that in fact two electron-accepting centres with signals at $g = 1.94$ are present with different midpoint potentials, $E_{m8.0}$=−50mV and $E_{m8.0}$=−290mV. Fig. 5 shows the effect of temperatures on the signal size, at constant power, of samples at −156mV and −411mV. This confirms that two different centres are involved, the maximum signal size being observed at 25–30°K for the high-potential ferredoxin, whereas the signal size of the low-potential ferredoxin increases to the lowest temperatures used. The difference between the two samples shows the contribution of the low-potential component. The potential–titration curves

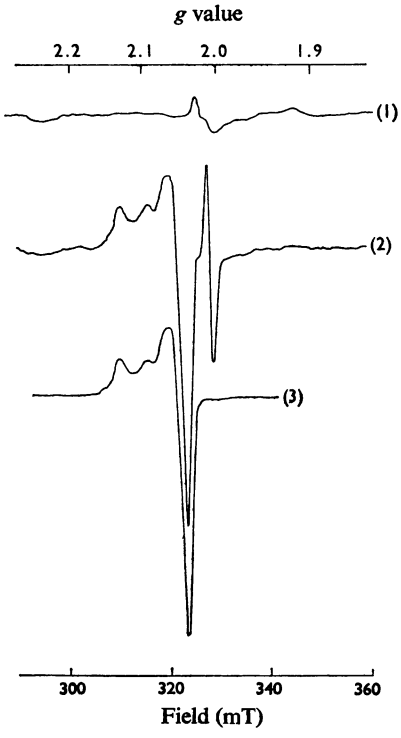

Fig. 1. *Low-temperature e.p.r. spectra of Chromatium chromatophores (3.5 mg of bacteriochlorophyll/ml) showing the photo-oxidation of the protein HIPIP*

Trace (1), sample prepared and frozen in the dark; trace (2), sample illuminated for 3min and frozen in the light (4×10^{-3} J/cm² per s); trace (3), e.p.r. spectrum of oxidized protein HIPIP from *Chromatium* (0.03mM). The spectra were recorded at 18°K with the following instrument settings: frequency 9.26GHz; power 20mW; modulation amplitudes 1mT; scan rate 100mT/min and gain 250.

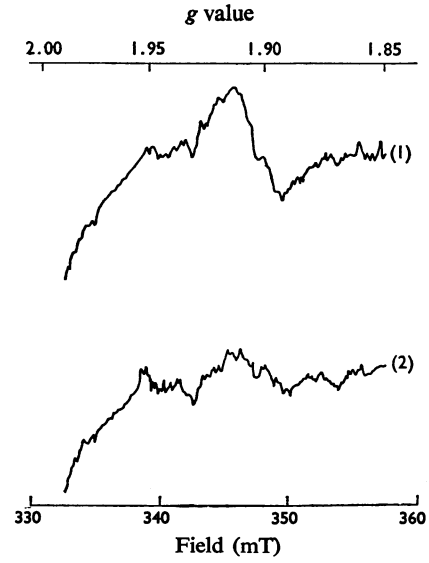

Fig. 2. *Low-temperature e.p.r. spectra of Chromatium chromatophores (3.5 mg of bacteriochlorophyll/ml) showing the photo-oxidation of the component with an e.p.r. signal at* g=1.90

Trace (1), sample prepared and frozen in the dark; trace (2), sample illuminated for 3min (4×10^{-3} J/cm² per s) and frozen in the light. The spectra were recorded as in Fig. 1 except that the gain was 2500 and the scan rate 50mT/min.

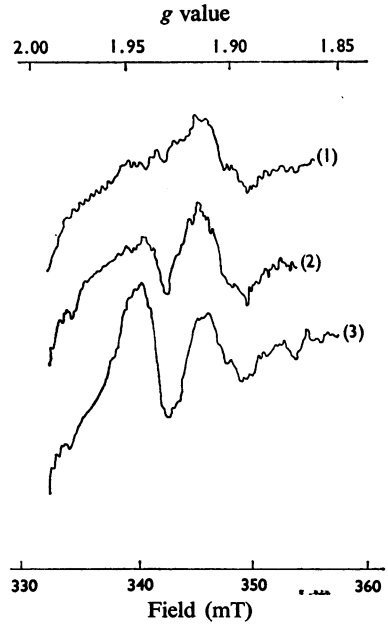

Fig. 3. *Effect of electron donors on the e.p.r. spectrum of Chromatium chromatophores*

Trace (1), chromatophores (3.5mg of bacteriochlorophyll/ml) with 20mM-sodium ascorbate and 0.1mM-dichlorophenol-indophenol frozen in the dark; trace (2), chromatophores as in trace (1) but illuminated (4×10^{-3} J/cm^2 per s) for 3min at room temperature, and frozen in the light; trace (3), chromatophores with 20mM-sodium dithionite in 100mM-Tris–HCl, pH8.0, frozen in the dark. The spectra were recorded as in Fig. 2.

