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Thermodynamic properties of the photochemical reaction center of *Heliobacterium chlorum*

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The thermodynamic and spectral properties of the photochemical reaction center components of *Heliobacterium chlorum* have been examined. The primary electron donor bacteriochlorophyll has $E_{m,7} = +225$ mV, and the 'primary acceptor' $E_{m,10} = -510$ mV. The former has an EPR signal in its oxidised form near $G = 2.0025$, $\Delta H = 0.95$ mT, reminiscent of the properties of the primary donor in bacteria containing bacteriochlorophyll *a*. The 'primary acceptor' has properties similar to those of the iron-sulfur cluster acceptors of green sulfur bacteria. *H. chlorum* contains a *c*-type cytochrome ($E_{m,7} = +170$ mV) that donates electrons to the photooxidised primary donor with $t_{1/2} \approx 6$ ms. The reaction center of *H. chlorum* is thus very similar to that found in representative green sulfur bacteria, but the cellular architecture and photopigments of this group are quite distinct from those of *H. chlorum*.

Introduction

Heliobacterium chlorum is a recently isolated, strictly anaerobic photosynthetic bacterium that possesses the previously unknown bacteriochlorophyll *g* [1,2]. The organism has a variety of novel properties, and may be only distantly related to other photosynthetic bacteria; despite its green color, for which it is named, its light absorption properties and membrane architecture are quite distinct from those of the currently recognised families of Green bacteria, the Chlorobiaceae and the Chloroflexaceae [3,4]. *H. chlorum* has a previously unknown type of photochemical reaction center, with the most prominent light-induced bleaching occuring at 798 nm [4]. In this paper we examine the thermodynamic and electron paramagnetic resonance properties of some of the components involved in light-driven electron flow in this reaction center.

Materials and Methods

Heliobacterium chlorum (ATCC 35205) was grown at Indiana University in medium 112 as previously described [1]. After anaerobic harvesting, the cells were sealed in an anaerobic pack, frozen, and shipped to Exxon in Annandale, NJ. The frozen cells were thawed, and all subsequent preparative manipulations took place, inside an anaerobic hood ($O_2 < 0.4$ ppm). The cells were suspended and homogenised in 20 mM *N*-morpholinopropane sulfonate, 100 mM KCl, 1 mM ascorbate, 1 mM $MgCl_2$ (pH 7) and passed through a French pressure cell at 138 MPa. Unbroken cells and large fragments were removed by centrifugation at $15000 \times g$ for 10 min, and membranes pelleted by centrifugation at $144000 \times g$ for 75 min. The pellet was resuspended in anaerobic

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buffer (as above, minus the ascorbate). All solutions were rigorously anaerobic (degassed three times and purged with argon), and membranes were kept in the dark, because of the extreme lability of bacteriochlorophyll *g* [2]. These precautions seemed to result in an even lower concentration of degradation product absorbing at 670 nm than in previous preparations ($A_{670}/A_{788} = 0.25$, see Ref. 4).

Redox titrations and time-resolved optical spectroscopy were essentially as described by Dutton et al. [5]. The actinic flash had a 'full width at half height' of about 10 μs, and was filtered through a Kodak Wratten 88A filter. A Varian E109 spectrometer equipped with an Oxford Instruments flowing helium cryostat was used for EPR spectroscopy.

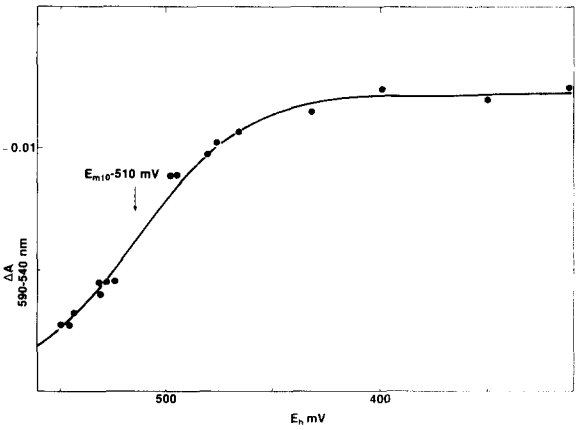

Fig. 2. Redox titration of the 'primary acceptor' of *H. chlorum*. Membranes were suspended as in Fig. 1, except that the buffer was 20 mM cyclohexylaminohydroxypropanesulfonate (pH 10.0). Photochemistry was monitored 0.6 ms after the flash at 590–540 nm.

