AN EPR ANALYSIS OF THE PARTIALLY PURIFIED CYTOCHROME *bf* COMPLEX OF HIGHER-PLANT CHLOROPLASTS

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Received 22 May 1980

1. Introduction

Quinol–cytochrome *bc* oxidoreductase systems occur in the electron-transport chains of mitochondria, photosynthetic bacteria and chloroplasts. The mitochondrial *bc* complex has been purified and its reactions extensively studied [1] and the analogous system in the intact electron-transport chain of photosynthetic bacteria has also been thoroughly investigated [2]. The corresponding *bf* complex, although it has been purified extensively [3], has received comparatively little attention.

This report describes an EPR investigation of a partially purified, catalytically active, *bf* preparation [3,4] isolated from lettuce chloroplasts. At least five components may be detected and these are compared to species identified in [5–7].

2. Materials and methods

2.1. *Cytochrome* bf *preparation*

Chloroplasts and cytochrome *bf* preparations were obtained from lettuce essentially as in [3], but taking the *bf* preparation only to the protamine sulphate supernatant stage. The particles were still active in the catalysis of the reduction of *Pseudomonas* cytochrome *c*-551 by plastoquinol-1 [4] and hence were considered to be still in a physiologically active state. Typically, concentrations of components at this stage were: cytochrome *f*, 0.8 nmol/ml; cytochrome *b*, 2.2 nmol/ml; chlorophylls A + B, 0.08 mg/ml. For EPR experiments, this preparation was concentrated 10–30-fold by ultrafiltration with a PM-30 filter (Diaflo products; 40 lb/in^2). This treatment did not cause loss of catalytic activity and the preparation was stored at 77 K until required.

Abbreviations: DDQ, 2,3-dicyano-5,6-dichloro-*p*-benzoquinone; DBMIB, 2,5 dibromo-6-methyl-3-isopropyl-*p*-benzoquinone; MES, 2-(*N*-morpholino)ethanesulphonate

2.2. *EPR samples and redox titrations*

The *bf* preparation was left in darkness for 30 min before preparation of samples since decay to a dark ground state was found to be rather slow. Samples were placed in quartz EPR tubes of ~3 mm i.d. and frozen rapidly in liquid nitrogen. Spectra were taken with a Jeol FE-1X spectrometer, and sample temperature was maintained with a flow of helium gas.

Oxidation–reduction potential titrations were performed essentially as in [8]. For titration of the high-spin haem signals the sample was made to 100 mM in potassium phosphate buffer at pH 6.8 and the following mediators were added: 20 μM *N*-methylphenazonium methosulphate; 50 μM pyocyanine; 40 μM 2-methyl-1,4-naphthoquinone; 20 μM 2-hydroxyl-1,4-naphthoquinone; 20 μM 5-hydroxy-1,4-naphthoquinone; 10 μM anthraquinone; 40 μM anthraquinone-2,6-disulphonate; 50 μM indigo-2-sulphonate; 50 μM phenosafranine; 50 μM methylviologen. The whole was kept anaerobic with a flow of deoxygenated nitrogen and potential was monitored with a platinum electrode read against a saturated KCl calomel electrode standard. Potential was varied by additions of solutions of sodium dithionite or potassium ferricyanide.

For titration of the high-potential components a buffer of 100 mM sodium MES (pH 6.0) was used together with the following mediators: 20 μM diaminodurene; 40 μM benzoquinone; 40 μM 2-methylbenzoquinone. Anaerobiosis and potential measurement

were as for the low-potential titration and the reductant used was dithionite. In this titration ferricyanide was unsuitable as an oxidant because of its large EPR signal in the $g = 3$ region of the spectrum. Instead, we used a new oxidant, DDQ, to raise potential. This compound had no signal in the $g = 3$ region of the spectrum and potentials >+550 mV were easily obtainable at this pH. Aliquots of a stock solution of 100 mM in absolute ethanol were used.

2.3. *Cytochrome estimation*

Cytochromes were estimated by the methods in [7] with a split-beam spectrophotometer and $E_{mM^{-1},cm^{-1}} = 20$ was assumed for the α-band maxima in the difference spectra for all species. Chlorophyll was estimated by the method in [9].

2.4. *Preparation of reagents*

Plastoquinol-1 was prepared as in [4] and other quinols by the methods in [10]. DBMIB was the kind gift of Dr A. Trebst and DDQ was purchased from Sigma Chemical Co.

