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Evidence for a unique Rieske iron-sulphur centre in *Heliobacterium chlorum*

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An iron sulphur centre ($g_z = 2.035$, $g_y = 1.89$, $g_x = 1.81$), which can be observed in both whole cells and isolated plasma membranes of *Heliobacterium chlorum*, was identified as a Rieske centre on the basis of its sensitivity to the inhibitors 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone (DBMIB), 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) and stigmatellin as well as on the basis of its orientation (g_x is oriented perpendicular and g_y is oriented parallel to the membrane plane). Its midpoint potential is unusually low ($E_{m,7} = +120 \pm 10$ mV) and does not depend on pH in the range between pH 6.5 and pH 8.0. The effects of the inhibitors on the EPR spectrum are altered compared to Rieske centres in other systems. The significance of the low E_m is discussed with regard to the overall midpoint potentials of the electron transfer chain in *Heliobacterium chlorum*.

Rieske center; *Heliobacterium chlorum*; Cytochrome *bc*-complex; Inhibitor; Q_O-site; Electron paramagnetic resonance spectrometry

1. INTRODUCTION

Only about six years ago Gest and Favinger [1] discovered a gram-negative, brownish-green, anoxygenic bacterium, designated as *Heliobacterium chlorum*. It soon became clear that *H. chlorum* did not fit in any of the hitherto known phyla of photosynthetic bacteria and it therefore attracted considerable interest. Three years later the closely related *Heliobacillus mobilis* was described [2] as the second member of the family *Heliobacteriaceae*. These organisms contain a previously unknown chemical form of bacteriochlorophyll, namely bacteriochlorophyll *g* (BChl *g*) as the principal pigment [3], localized in the cytoplasma membrane. The organism lacks chlorosomes as well as a developed intracytoplasmic membrane system. Even though it is gram-negative, comparisons based on 16 S rRNA sequences demonstrated that *H. chlorum* is phylogenetically close to gram-positive bacteria [4].

Since the discovery of *H. chlorum*, the reaction centre has been studied [5,6] but little or no attention was paid to the electron transfer chain outside the reaction centre. No soluble cytochromes have been detected, however, a membrane-bound cytochrome c_{553} was described [5], which donates directly and rapidly electrons to P_{798}^+, the photooxidized primary donor of *H. chlorum* [7,8].

The presence of membrane-bound hemeproteins with 20 and 33 kDa apparent molecular weight on SDS-PAGE in addition to cytochrome c_{553} (which is probably associated to the reaction centre) was taken as an indication for the existence of a cytochrome bc_1 complex, yet, so far no signal arising from a Rieske FeS centre, characteristic for cytochrome bc_1 complexes, was detected [6]. Furthermore, it has been shown that reaction centre-associated cytochrome subunits are often proteolytically degraded, resulting in two additional (heme-staining) bands at about 20 and 30 kDa [9], which could provide an alternative explanation for the bands attributed to the cytochromes of a putative cytochrome bc_1 complex [5]. Moreover, recently published data seem to indicate, that photosynthetic electron transport in *H. chlorum* could be mainly non-cyclic [7].

However, the recent discovery of quinones (menaquinone) in *H. chlorum* [10], a prerequisite of cytochrome *bc* complex function, anew raised the question of cyclic electron transfer in this organism.

Our EPR investigations unambiguously demonstrate the existence of a Rieske FeS centre and thus that of a cytochrome bc_1 complex. The electrochemical properties, orientation and inhibitor-sensitivity of this centre are detailed in this work.

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone; EDTA, ethylenediaminetetraacetate; FeS centre, iron-sulphur centre; Mops, 4-morpholinepropanesulphonic acid; Q_O-site, the quinone-oxidizing site of cytochrome *bc* complexes; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole

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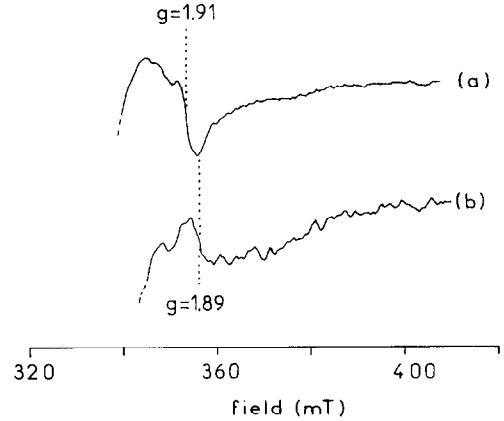

Fig.1. EPR spectra of whole cells of *H. chlorum* (a) in the presence of 100 mM sodium dithionite and (b) without additions. Spectrum (b) is expanded 4-fold in the vertical direction. EPR conditions: microwave power, 6.3 mW; modulation amplitude, 1.6 mT; frequency, 9.44 GHz; temperature, 25 K.