Results

Membranes of *H. chlorum* exhibit a prominent light-induced bleaching at 798 nm, and a small increase in absorbance at 778 nm, which have been attributed to reaction center bacteriochlorophyll photo-oxidation [4]. Fig. 1 shows the spectral changes occurring in the visible region of the spectrum. These changes were measured under conditions such that reactants outside the reaction center, such as cytochromes, would not be expected to contribute, and were unaffected by the addition of valinomycin; the latter would have collapsed any membrane potential that might have caused electrochromic shifts of the pigments (see Ref. 6). Similar spectra were obtained at $E_h = +225$ and $E_h = -400$ mV. The split band near 600 nm (usually attributable to the Q_x transition of reaction center bacteriochlorophyll) is unusual; it is not seen in purple bacteria (e.g., *Rhodopseudomonas sphaeroides* [5]), or green bacteria (e.g., *Chlorobium limicola* f. *thiosulfatophilum* [7] and *Chloroflexus aurantiacus* [8,9]). The photobleaching at 570 nm is at the same wavelength as the Q_x band of the antenna bacteriochlorophyll *g* absorption (see Ref. 4). The 590 nm bleaching presuma-

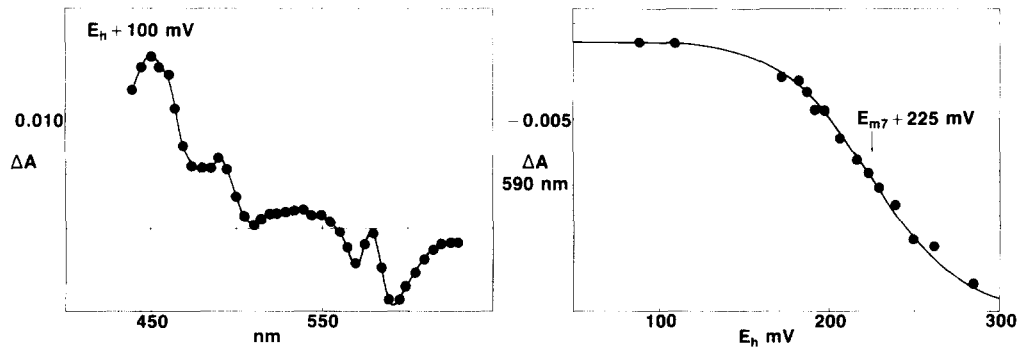

Fig. 1. Flash-induced absorption changes in *H. chlorum*. Membranes ($A_{788} = 3.0$) were suspended in 20 mM *N*-morpholinopropanesulfonate/100 mM KCl (pH 7.0) with 10 μM *N*-methylphenazonium methosulfate, 2,3,5,6- and *N,N,N',N'*-tetramethylphenylenediamines, 2-hydroxy-1,4-naphtho- and anthra-quinones, benzyl and methyl viologens and *o*-naphthoquinone-4-sulfonate. The spectrum (on the left) was measured 0.6 ms after the flash, at an E_h of +100 mV, and the redox titration (on the right) was monitored at 590 nm.

bly arises from a reaction center pigment that is present in only very small quantities relative to the antenna pigments, and is therefore undetected in the ground state absorption spectrum. Whether the 570 nm absorbing species is an antenna pigment whose absorbtion properties are affected by reaction center photochemistry, or a separate reaction center pigment that happens to have an absorbtion spectrum similar to the antenna pigments is not yet clear. The two spectral components exhibited identical kinetic properties in our measurements at all redox potentials studied.

Clearly, the reaction center can undergo useful photochemistry only if the primary donor is reduced, and the 'primary acceptor' * is oxidised,

* We are using the term 'primary acceptor' in the sense in which it was originally used in studies of bacterial photochemical reaction centers (for a review, see Ref. 10), where the reduction of this component prevented useful photochemistry, as measured on a microsecond-to-millisecond timescale. The introduction of picosecond spectroscopy revealed that there was at least one 'intermediary acceptor' between the primary electron donor bacteriochlorophyll and the 'primary acceptor' (see Refs. 10, 12 and 15).

