A thermodynamic analysis of the plasma membrane electron transport components in photoheterotrophically grown cells of *Chloroflexus aurantiacus*

An optical and electron paramagnetic resonance study

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The thermodynamic and spectroscopic properties of the membrane bound iron-sulphur centers and cytochromes in phototrophically grown cells of the thermophilic facultative photosynthetic green bacterium *Chloroflexus aurantiacus* have been examined. The present study shows that *Chloroflexus* contains several cytochromes of *b* and *c* type, along with multiple ferredoxin-like centers with resonances centered at $g = 1.93$, $g = 2.017$ (HiPIP-like center) and $g = 1.90$ (Rieske-type protein). The $g = 1.90$ centre appears to be actively involved in photooxidative reactions. These observations suggest the presence of a previously undetected cytochrome b/c_1 complex in this bacterium.

Cytochrome b *Cytochrome* c *Iron-sulfur center* *Potentiometry* *Electron transport* *Thermophilic bacterium* (Chloroflexus aurantiacus)

1. INTRODUCTION

Chloroflexus aurantiacus, a thermophilic photosynthetic green bacterium, is capable of photoheterotrophy, photoautotrophy and chemoheterotrophy [1], and in this respect resembles the family of purple non-sulphur bacteria, the Rhodospirillaceae. As with all green bacteria, *Chloroflexus* contains chlorosomes, which are oblong bodies in close association with the inner surface of the cytoplasmic membrane [2] and include all of the bacteriochlorophyll *c* (Bchl *c*) within the cell. *Chloroflexus* also contains BChl *a* which functions as antenna bacteriochlorophyll (B800–850) and reaction centre (RC-BChl). Thermodynamically, the RC-BChl of *Chloroflexus* is different from that found in either the green or purple bacteria, with a mid-point potential ($E_{m,8.1} = 360$ mV) nearly halfway between the 2 bacterial groups ($E_{m,7.0}$ of 450 and 240 mV for purple and green bacteria, respectively) [3,4], Conversely, the 2-quinone primary acceptor system has an $E_{m,8.1}$ of -50 mV [3,5,6] which is quite different from that found in *Chlorobium* ($E_{m,7.0} = -550$ mV) [7] but very similar to the primary acceptor of all purple bacteria studied to date [4]. Reduced-minus-oxidized cytochrome (cyt.) difference spectra of light-grown cells of *Chloroflexus* revealed the presence of a cyt. *c* (α-peak at 554 nm, $E_{m,8.1} = 260$ mV) thought to be analogous to the high-potential cyt. *c*-555 found in *Chromatium* [8], with no evidence of *b*-type cytochromes (but see also [9]). Thus, except for cyt. *c*-554, no information is yet available about other membrane-bound redox components which are presumably involved in delivering electrons from the primary acceptor system to the photochemical reaction centre.

It was the aim of this study to determine what

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types of cytochromes and iron-sulphur centres were present in membranes from phototrophically grown cells of *C. aurantiacus* and to examine the thermodynamic and spectroscopic properties of these electron transport components.

2. MATERIALS AND METHODS

2.1. *Organism and cultivation*

The medium used for phototrophic growth of *C. aurantiacus* strain J-10-f1 was that described by Pierson and Castenholz [10]. Phototrophic cells were cultivated in a 14-l fermentor (Microferm; New Brunswick Scientific, NJ) at 55°C with an incident light intensity of 2000 $W \cdot m^{-2}$ for 20 h with stirring at 400 rpm.

2.2. *Membrane isolation*

The harvested cells were suspended in 50 mM Tris-HCl buffer, pH 8.2, to a concentration of 0.2–0.3 g/ml (wet wt) and passed through a precooled (5°C) French pressure cell at 2×10^6 Pa. Whole cells and debris were removed from the crude extract by centrifugation at $20\,000 \times g$ for 20 min. The clear supernatant was centrifuged at $106\,000 \times g$ for 90 min. The sediment was washed once and used at about 30–40 mg/ml of proteins.