2. MATERIALS AND METHODS

Heliobacterium chlorum was obtained from the Deutsche Sammlung von Mikroorganismen (DSM), Braunschweig/FRG and cultured in 1 and 2 l bottles in an anaerobic hood.

Media 112 [1] and 1552 [7] of the American Type Culture Collection containing 2.5 mM sodium ascorbate were used with equal yields of cell material.

For the preparation of membranes, cells were washed in 20 mM Mops, pH 7.0 and broken by three passages through a French pressure cell (20000 psi) in the presence of DNase. Cell debris was removed by centrifugation at 10000 × *g* for 10 min. Membranes were separated from soluble components by a centrifugation step at 260000 × *g* for 2 h. All steps were carried out at 4°C in the presence of 5 mM sodium ascorbate.

Redox titrations were carried out (in 100 mM Mops, pH 7.0, 5 mM EDTA) in near darkness as described by Dutton [11] using a membrane suspension at a ΔA of 90 at 780 nm.

The following redox mediators were used: benzoquinone, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, diaminodiurol, 5'-H-1,4-naphthoquinone, 2,5-dimethylbenzoquinone, methylene blue, pyocyanine, indigodisulfonate, indigotetrasulfonate, 1,4-dihydroxynaphthoquinone, 2,5-dihydroxy-*p*-benzoquinone, anthraquinone-2,6-disulphonate, variamine blue, toluylene blue, vitamin K_3 and duroquinone at 100 μM and phenazine methosulphate and phenazine ethosulphate at 50 μM. Reductive titrations were carried out using sodium dithionite and oxidative titrations were done using porphyrexide.

Oriented membrane multilayers were produced by partial dehydration in a humidity-controlled atmosphere [12].

DBMIB was obtained from Sigma and UHDBT from Dr B.L. Trumpower, Hanover, USA.

EPR spectra were recorded on Bruker 200 and 300 X-band spectrometers fitted with an Oxford Instruments cryostat and temperature control system.

3. RESULTS

The EPR spectra at low temperature of whole cells of *H. chlorum* at two different redox states are shown in fig.1. The dithionite treated sample shows the presence of a number of FeS centres (fig.1a) similar to those detected by Prince et al. [6] in membrane preparations of *Heliobacterium*. However, already the untreated sample (note that the growth medium contains 2.5 mM sodium ascorbate) shows a weak signal of a reduced FeS centre at $g = 1.89$ (fig.1b). The g-value of the signal as well as the fact that the centre can be reduced even by weakly reducing agents such as ascorbate is characteristic for the so-called Rieske centre which is one of the essential redox centres of the cytochrome bc_1 complexes [13].

To determine the exact midpoint potential of this centre a redox titration was carried out on isolated membranes from *H. chlorum* (fig.2). The better signal to noise ratio in these samples (see fig.2, inset) allowed