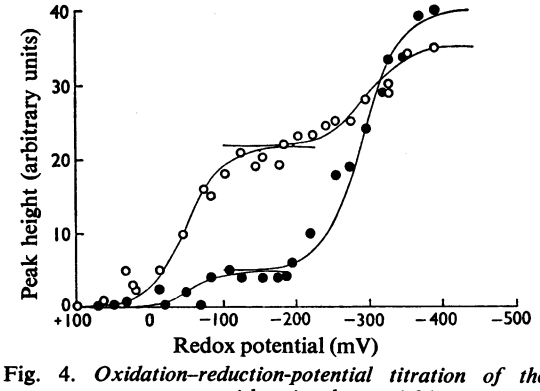

Fig. 4. *Oxidation–reduction-potential titration of the component with a signal at $g=1.94$*

Measurements were made at two temperatures: ○, 20°K; ●, 8°K. The titrations were carried out as described in the Materials and Methods section. The spectra were recorded as in Fig. 2, and the peak height of the signal taken as a measure of the signal size. The experimental points are plotted. The curves drawn are the theoretical curves for a one-electron-accepting centre with the observed midpoint potential.

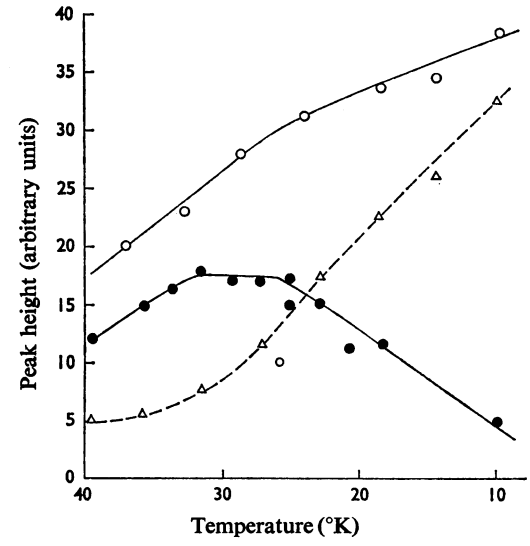

Fig. 5. *Temperature-dependence of the two components with signals at $g=1.94$*

●, Sample poised at −156mV; ○, sample poised at −411mV; △, difference. The spectra were recorded as in Fig. 2 and the peak height taken as a measure of the signal size.

for both of these components fit the theoretical curve for single electron acceptors. Neither of these components is reduced by succinate; it is therefore unlikely that either is a component of succinate dehydrogenase.

The signal induced on illumination in the presence of ascorbate and dichlorophenol-indophenol shows a temperature-dependence similar to that of the high-potential ferredoxin; however, the optimum is very broad and there may be a small contribution from the low-potential component. The photoreduction of the $g=1.94$ component is not inhibited by the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone, or by the electron-transport inhibitors hydroxyquinoline *N*-oxide or *o*-phenanthroline.

At very low temperatures (6–10°K; Fig. 6) two other signals are observed in dithionite-reduced samples. One of these, centred at $g=1.82$, corresponds to that reported by Leigh & Dutton (1972); the other, at $g=1.98$ and $g=2.04$, which is only observed in samples that have been frozen under illumination, has not previously been reported. The $g=1.82$ signal is observed in dithionite-reduced samples prepared in the light or dark; however, with ascorbate and dichlorophenol-indophenol as electron donor it is seen only in illuminated examples. Leigh & Dutton (1972) reported that this signal is induced on illumination of reaction centres at 10°K in samples poised at around 0mV before freezing. We have also

observed the low-temperature photoreduction of this component in chromatophore preparations, poised around 0mV. However, the signal induced by illumination at 8°K in our experiments is very small. This is probably in part due to the relatively low concentration of reaction centres in chromatophores, and in part to difficulties in maintaining very low temperatures in our apparatus during illumination when the light-source raises the temperature of the samples. The signal disappears when the light is turned off, showing that the reaction is reversible. Oxidation–reduction-potential titration (Fig. 7) shows that the component with a signal at $g = 1.82$ has $E_{m8.0} = -135$mV, and the titration curve fits a theoretical curve for a one-electron acceptor. If the titration is carried out in the presence of *o*-phenanthroline, which inhibits the transfer of electrons from the primary acceptor to secondary acceptors in *Chromatium* (Parson & Case, 1970), the $E_{m8.0} = +50$mV, and the shape of the curve is unaffected, indicating that it is still a one-electron acceptor. At pH6.5 the midpoint potential is also shifted to a more oxidized value ($E_{m6.5} = +12$mV), a shift of +98mV/pH unit.