3. Results and discussion

3.1. *Characterisation of spectra*

The EPR spectrum of a typical dark-adapted *bf* preparation is illustrated in the upper trace of fig.1.

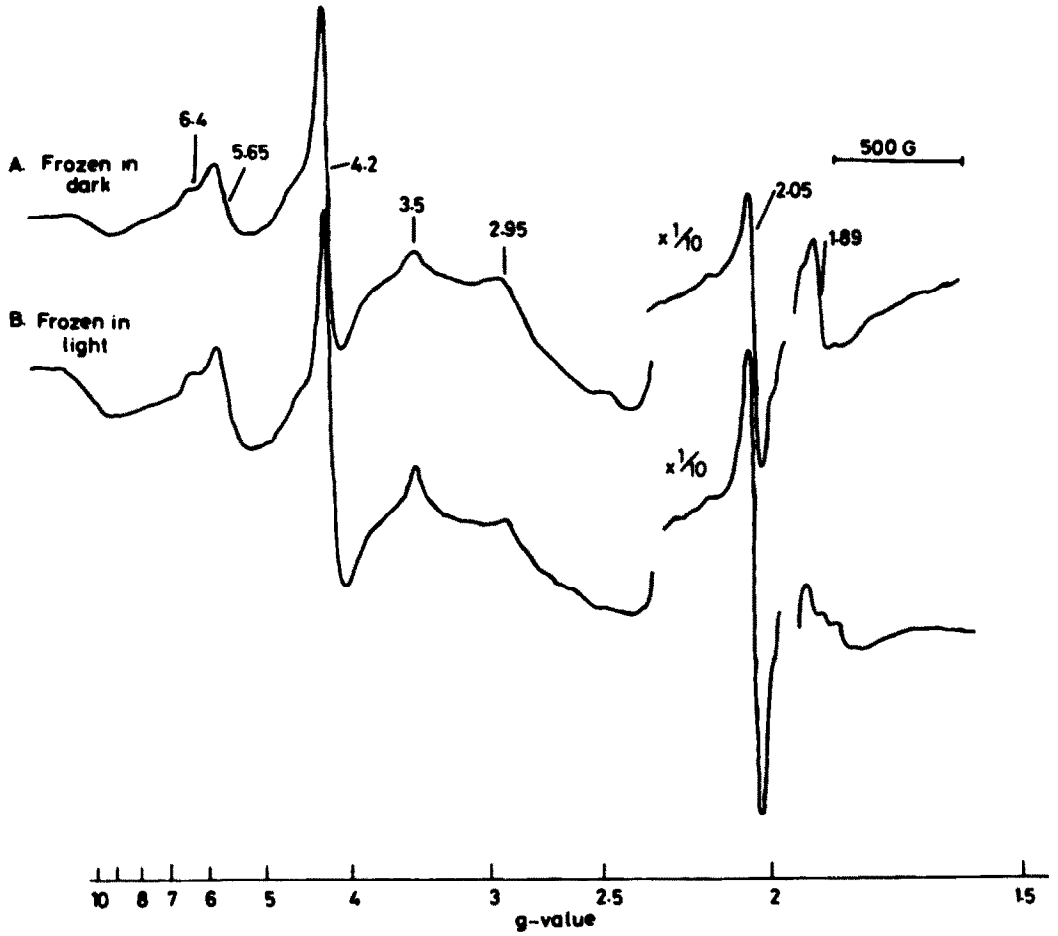

Fig.1. EPR spectra of dark-adapted and illuminated cytochrome *bf* preparations. Identical samples of a *bf* preparation in 100 mM MES at pH 6 (cytochrome *f* ~6 μM) were placed in matched quartz EPR tubes. One sample (top trace) was left in darkness for 30 min at 4°C and was then frozen in liquid nitrogen, while the other sample (lower trace) was illuminated with strong white light for 30 s prior to, and during, freezing in liquid nitrogen. EPR instrument conditions were: microwave power, 20 mW; modulation amplitude, 10 G; modulation frequency, 100 kHz; temperature 12 K.

The spectrum contains the following features: (i) a high-spin haem species with g_x and g_y at 6.4 and 5.65; (ii) two low-spin haem species with g_z values of 3.5 and 2.95; (iii) a large copper signal centred around $g = 2$ and with $g_\perp$ at 2.05; (iv) an iron–sulphur centre with g_y at 1.89.