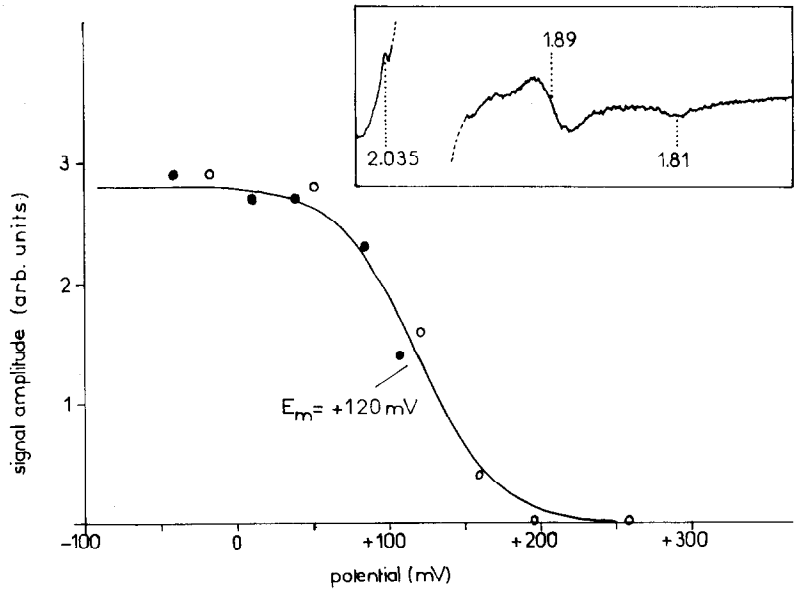

Fig.2. Redox-titration of *H. chlorum* membranes at pH 7.0. The signal size of the g_y line is plotted vs ambient potential. Data points obtained on titrating in the positive and negative directions are denoted by open and closed symbols, respectively. Inset: EPR spectrum of a sample titrated to +50 mV. EPR conditions were as described in fig.1.

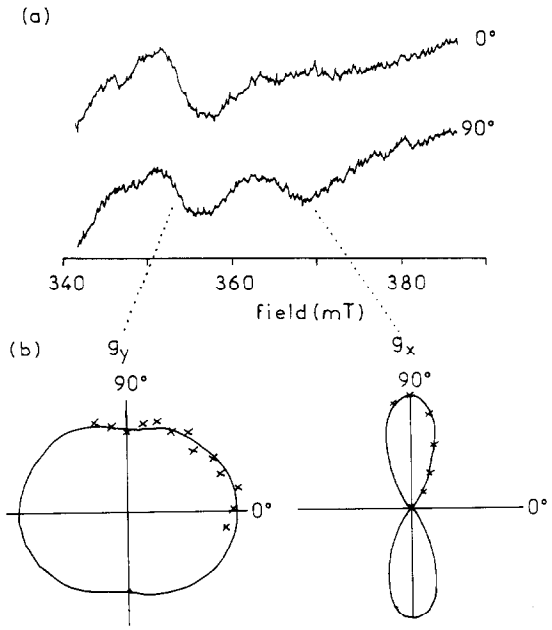

Fig.3. Orientation dependence of the g_y and g_x signals. (a) EPR spectra of partially ordered membrane multilayers from *H. chlorum* at 0° and 90° angles between the plane of the multilayer and the magnetic field are shown. A solution containing 5 mM EDTA and 50 mM sodium ascorbate was applied to the dried membranes followed by renewed drying under a stream of argon gas in darkness. (b) Polar plot evaluation of (a). EPR conditions were as described for fig.1.

for the determination of all three g-values (g_z = 2.035, g_y = 1.89 and g_x = 1.81). An E_m of +120 ± 10 mV is determined from the experimental data points. This E_m was found to be pH-independent in the range from pH 6.7 to pH 8.0 (data not shown).

No further FeS centre is reduced at ambient potentials between +200 mV and −50 mV. It is of note that the relaxation properties of the spectrum are again similar to those of a typical Rieske centre in that the signal becomes saturated under conditions where most other FeS centres are still far from saturation.

The orientation of this FeS centre with respect to the membrane plane was examined in partially ordered membrane multilayers. Fig.3 shows two representative spectra taken at orientations of 0° and 90° angle between the magnetic field and the membrane plane. The full set of data is depicted in the polar plots of fig.3b demonstrating that g_x is well-ordered perpendicular to the membrane plane, whereas the g_y peak is maximal in the plane of the membrane, however with a rather poor dichroic ratio. Unfortunately, due to the large signal in the g = 2 region, the orientation of the g_z direction could not be determined.

As a definitive test concerning the identification of the observed FeS centre with a Rieske centre, the effects of Q_O-site inhibitors of the cytochrome bc_1 complexes on the EPR spectrum were studied. All inhibitors tested affected the EPR spectrum of the centre as shown in fig.4.