2.3. *Optical spectroscopy*

Pigment absorption spectra and reduced-minus-oxidized difference spectra of cytochromes were obtained at 25°C with a Jasco Uvidec-610 UV/VIS spectrophotometer. Dark equilibrium potentiometric titrations were performed on a dual-wavelength spectrophotometer (Sigma ZWS-II) according to Dutton and Jackson [11]. All titrations were performed in a medium containing 50 mM KCl and 100 mM Hepes buffer, pH 7.0. E_0' values were assigned on the basis of a computer-assisted analysis, as in [11].

2.4. *EPR spectroscopy*

EPR spectra were obtained using a Bruker 200 D EPR spectrometer (Bruker Analyt. Messtechnik, Forchleim, FRG) equipped with a cryostat (Oxford Instruments, Oxford, England) and a liquid-helium transfer system. The temperature was measured with a termocouple.

2.5. *Protein and pigment determinations*

Proteins were assayed by using the method of Lowry et al. [12]. BChl *a* and *c* concentrations were measured in acetone/methanol extracts (7:2, v/v) at 769 and 666 nm using extinction coefficients of 68.6 and 74 $mM^{-1} \cdot cm^{-1}$, respectively [13].

3. RESULTS

3.1. *Optical spectroscopy*

As previously reported, the cellular amount of BChl *c* in *C. aurantiacus* decreases up to 20-fold as the light intensity is increased, whereas the BChl *a* content decreases only slightly [13]. Since the BChl *a* (B800–850 plus RC-BChl) is associated with the cytoplasmic membrane, cells cultivated at extremely high light intensity, i.e. 2000–3000 $W \cdot m^{-2}$, are relatively enriched in cytoplasmic membrane [13]. As a consequence, the 'whole membrane fraction' which can readily be isolated by differential centrifugation (see section 2) is mainly composed of cytoplasmic membrane proteins (presumably involved in electron transport reactions) with little contamination by chlorosome membrane proteins. The membrane fragments routinely used here contained a BChl *c*/BChl *a* ratio of about 0.5–0.8 on a molar basis.

As shown in fig.1 (inset) membrane fragments from high-light intensity grown cells of *Chloroflexus* contain cytochromes of *b* and *c* type (α-bands at 552, 554 and 562 nm for cyt. *c* and cyt. *b*, respectively). No evidence for *a*-type cytochromes was present. The absorption spectrum of the CO-plus-dithionite minus dithionite-reduced membrane fraction had a typical pattern of peaks and troughs for a cyt. *c*-CO complex (troughs at 552–553 and 423 nm and a peak at 414–415 nm) (not shown). No indication for CO-binding cyt. *b* was obtained. Dark potentiometric titrations of such membrane preparations at 562–575 nm revealed the presence of both high- and low-potential *b*-type hemes ($E_{m,7.0}$ of $+240 \pm 5$, 160 ± 2, 65 ± 2 and -70 ± 2 mV). Each component fitted closely to theoretical single-electron equivalent ($n = 1$) curve. The $E_{m,7.0}$ values for these apparent *b*-type cytochromes were calculated from the results of several titrations and their relative contributions to the total absorption change at