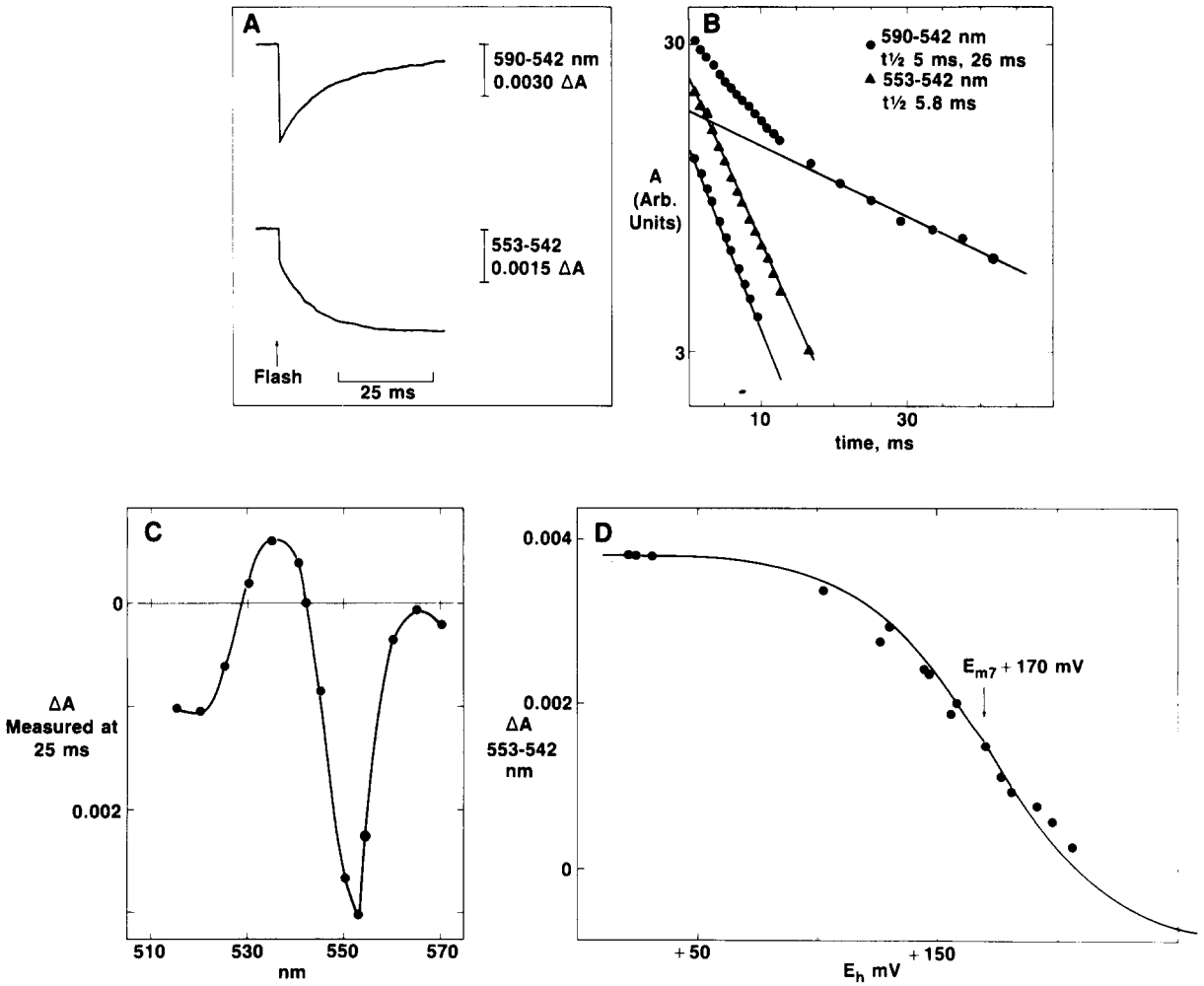

Fig. 3. Photo-oxidation of cytochrome *c*-553 in *H. chlorum*. Membranes were suspended as in Fig. 1, but the redox mediators were only added for the experiment of panel D. For the other panels, the ambient potential was lowered to near −300 mV with a small addition of sodium dithionite. Panel A shows the kinetics of the reaction center 'primary donor' and the cytochrome *c*. Panel B shows a semi-log plot of the data, while panel C shows the spectrum of the photo-oxidised cytochrome. Panel D shows a redox titration of the photo-oxidation of the cytochrome, monitored 25 ms after the flash at 553–542 nm.

prior to illumination. Indeed, the oxidation-reduction midpoint potentials of these components can be assessed by monitoring photochemistry as a function of ambient potential (see Ref. 10). Figs. 1 and 2 show that these potentials are +225 mV and −510 mV for the primary donor and 'acceptor', respectively. Note that in order to reduce chemically the 'primary acceptor' at equilibrium, the pH had to be raised to 10; it is not chemically reducible, at equilibrium, at pH 7.