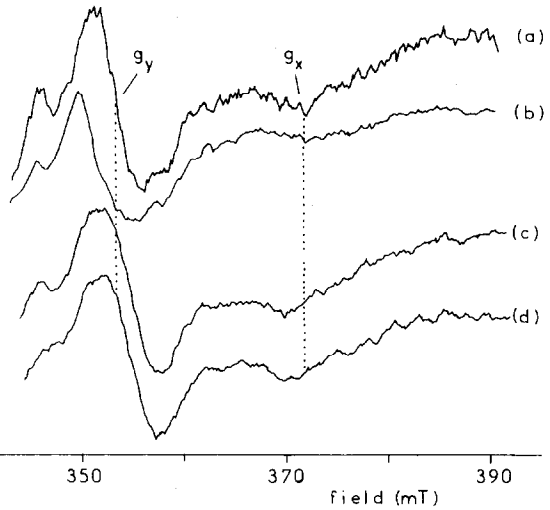

Fig.4. Effect of Q_O-site inhibitors on the g_y and g_x signals. EPR spectra of *H. chlorum* membranes were taken as described in fig.1 in the absence of inhibitors (a), in the presence of 500 μM DBMIB (b), 400 μM UHDBT (c) and 400 μM stigmatellin (d). All samples contained 5 mM EDTA and 50 mM sodium ascorbate.

4. DISCUSSION

From its spectral parameters, orientation dependence and inhibitor sensitivity it is clear that the observed EPR spectrum arises from a Rieske centre. However, it shows a number of unusual features:

(i) its E_m is by about 200 mV more negative than that of most Rieske FeS centres [14]. This low midpoint potential is necessary for the Rieske centre in *H. chlorum* to function as a secondary electron donor for the photooxidized P_{798}^{+} of the reaction centre, which has an E_m of about +225 mV [6]. This situation is remarkably similar to that encountered with the green sulphur bacteria, where E_m's of +250 mV [15,16] and +160 mV [17] have been determined for the primary donor and the Rieske centre, respectively. Thus it appears as if the redox span between both compounds is conserved between different species and amounts to between 100 and 150 mV. It is of note that the same holds true for the analogous components in photosynthetic membranes of cyanobacteria and plants [13,18].

However, it should be pointed out that so far no soluble electron carrier has been found in *H. chlorum* which could function between the cytochrome bc_1 complex and the reaction centre-associated cytochrome c_{553}. Thus it cannot be excluded that a direct electron transfer operates between both membrane-bound complexes.

(ii) DBMIB affects the g_y peak, shifting it by 2.7 mT towards lower magnetic field (fig.4b). This effect, however, is much less marked than in 'usual' cytochrome bc_1 and b_6f complexes [19], where the modified g_y peak is at g = 1.94, i.e. shifted by about 9 mT (under X-band conditions). UHDBT (fig.4c) and stigmatellin (fig.4d) shift the g_y-line to higher field values, just as has been reported for the Rieske centres

from mitochondria [20,21] and plants [22,23]. However, in *H. chlorum* both these inhibitors induce a shift of the g_x-trough to lower field position, whereas the opposite effect is usually observed both in cytochrome bc_1 [20,21] and b_6f complexes (Nitschke, unpublished). Thus, the effects of the Q_O-site inhibitors on the EPR spectrum of the centre are comparable but not identical with what has been reported for other Rieske proteins. One might speculate that this is due to the fact that the electron donor which reacts at the Q_O-site must be a menaquinone in the case of *H. chlorum* (as this is the only quinone present [10]), whereas it is ubiquinone or plastoquinone in most other systems. However, this cannot be the only reason as in the case of green sulphur bacteria, where also only menaquinone can be detected in the membrane, DBMIB shows the usual shift of g_y to $g = 1.94$ (Nitschke, unpublished data).

(iii) Whereas both the g_y and the g_x directions are oriented with respect to the membrane in the same way as has been reported for other cytochrome bc_1 complexes [24,25], the dichroism of the g_y peak is much less marked than is usually reported for the other systems. This finding is reminiscent of the situation reported for the cytochrome b_6f complex [26,27], where the g_y peak appears to be almost unoriented. The Rieske centre of *H. chlorum* represents in this respect an intermediate case between the cytochrome b_6f and the bc_1 systems (an almost identical orientation dependence of the g_y peak is observed in green sulphur bacteria; Nitschke and Rutherford, unpublished).

Thus, the Rieske centre from *H. chlorum* seems to be related to that of the green sulphur bacteria. However, with respect to its even lower E_m and its individual inhibitor sensitivity it constitutes an unusual, unique member of this group of FeS proteins.

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