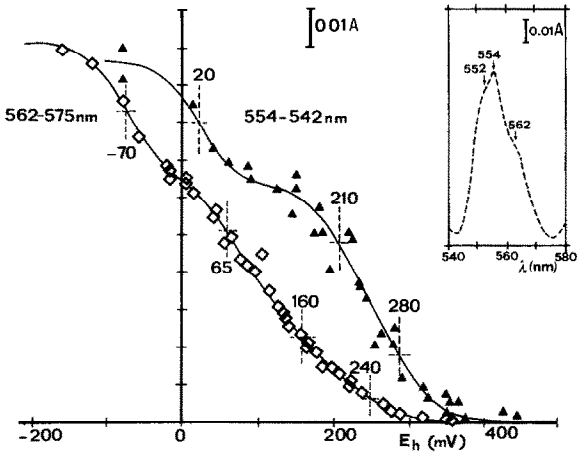

Fig.1. Dark equilibrium potentiometric titrations and reduced-minus-oxidized spectrum of membranes from photoheterotrophically grown *C. aurantiacus*. Redox titrations at 554–542 nm (▲——▲) and 562–575 nm (◇——◇) are shown. Mid-point potentials from best-fit procedures are indicated. In (□——□) and (▲——▲), the protein concentrations were about 5.7 and 3.4 mg/ml, respectively. (Inset) Dithionite-reduced minus ferricyanide-oxidized difference spectrum of membranes suspended at a protein concentration of 2,8 mg/ml. Spectrophotometer settings were as follows: *t*,25°C; bandwidth, 1nm; time constant, 1 s; scanning speed, 20 nm/min.

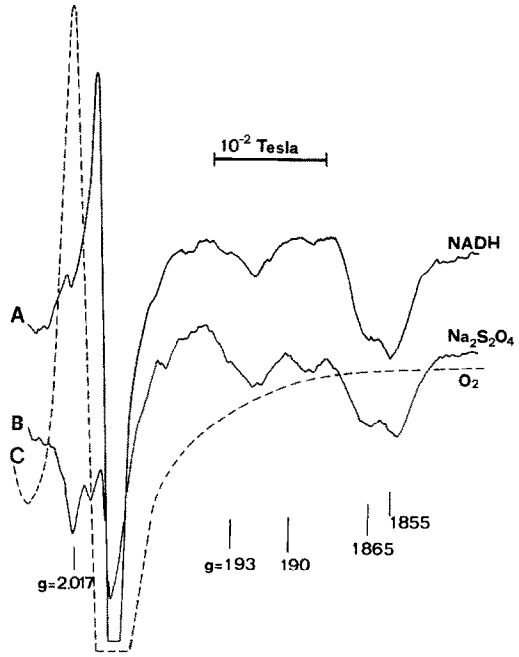

Fig.2. EPR spectra of membranes from *C. aurantiacus*. Membrane samples were NADH-reduced (A), dithionite-reduced (B) or oxidized (C). Spectra were taken at 14 K. The concentration of proteins was 38 mg/ml. EPR conditions: microwave power, 22 mW; modulation amplitude, 10^{-3} T; microwave frequency, 9.49 GHz; modulation frequency, 100 kHz.

562–575 nm were approx. 11,20,31 and 38% for *b*240, *b*160, *b*65 and *b*-70, respectively.

With regard to the cytochrome(s) *c*, the results of titrations at 554–542 nm could be fitted most readily to a 3-component Nernst curve ($n=1$), to which were assigned $E_{m,7.0}$ values of $+280\pm5$, 210 ± 2 and 20 ± 4 mV. Their relative contributions to the total absorbance signal at 554–542 nm were approx. 32,31 and 37%, respectively.

3.2. *EPR analysis*

EPR spectra of *C. aurantiacus* membranes either oxidized, NADH-reduced (anaerobic) or dithionite-reduced, are shown in fig.2. A number of overlapping lines attributable to iron-sulphur centres are seen after reduction with these reducing agents. The major resonances are centred at $g=1.93$, $g=1.865$ and $g=1.855$. The last 2 signals are considerably more intense after reduction by NADH than by dithionite in contrast to the first which is better seen with dithionite. In both preparations an additional signal is observed at $g=1.90$, together with other less well-resolved components at higher field values. When spectra obtained over a range of temperatures were examined (not shown) it was observed that: (a) the $g=1.90$ resonance was seen only at >10 K; (b) the $g=1.865$ and $g=1.855$ signals were seen at <15 K; (c) the line shape around the $g=1.93$ centre was different when comparing the dithionite-reduced sample with the NADH-reduced sample and (d) the ratio of the signal amplitudes of the NADH- and dithionite-reduced samples at $g=1.93$ was different at temperatures >15 K compared to temperatures <10 K. Points (c) and (d) suggest the presence of two $g=1.93$ centres, one being substrate reducible (NADH), best observed at the higher temperatures and heavily saturated at <10 K (microwave power 22 mW), and the other not being NADH reducible but dithionite reducible and best observable at low temperatures. Studies of the temperature dependence of the respective

spectra and the analysis of redox titrations (see below, fig.3), which resolved the $g = 1.93$ centre into 2 components, confirmed this suggestion.