H. chlorum has a membrane-bound *c*-type cytochrome that donates an electron to the photo-oxidised reaction center [4]. Fig. 3 shows the kinetics of this event; approximately half of the total photo-oxidised reaction center bacteriochlorophyll (measured at 590–542 nm, see Fig. 1) is reduced by the cytochrome *c* (measured at 553–542 nm) with a half-time close to 6 ms. As shown in Fig. 3, the cytochrome α-band is at 553 nm, and its $E_{m,7}$ is +170 mV. No other cytochromes were detected in the membrane or soluble fractions [4].

The EPR properties of the reaction center bacteriochlorophyll primary donor are shown in Fig. 4. Illumination of samples reduced with dithionite at pH 7 (hence with the 'primary acceptor' oxidised in the dark, see Fig. 2) generates a signal near $g = 2.0025$ with a line-width of 0.95 mT. Approximately half of this signal decays when the light is turned off, and the reversible signal can then be reversibly induced on successive illuminations. The signals were unaffected by whether the *c* type cytochrome was oxidised or reduced prior to freezing, and we conclude that the cytochrome is unable to donate electrons to the reaction center at cryogenic temperatures. The reversible and irre-

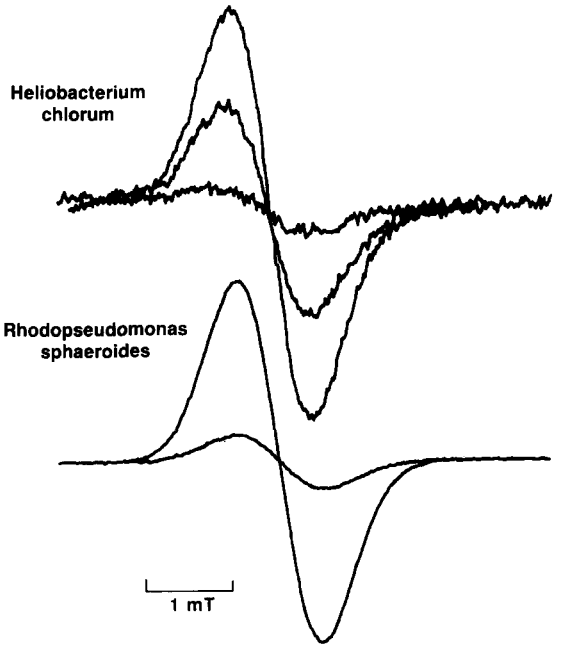

Fig. 4. Light-induced signals near g_2 in *H. chlorum*. Membranes ($A_{788} = 113$) in the buffer of Fig. 1 were reduced with dithionite and frozen in the dark. The sample was measured in the dark (smallest signal), during illumination (largest signal), and after illumination. For comparison, reaction centers from *Rps. sphaeroides* R26 were measured under similar conditions. Spectrometer conditions; 1 mW applied power, 0.25 mT modulation amplitude, 11 K.