Also present in the samples was a $g = 4.2$ iron signal, characteristic of a ferric iron species, and a manganese signal centred at $g = 2$ but split over the g 2.2–1.8 range. Both of these signals occur in many biological samples and are generally considered to be non-functional in electron transport. They were not considered further here.

When an identical sample was frozen under conditions of strong illumination, the spectrum shown in fig.1, lower trace, was obtained. It may be seen that the $g = 3.5$ haem, the copper signal and the $g = 1.89$ iron–sulphur centre become more oxidized by such treatment. The oxidation is presumably mediated by residual amounts of photosystem I activity which remain in this preparation and such an observation allows us to tentatively identify the $g = 3.5$ species as cytochrome *f*, the copper signal as plastocyanin and the iron–sulphur signal as the Rieske centre, since all of these components are photooxidized by photosystem I in the more intact systems. A spectrophotometric study confirmed that hydroquinone-reduced cytochrome *f* in these particles became photooxidized on illumination in the presence of methylviologen as a photosystem I acceptor (not shown).

These assignments are also consistent with the results for cytochrome *f* [5], the Rieske centre [11] and plastocyanin [12], and for cytochrome *f* [6].

3.2. *The identification of signals by selective reduction*

To identify further the EPR signals in this preparation, we exploited the differences in redox potentials of the known components and used a variety of reductants of different potentials.

Addition of 1 mM hydroquinone (E_{m7} (Q/QH_2) = +280 mV [13]) to a *bf* preparation at pH 6 caused the $g = 3.5$ haem signal and the copper signal to be lost (i.e., reduced), and caused full appearance (reduction) of the $g = 1.89$ iron–sulphur centre signal. This is consistent with the assignment of the $g = 3.5$ signal to cytochrome *f* (E_{m7} = +370 mV [14]), the copper signal to plastocyanin (E_{m7} = +370 mV [15]) and the $g = 1.89$ signal to the Rieske centre (E_{m7} = +290 mV [11]). A similar result was obtained when plastoquinol-1 (E_{m7} (QH_2/Q) $\simeq$ +110 mV [16]) was used as a reductant.

When 1 mM menadiol (E_{m7} (QH_2/Q) = 0 [13]) was used as a reductant, these same three components became reduced, but in addition the $g = 2.95$ feature was lost (fig.2B). This observation is consistent with its identification as a feature of cytochrome *b*-559 a component which has been detected in *bf* particles [7,17,18] and which is menadiol-reducible (E_{m7} of *b*-559 in *bf* preparations = +80 mV [7]).

Only when dithionite was used as reductant did the high-spin haem species become reduced (fig.2C). This behaviour is consistent with its identification as cytochrome *b*-563 (E_{m7} = –90 mV in *bf* preparations [7]), although the high-spin haem complement of chloroplasts may not be significant [5].

To investigate these assignments further, we carried out oxidation–reduction potential titrations of the *bf* preparation as in section 2 and the results are presented in fig.3. In the high-potential region, the E_{m6} values which we obtained were +350 mV for cytochrome *f*, +330 mV for the Rieske centre and +370 mV for plastocyanin. These are all close to the published values of +370 mV for cytochrome *f* obtained optically at room temperature [14], +290 mV for the Rieske centre [11] and +370 mV for plastocyanin [15] and hence confirm the assignments given.

The high-spin haem titration proved much more difficult. We noticed significant changes in line shape and also the appearance of a somewhat more axial, very low-potential, high-spin haem signal during the redox titration. These effects caused significant scatter in our data (fig.3D). Even so, the majority of the high-spin haem signal titrated with an $E_{m6.8}$ of ~–70 mV. This is close to the value of –90 mV which we obtained spectrophotometrically for cytochrome *b*-563 in identical *bf* preparations [7] and leads us to suspect that at least part of the high-spin haem signal is caused by cytochrome *b*-563, a result consistent with the original suggestions [6,19].

We have been unable to obtain concentrated enough *bf* preparations to determine the oxidation–reduction potential of the $g = 2.95$ feature which we have tentatively identified as cytochrome *b*-559.