In the oxidized sample at 14 K of fig.2 a large EPR signal with a peak at $g = 2.017$ was observed. This centre was similar to centres described in succinate dehydrogenase, fumarate reductases and some other enzymes such as aconitase, thought to be due to a 4 or 3 iron-sulphide cluster which is paramagnetic in its physiologically oxidized form (designated HiPIP). The potentiometric analysis of the EPR-detectable signals at $g = 1.93$, $g = 1.90$ and $g = 2.017$ in *Chloroflexus* membrane fragments is shown in fig.3. Curves a–c show the results at 3 temperatures of a redox titration of the $g = 1.93$ signal. Curves e and d show the behaviour of the HiPIP ($g = 2.017$) centre at 14 K and the $g = 1.90$ signal at 40 K, respectively. It is apparent that the $g = 1.93$ ferredoxin titrates biphasically suggesting the contribution of 2 components with $E_{m,7.0}$ of -50 and -320 mV. The centre with $E_{m,7.0}$ of -50 mV makes a relatively large contribution at 40 K in contrast to the -320 mV centre which is optimally

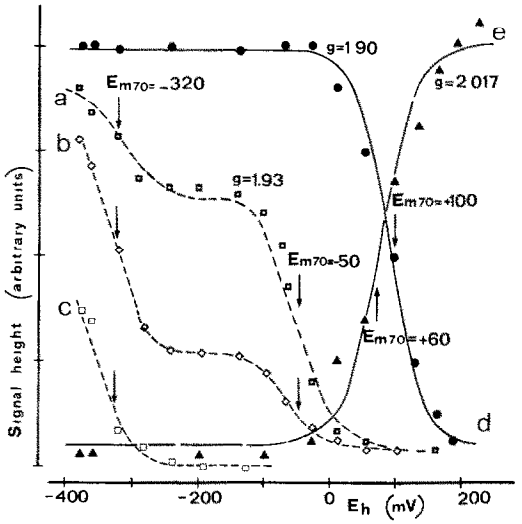

Fig.3. Analysis of oxidation-reduction titrations of the $g = 1.93$, $g = 1.90$ and $g = 2.017$ centres in membranes from *C. aurantiacus*. The curves are drawn for $n = 1$ oxidation-reduction reactions. Mid-point potentials at pH 7.0 are indicated by the arrows. The titration of the $g = 1.93$ signal is shown from spectra taken at 40 K (a), 14 K (b) and 5 K (c). The Rieske data (d) ($g = 1.90$) were obtained from 40 K spectra while the $g = 2.017$ data (e) are from spectra at 14 K.

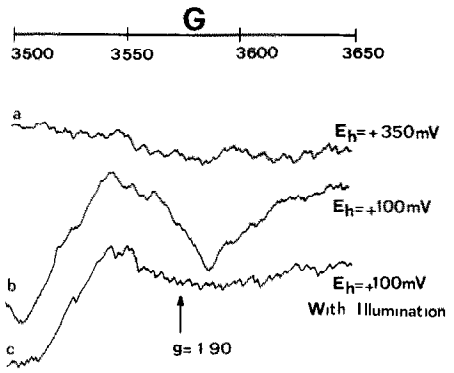

Fig.4. The light effect on the oxidation-reduction state of the $g = 1.90$ signal of *C. aurantiacus* membrane fragments. Membranes were suspended to 35 mg protein/ml in 50 mM KCl, 100 mM Hepes buffer (pH 7.0), 2 μM phenazine methosulphate (PMS), 20 μM phenazine ethosulphate (PES), 30 μM diaminodurene (DAD), and the samples taken and rapidly frozen in the dark at the potentials indicated (samples a and b). A sample (indicated as c) was taken at 100 mV and illuminated for 30 s under controlled ambient redox potential (light intensity 500 W · m^{-2}), prior to freezing. Spectrometer settings: temperature, 22 K; microwave power, 10 mW.