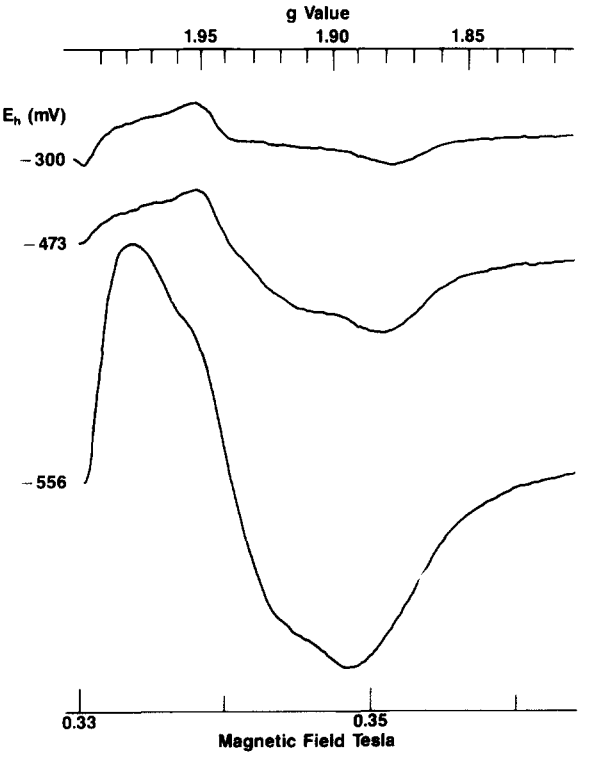

Fig. 5. Iron-sulfur clusters in membranes of *H. chlorum*. Membranes ($A_{788} = 113$) were suspended in 20 mM cyclohexylaminohydroxy propane sulfonate, 100 mM KCl (pH 10.0) with 40 μM 2-hydroxy-1,4-anthraquinone, *o*-naphthoquinone-4-sulfonate, tetramethyl-*p*-benzoquinone, indigodisulfonate and methyl viologen, at the E_h values indicated. Spectrometer conditions; 10 mW applied power, 1.6 mT modulation amplitude, 20 K.

versible signals have similar g values and linewidths, which are in turn similar to the light-induced signal seen in isolated reaction centers from *Rps. sphaeroides*. In the latter case, the light-induced signal decays completely in the dark (Fig. 4).

We were unable to carry out a redox titration of the appearance of the $g = 2.0025$ signals, because poising samples at $E_h > +200$ mV generated very large free radical signals, even in the dark, which prevented the observation of light-induced signals. However, poising samples at $E_h = -560$ mV at pH 10 eliminated almost all the light-induced signals.

Membranes of *H. chlorum* contain substantial amounts of iron sulfur clusters, as shown in Fig. 5. We were unable to see any light-induced changes in such signals due to light-induced heating artifacts, but it is clear from Fig. 5 that there are iron sulfur clusters that may have appropriate thermodynamic properties to function as the 'primary acceptor' in the reaction center (i.e., with $E_{m,10}$ of -510 mV, see Fig. 2).

Discussion

The thermodynamic properties of the primary electron donor, 'primary acceptor' and the membrane-bound cytochrome *c* of *H. chlorum* are almost identical to those of *C. limicola* f. *thiosulfatophilum* [7,11,12] and *Prosthecochloris aestuarii* [12], as shown in Fig. 6; they are rather different from those of purple bacteria, such as *Rps. sphaeroides* [13].