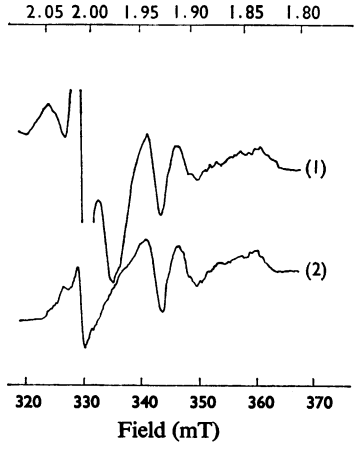

Fig. 6. *E.p.r. spectra of Chromatium chromatophores at* 8°K

Trace (1), sample frozen under illumination in the presence of 20mM-sodium dithionite in 100mM-Tris–HCl buffer at pH8.0; trace (2), sample as in trace (1) but frozen in the dark. The spectra were recorded as in Fig. 2, but the temperature was 8°K.

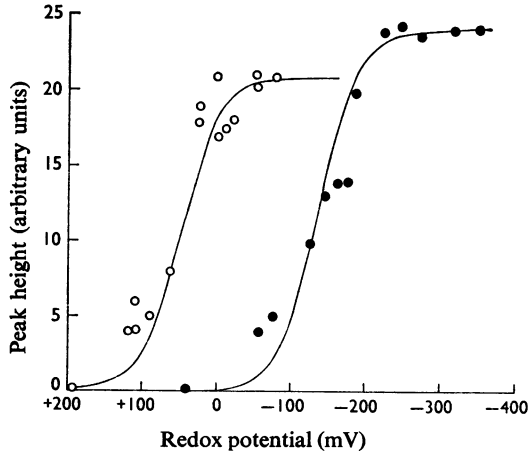

Fig. 7. *Oxidation–reduction-potential titration of the component with a signal at* $g = 1.82$, *in the presence and absence of o-phenanthroline*

●, Chromatophores with no *o*-phenanthroline; ○, chromatophores plus 2mM-*o*-phenanthroline. The experimental procedure was as in Fig. 4.

The other signal observed in illuminated samples at $g = 1.98$ and $g = 2.04$ (Fig. 6) has proved extremely difficult to study. The signal is observed only in samples illuminated in the presence of an electron donor and it is larger if dithionite is the donor than with reduced dichlorophenol-indophenol. The signal decays very rapidly in the dark at room temperature; samples frozen immediately after turning off the light do not have the signal, and rapid-freezing techniques would be required to follow its decay. It also decays at liquid N_2 temperature with a half-life of about 24h; this decay is accompanied by a decrease in the size of the free-radical signal ($g = 2.0$) also seen in these spectra. The requirements for an electron donor to obtain the signal suggests that it is due to the reduction of a chromatophore component; however, we have been unable to obtain the signal by chemical reduction in the presence of excess of dithionite and a mediating dye with $E_0 = -650$mV or during potential titration over a wide range. We have not observed any photoreduction of this component at low temperatures.

Discussion

The results that we have obtained show that the photosynthetic lamellae of the purple photosynthetic bacterium *Chromatium* contain a number of e.p.r.-detectable electron-transport components, and that the steady-state redox condition of some of these components is affected by light. Table 1 summarizes the information we have obtained about the midpoint potentials and the effect of illumination on these components. The nature of the experiments, in which electron transport occurs at room temperature although the measurement is made at liquid-helium temperature, means that no kinetic studies can be made; the effects of illumination provide an indication of the possible function of the components.

Table 1. *Midpoint potentials of e.p.r.-detectable components in Chromatium chromatophores*

g value	Type of component	Condition for oxidation or reduction	Condition of measurement	E_m
2.04	High-potential iron protein	Oxidized on illumination at room temperature. Chemically reduced and oxidized	pH8.0	+325mV
1.90	Probably iron–sulphur protein	Oxidized on illumination at room temperature. Chemically reduced and oxidized	pH8.0	+285mV
1.94	Probably iron–sulphur protein	Reduced on illumination at room temperature. Chemically oxidized or reduced	pH8.0 pH8.0+*o*-phenanthroline	−50mV −50mV
1.82	Probably iron protein	Reversibly reduced on illumination at 8°K. Chemically oxidized or reduced	pH8.0 pH8.0+*o*-phenanthroline pH6.5	−135mV +50mV +12mV
1.94	Probably iron–sulphur protein	Chemically oxidized or reduced	pH8.0 pH8.0+*o*-phenanthroline	−290mV −290mV
1.98 and 2.04	Unknown	Reduced on illumination at room temperature in presence of electron donor	—	—

One of these components, with a signal at $g = 2.04$ in the oxidized state can be identified with the well-characterized soluble protein HIPIP. This and a component with a signal at $g = 1.90$ have high potentials and are oxidized on illumination. It seems likely that they are involved in an electron-transport chain which donates electrons to the photochemical system through cytochrome c_{555}. Although the protein HIPIP was first discovered by Bartsch (1963), who showed that chromatophores oxidized added protein HIPIP, there has not previously been any direct evidence for its presence in chromatophores or involvement in electron transport. J. S. Leigh & P. L. Dutton (personal communication) have recently obtained similar results indicating the involvement of the protein HIPIP in chromatophore electron transport. The properties of the $g = 1.90$ component suggest that it is an iron–sulphur protein. It has the most oxidized midpoint potential of any protein of this type so far identified. It suggests that this group of proteins has as wide a range of redox potentials as the cytochromes.