observed at lower temperature. The $g = 2.017$ and $g = 1.90$ signals titrate with $E_{m,7.0}$ of 55 and 100 mV, respectively. The latter resonance was therefore similar to those of the Rieske iron-sulphur centres observed in other bacterial and mitochondrial systems in terms of its relatively high E_0', g values and temperature dependence [14,15]. The light effect on the oxidation-reduction state of the $g = 1.90$ signal in a sample poised at 100 mV (Rieske centre 50% reduced) is shown in fig.4. The lower trace indicates that the $g = 1.90$ centre can be photo-oxidized if the membrane fragments are illuminated briefly immediately prior to freezing (30 s, light intensity 500 W · m^{-2}). We may thus assign a functional role to the $g = 1.90$ centre in photosynthetic electron flow.

4. DISCUSSION AND CONCLUSIONS

In this study we have shown that at least 4 *b*-type cytochromes ($E_{m,7.0}$ values of 240, 160, 65 and -70 mV) and 3 *c*-type cytochromes ($E_{m,7.0}$ values of 280, 210 and 20 mV) characterize the membrane-bound cytochrome complement of photoheterotrophically grown *C. aurantiacus*. At

present, except for cyt. *c*280 thought to be homologous to cyt. *c*-554 ($E_{m,8.1}$ = 260 mV) [3,16], the actual role of these cytochromes is largely obscure although for many of them tentative conclusions can be made by analogy with equivalent components found in either purple or green bacteria. Indeed, if we assume that cyt. *c*280 is the direct electron donor to the RC-BChl (see [3]) the similarity between cyt. *c*210 and cyt. c_1 of other organisms, e.g. *Chromatium* [17], is compelling. In *Chloroflexus,* cyt. *c*210 might operate in mediating electron flow between the Rieske centre ($E_{m,7.0}$ = 100 mV) (discussed below) and cyt. *c*280. Additional similarities between *Chloroflexus* and purple sulphur bacteria are found in the presence of a low-potential ($E_{m,7.0}$ = 20 mV) membrane-bound cyt. *c*, i.e. *Chromatium* cyt. *c*-552, $E_{m,7.0}$ = 10 mV [18]). *Chromatium* cyt. *c*-552 has a molecular mass of 72 kDa, is readily auto-oxidizable and can combine with CO [19]. The recent finding that membranes of *C. aurantiacus* contain a haem-carrying peptide of 79 kDa [16], along with our evidence that this bacterial species contains a typical cyt. *c*-binding CO activity, seems to stress the analogy between cyt. *c*-552 of *Chromatium* and cyt. *c*20 of *Chloroflexus.* A noteworthy observation reported above is the detection of 2 *b*-type haems with $E_{m,7.0}$ values of 65 and −70 mV. These values correspond to those of cyt. *b*-562 and cyt. *b*-566, respectively, the 2 haems associated with mitochondrial and bacterial cyt. b/c_1 complexes [20]. The presence of the cyt. *b*-type elements of a cyt. b/c_1 is also suggested by the observation (Zannoni et al., in preparation) that electron flow in *C. aurantiacus* is sensitive to antimycin A and myxothiazol, 2 specific inhibitors of electron flow through such complexes [20].

C. aurantiacus membranes contain 2 $g = 1.93$ centres distinguishable by temperature dependence and mid-point potential (−50 and −320 mV), a HiPIP-type centre ($E_{m,7.0}$ = 55 mV), and a Rieske iron-sulphur centre ($E_{m,7.0}$ = 100 mV). Additional centres at $g = 1.855$ and $g = 1.865$, although present, have not been examined in the present study.

The $g = 1.93$ iron-sulphur centres plus the HiPIP centre could be tentatively assigned to succinic dehydrogenase since all known succinate dehydrogenases contain 2 $g = 1.93$ centres and a HiPIP centre with E_0' close to those reported here [15]. An additional similarity between these centres and those of known succinate dehydrogenases is the difference in temperature dependence between the substrate-reducible $g = 1.93$ centre ($E_{m,7.0}$ = −50 mV) and the dithionite-reducible centre ($E_{m,7.0}$ = −320 mV).