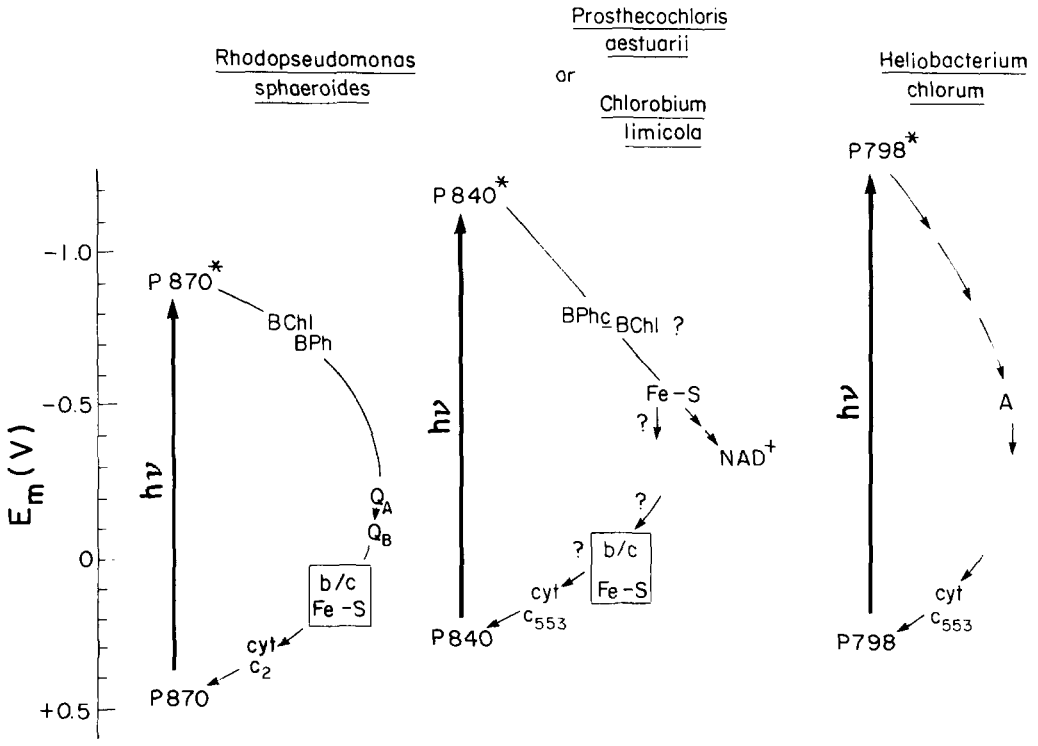

Fig. 6. A comparison of *Rps. sphaeroides*, *P. aestuarii* or *C. limicola* and *H. chlorum*. This figure shows a somewhat simplified scheme of the thermodynamic properties of electron carriers associated with the reaction centers of these organism. While the figure is discussed in the text, a more detailed consideration can be found in Refs. 10, 12, 13, 15 and 28. We have indicated both bacteriochlorophyll (BChl) and bacteriopheophytin (BPh) as being involved in the electron pathway within the reaction center of *Rps. sphaeroides*. There is some controversy about this point; as discussed recently by Kirmaier et al. [31], there is no convincing kinetic or spectroscopic evidence that bacteriochlorophyll is involved, yet the X-ray crystal structure of Diesenhofer et al. [18] suggests that it should be.

EPR, ENDOR and optical data on a variety of photosynthetic organisms have been interpreted as evidence of a dimeric nature of the photoactive pigments in the reaction center [14,15]. While this has been questioned in some systems [16,17], the electron density map of the photosynthetic reaction center of the purple bacterium *Rps. viridis* has suggested that the dimeric nature is achieved by the acetyl groups on the bacteriochlorophyll ring I acting as fifth ligands for the Mg atoms of the bacteriochlorophylls, with close contact between the side groups of rings II [19].

In those organisms containing bacteriochlorophyll *a*, the line width of the EPR signal attributed to the oxidised form of the primary donor is close to 0.95 mT, which is approximately $\sqrt{2}$ narrower than the signal arising from the monomeric bacteriochlorophyll *a* cation radical [14,15,19,20]. However, the $\sqrt{2}$ narrowing of the primary donor radical signal compared with the in vitro monomer is not always seen. For example, bacteriochlorophyll *b*-containing organisms, such as *Rps. viridis* [21,22] and *Thiocapsa pfennigii* [23], display a linewidth only slightly narrower than that of the monomeric form [24]. Yet optical data (see Ref. 15) and now the electron-density map [18] seem to clearly indicate a dimeric structure. The absence of the expected narrowing in these organisms has been ascribed either to slow electron hopping over the dimer (on an EPR time scale), or perhaps to a binding-site-induced twisting of the molecules (see Ref. 15). Presumably interactions with the binding site could also narrow the signal of a monomeric species.

The EPR properties of the oxidised primary donor of *H. chlorum* are very similar to those of the bacteriochlorophyll-*a*-containing purple and green bacteria, with a *g*-value close to $g = 2.0025$, and a line width of 0.95 mT (Fig. 4). We might thus predict that the primary donor would be a dimeric species, although of course this presupposes that the line width of the monomeric bacteriochlorophyll *g* cation radical is similar to that of bacteriochlorophyll *a*. However, as Hanson and Fajer [25] point out, the optical properties of the primary donor of *H. chlorum* are rather more consistent with a monomeric structure. Furthermore, bacteriochlorophyll *g*, which is apparently the only chlorophyll in *H. chlorum,* has a vinyl group in place of the acetyl group on ring I [2] which is proposed to be involved intimately in the dimeric structure [18]. We are thus left with optical spectroscopy suggesting a monomer, and EPR suggesting a dimer as the structure of the primary donor.