The nature of the primary electron acceptor for the photochemical system in photosynthetic bacteria has been the subject of much speculation. Leigh & Dutton (1972) showed that a component with an e.p.r. signal at $g = 1.82$ was photoreduced at liquid-helium temperatures, in preparations of reaction centres. We have confirmed their experiments showing that this component can be detected in chromatophores and is photoreduced at low temperature. We have also measured its midpoint potential $E_{m8.0} = -135$mV, and have shown that this agrees very closely with the potential measured indirectly by a number of workers who have followed the potential-dependence of cytochrome photo-oxidation (Cusanovich *et al.*, 1968; Dutton, 1971; Case & Parson, 1971). We have also shown that the redox potential of this component is sensitive to pH and to *o*-phenanthroline. These results agree with those of Jackson *et al.* (1973) obtained by indirect measurement, and provide good evidence to support the suggestion that the $g = 1.82$ component is the primary electron acceptor in chromatophores. The pH dependence of the midpoint potential of the $g = 1.82$ component suggests that a proton is involved directly in its reduction. However, the results of Evans & Crofts (1973) show that the first detectable proton uptake during electron flow in chromatophores is associated with the reduction of the secondary electron acceptor ubiquinone. The effect of pH on the primary acceptor must therefore be indirect.

Two other e.p.r.-detectable components, ubiquinone (Feher *et al.*, 1972) and an unidentified free radical (Loach & Hall, 1972) have been observed to undergo photoreduction at low temperatures in reaction centres. These components would not be seen in our experiments, as they were only observed after removal of most of the electron-transport components from the chromatophores. We cannot therefore exclude their involvement in the primary electron-acceptor complex. However, the conditions used to prepare reaction centres in which they are observed could possibly disrupt the normal electron-transfer sequence. Our results show that in untreated membranes the $g = 1.82$ component has the properties expected for the primary electron acceptor. The chemical composition of this centre is unknown but

its reaction with *o*-phenanthroline suggests that it may be an iron compound.

The two components with signals at $g = 1.94$ in the reduced state are presumably iron–sulphur proteins, probably of the ferredoxin type, although we have not been able to observe other g values expected in ferredoxin spectra. The high-potential component ($E_{m8.0} = -50$mV) has a midpoint potential that would suggest that it might function as a secondary electron carrier accepting electrons from the primary acceptor. However, it is photoreduced in the presence of *o*-phenanthroline when its midpoint potential is lower than that of the primary acceptor. The low-potential $g = 1.94$ component has a midpoint potential ($E_{m8.0} = -290$mV) considerably lower than that of the primary acceptor, which has a signal at $g = 1.82$. Although it might be expected that this component could be involved in electron transport to NAD^+, we have no evidence for any light-dependent reduction of the low-potential iron–sulphur proteins. We have not detected a ferredoxin in chromatophores with midpoint potential as low as that of the soluble ferredoxin from *Chromatium* (−490mV; Tagawa & Arnon, 1968). J. S. Leigh & P. L. Dutton (personal communication) have shown the presence of the high-potential $g = 1.94$ component in their preparations but had not investigated the possible occurrence of a lower-potential component.

The $g = 1.98$ signal cannot at present be identified. It has some of the properties that would be expected in the primary electron acceptor of a photochemical system; it is observed only in illuminated samples and decays rapidly in the dark. It decays at liquid-N_2 temperature, and this decay is accompanied by the disappearance of a free radical which may represent the oxidized chlorophyll of the reaction centre. Its properties suggest that it may have a very low potential and that it is very inaccessible to added reagents, presumably being in a highly lipophilic region of the membrane. However, as we have not observed its reduction at low temperature and we have been unable to measure its oxidation–reduction potential, we cannot define its function.

Our results show that the component with an e.p.r. signal at $g = 1.82$ has the properties of the primary electron acceptor of the photochemical system of the cyclic electron-transport system in *Chromatium*, and that the protein HIPIP and the component with an e.p.r. signal at $g = 1.90$ are involved in electron transfer to a photochemical system. We have also shown that very-low-potential components are present in the electron-transport system. However, the mechanisms by which these components are reduced is unclear.

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