The $g = 1.90$ (Rieske-type protein) centre in *Chloroflexus* membranes has a mid-point potential of 100 mV (at pH 7.0). This value is approx. 60 mV more negative than thevalue reported for the $g = 1.90$ centre in *Chlorobium* [21] and considerably more negative than the value reported for the the centre in purple sulphur [22] and purple non-sulphur bacteria [23]. Nevertheless the E_0' of the $g = 1.90$ centre in *Chloroflexus* is consistent with this component functioning as an electron carrier that donates electrons to the RC-BChl ($E_{m,8.1}$ = 360 mV) through cyt. *c*210 and cyt. *c*280. Our finding that the $g = 1.90$ centre can be photo-oxidized under steady-state illumination at controlled ambient redox potential (shown in fig.4) provides a clear indication of its role in photosynthetic electron transport.

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REFERENCES

[1] Pierson, B.K. and Castenholz, R.W. (1978) in: The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R. ed) pp. 179–197, Plenum, New York.
[2] Staehelin, L.A., Golecki, J.R., Fuller, R.C. and Drews, G. (1978) Arch. Microbiol. 119, 269–277.
[3] Bruce, B.D., Fuller, R.C. and Blankenship, R.E. (1982) Proc. Natl. Acad. Sci. USA 79, 6532–6536.
[4] Parson, W.W. (1978) in: The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R. eds) pp. 455–469, Plenum Press New York.
[5] Hale, M.B., Blankenship, R.E. and Fuller, R.C. (1983) Biochim. Biophys. Acta 723, 376–382.
[6] Mancino, L.J., Hansen, P.L., Stark, R.E. and Blankenship, R.E. (1985) Biophys. J. M-AM-A1.

[7] Olson, J.M., Prince, R.C. and Brune, D.C. (1977) Brookhaven Symp. Biol. 28, 238–248.
[8] Bartsch, R.G. (1978) in: The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R. eds) pp. 249–279, Plenum, New York.
[9] Pierson, B.K. (1979) Proceedings of the Third International Symposium on Photosynthetic Prokaryotes, B40, Oxford, England.
[10] Pierson, B.K. and Castenholz, R.W. (1974) Arch. Microbiol. 100, 5–24.
[11] Dutton, P.L. and Jackson, J.B. (1972) Eur. J. Biochem. 30, 495–510.
[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[13] Feick, R.G., Fitzpatrick, M. and Fuller, R.C. (1982) J. Bacteriol. 150, 905–911.
[14] Rieske, J.S., Zang, W.S. and Hansen, R.E. (1964) J. Biol. Chem. 239, 3023–3036.
[15] Malkin, R. and Bearden, A.J. (1978) Biochim. Biophys. Acta 505, 147–181.
[16] Blankenship, R.E., Huynh, P., Gabrielson, H. and Mancino, L.J. (1985) Biophys. J., M-Am-A2.
[17] Gaul, D.F. and Knaff, D.B. (1978) FEBS Lett. 163, 69–75.
[18] Cusanovich, M.A., Bartsch, R.G. and Kamen, M.D. (1968) Biochim. Biophys. Acta 153, 397–417.
[19] Van Holde, K.E. and Baldwin, R.L. (1958) J. Phys. Chem. 62, 734–743.
[20] Cramer, W.A. and Crofts, A.R. (1982) in: Energy Conversion by Plants and Bacteria (Govindjee, ed.) Vol.1, pp. 387–467, Academic Press, New York.
[21] Knaff, D.B. and Malkin, R. (1976) Biochim. Biophys. Acta 430, 244–252.
[22] Dutton, P.L. and Leigh, J.S. (1973) Biochim. Biophys. Acta 314, 178–190.
[23] Prince, R.C., Leigh, J.S. and Dutton, P.L. (1974) Biochem. Soc. Trans, 2, 950–953.