The E_m value of the primary donor of *H. chlorum* is very similar to that of the green sulfur bacteria, about 200 mV less positive than that found in the purple bacteria, and about 125 mV less positive than that found in the green gliding organism *C. aurantiacus* (see Ref. 12). The reasons for these differences are not yet apparent, but they emphasise that in a number of systems the redox properties of the primary donors appear to be be more dependent on the nature of the protein environment than on the chemical nature of the pigment. When comparing organisms which, except for pigment composition, seem quite similar (e.g., *H. chlorum* and *P. aestuarii*), major changes in the chemical structure of the reaction center pigments are not reflected in their redox properties in vivo. On the other hand, apparently identical pigments in different systems can display significantly different redox properties (e.g., *Rps. sphaeroides* and *P. aestuarii*).

We were not able to detect the characteristic radical pair spin polarised triplet state of the primary donor of *H. chlorum* [26], despite trying a variety of techniques that have proven useful in other systems, such as illuminating at 200 K, or illuminating while freezing chemically reduced samples. This may be similar to the situation in Photosystem I, where the very low redox potentials necessary to reduce the acceptors make observation of the triplet more difficult than in purple bacteria [27].

The initial acceptor of the electron that leaves the reaction center primary donor seems to be a monomeric (bacterio-)chlorophyll or (bacterio-)pheophytin molecule, depending on the system (see Refs. 12 and 15). This component, often known as the 'intermediary carrier' (I), is presumably present in *H. chlorum*, but we have been unable to trap it in its reduced form.

The 'primary acceptor' of *H. chlorum* is clearly very different from that of the purple and green gliding bacteria, but apparently rather similar to that of the green sulfur organisms. The former

have a quinone-iron complex, while the latter seems to have an iron-sulfur cluster with a far more negative potential (Fig. 6, and see also Ref. 12). Indeed the green sulfur bacteria are the only bacteria capable of direct photoreduction, via ferredoxin, of NAD^+ in a manner apparently analogous to that of chloroplast Photosystem I (see Ref. 12). The chloroplast Photosystem I 'primary acceptor' complex is complicated, with at least three iron-sulfur like clusters (see Ref. 15); such a complicated system has not been seen in the green sulfur organisms, and to date the available data fit the hypothesis that the 'primary acceptor' of these organisms is a 'ferredoxin-like' (i.e., visible by EPR in its reduced form, with $g_{av} = 1.96$) iron-sulfur cluster with an E_m of approximately -540 mV (see Ref. 12). We find a similar E_m in *H. chlorum* (Fig. 3), and membranes of the organism contain iron-sulfur clusters with appropriate thermodynamic properties (Fig. 5), so we conclude that the acceptor system of *H. chlorum* is very similar to that of the green sulfur bacteria. As such, we would predict that this organism would be able to catalyse the direct photoreduction of NAD^+, but there is as yet no evidence to support this.

A single *c*-type cytochrome has been detected in membranes of *H. chlorum*, with no soluble cytochromes yet seen [4]. As we show in Fig. 3, this cytochrome has an α-band λ_{max} near 553 nm, and an $E_{m,7}$ of $+170$ mV. These are very similar properties to those found for the green sulfur organisms (see Ref. 12), although in *H. chlorum* the photo-oxidation of the cytochrome ($t_{1/2} \approx 6$ ms, Fig. 3) is much slower ($t_{1/2}$ faster than 100 μs in *C. limicola* f. *thiosulfatophilum* and *P. aestuarii* [7,28]).

Cytochrome bc_1 complexes, and their analogs, are widely distributed in procaryotes and eukaryotes (see Ref. 30 for a recent review) and play a vital role in energy-conserving electron-transfer systems. Hurt and Hauska have recently isolated what may be such a complex from *C. limicola* f. *thiosulfatophilum* [30]. This raises the question of how electron flow is directed in this organism, which as discussed above is capable of direct photoreduction of NAD^+, and which would presumably use a cytochrome bc_1 complex in cyclic electron flow around the reaction center. We did not detect a Rieske iron-sulfur cluster in membranes of *H. chlorum* (see Fig. 5), nor any evidence for rapid cyclic electron flow (see Fig. 3), but this by no means rules out the possibility that *H. chlorum* may contain a cytochrome bc_1 complex.

Since completing this work, Nuijs et al. [32] have reported picosecond spectroscopic data that are in broad agreement with the findings reported here, although they do not report the split absorption band near 600 nm (see Fig. 1). They conclude that the 'intermediary electron acceptor' within the reaction center is a pigment absorbing at 670 nm, possibly a bacteriochlorophyll *c*-like species. They also conclude that the reaction center of *H. chlorum* is similar to that of Green sulfur bacteria.

Thus in summary, our data indicate that the reaction center and associated *c*-type cytochrome of *H. chlorum* are very similar to those of the green sulfur bacteria. Yet the pigment composition and cellular organisation of this group of organisms are totally distinct from those of *H. chlorum* [4].

Acknowledgements

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References

1 Gest, H. and Favinger, J.L. (1983) Arch. Microbiol. 136, 11–16
2 Brockmann, H. and Lipinski, A. (1983) Arch. Microbiol. 136, 17–19
3 Truper, H.G. and Pfennig, N. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 19–27, Plenum Press, New York
4 Fuller, R.C., Sprague, S.G., Gest, H. and Blankenship, R.E. (1985) FEBS Lett. 182, 345–9
5 Dutton, P.L., Petty, K.M., Bonner, H.S. and Morse, S.D. (1975) Biochim. Biophys. Acta 387, 536–556
6 Jackson, J.B. and Crofts, A.R. (1971) Eur. J. Biochem. 18, 120–130

7 Prince, R.C. and Olson, J.M. (1976) Biochim. Biophys. Acta 423, 357–362
8 Bruce, B.D., Fuller, R.C. and Blankenship, R.E. (1982) Proc. Natl. Acad. Sci. USA 79, 6532–6536
9 Pierson, B.K. and Thornber, J.P. (1983) Proc. Natl. Acad. Sci. USA 80, 80–84
10 Prince, R.C. and Dutton, P.L. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 439–453, Plenum Press, new York
11 Olson, J.M., Prince, R.C. and Brune, D.C. (1976) Brookhaven Symp. Biol. 28, 238–246
12 Blankenship, R.E. (1984) Photochem. Photobiol. 40, 801–806
13 Prince, R.C., O'Keefe, D.P. and Dutton, P.L. (1982) in Electron Transport and Photophosphorylation (Barber, J., ed.), pp. 197–248, Elsevier Science Publishers, Amsterdam
14 Norris, J.R. and Katz, J.J. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 397–418, Plenum Press, New York
15 Hoff, A.J. (1982) Biophys. Struct. Mech. 8, 107–150
16 Davis, M.S., Forman, A. and Fajer, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4170–4174
17 O'Malley, P.J. and Babcock, G.T. (1984) Proc. Natl. Acad. Sci. USA 81, 1098–1101
18 Diesenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1984) J. Mol. Biol. 180, 385–398
19 Knaff, D.B., Olson, J.M. and Prince, R.C. (1979) FEBS Lett. 98, 285–289
20 Swarthoff, T., Gast, P. and Hoff, A.J. (1981) FEBS Lett. 127, 83–86
21 McElroy, J.D., Feher, G. and Mauzerall, D.C. (1972) Biochim. Biophys. Acta 267, 363–374
22 Prince, R.C., Leigh, J.S. and Dutton, P.L. (1976) Biochim. Biophys. Acta 440, 622–636
23 Prince, R.C. (1978) Biochim. Biophys. Acta 501, 195–207
24 Fajer, J., Davis, M.S., Brune, D.C., Spaulding, L.D., Borg, D.C. and Forman, A. (1977) Brookhaven Symp. Biol. 28, 74–104
25 Hanson, L.K. and Fajer, J. (1985) Biophys. J. 47, 422a. 20
26 Dutton, P.L., Leigh, J.S. and Seibert, M. (1972) Biochem. Biophys. Res. Commun. 46, 406–413
27 Frank, H.A., McLean, M.B. and Sauer, K. (1979) Proc. Natl. Acad. Sci. USA 76, 5124–5128
28 Swarthoff, T., Van der Veek-Horsley, K.M. and Amesz, J. (1981) Biochim. Biophys. Acta 635, 1–12
29 Hauska, G., Hurt, E., Gabellini, N. and Lockau, W. (1983) Biochim. Biophys. Acta 726, 97–133
30 Hurt, E.C. and Hauska, G. (1984) FEBS Lett. 168, 149–154
31 Kirmaier, C., Holten, D. and Parson, W.W. (1985) FEBS Lett. 185, 76–82
32 Nuijs, A.M., Van Dorssen, R.J., Duysens, L.N.M. and Amesz, J. (1985) Proc. Natl. Acad. Sci. USA, in the press