3.3. *The effects of DBMIB and antimycin A*

Fig.4 illustrates the effects of the electron-transport inhibitor DBMIB on the observed EPR spectra. When

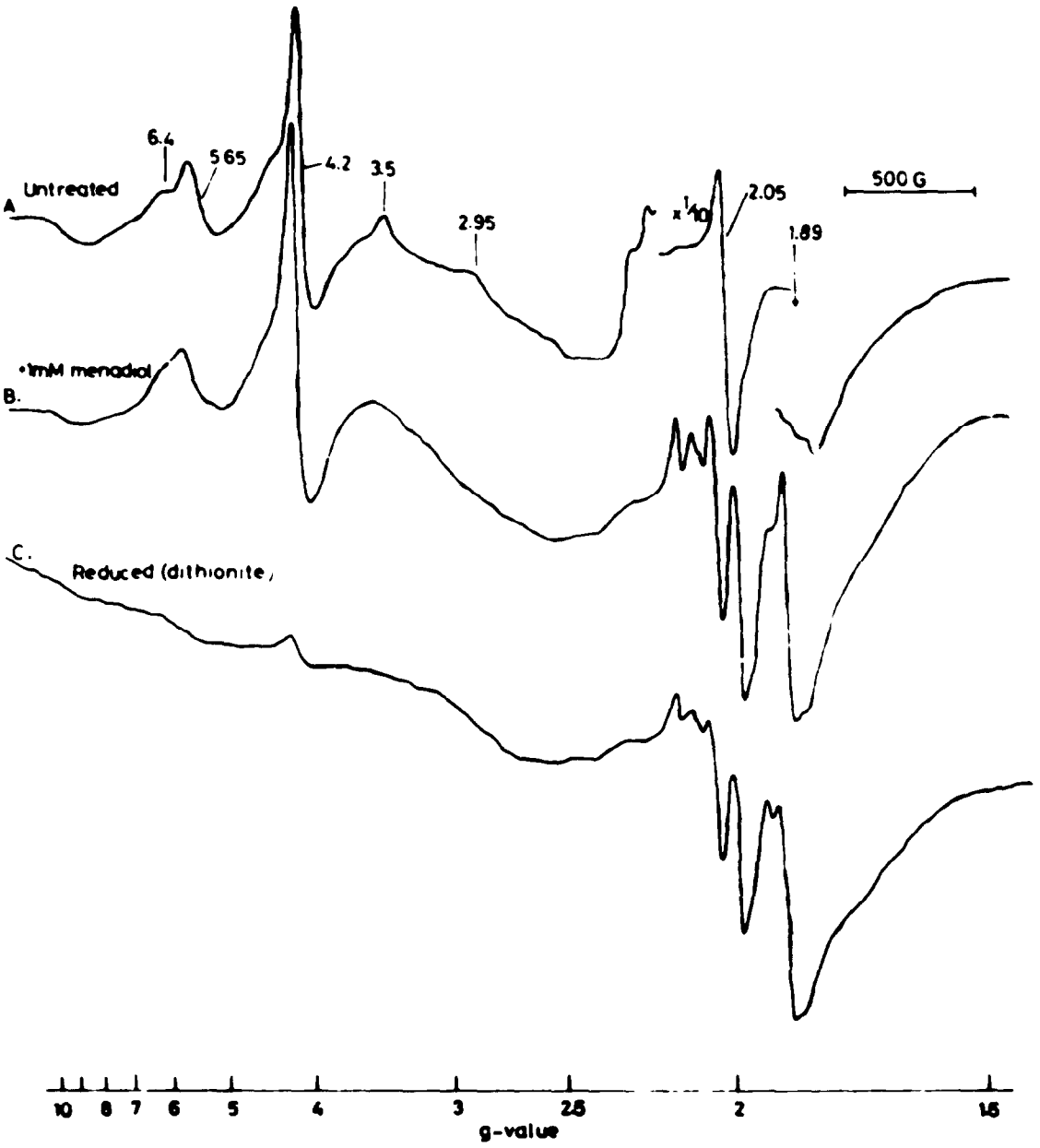

Fig.2. The effects of a variety of reductants on the *bf* preparation EPR spectrum. Identical samples of a *bf* preparation in 100 mM MES at pH 6 (cytochrome *f* ~6 μM) were placed in matched quartz EPR tubes and all were dark-adapted for 30 min at 4°C before being frozen in liquid nitrogen. (A) untreated control; (B) a sample to which 1 mM menadiol was added 30 s before freezing; (C) a sample to which a few crystals of solid sodium dithionite were added 30 s before freezing. EPR conditions were as in fig.1.

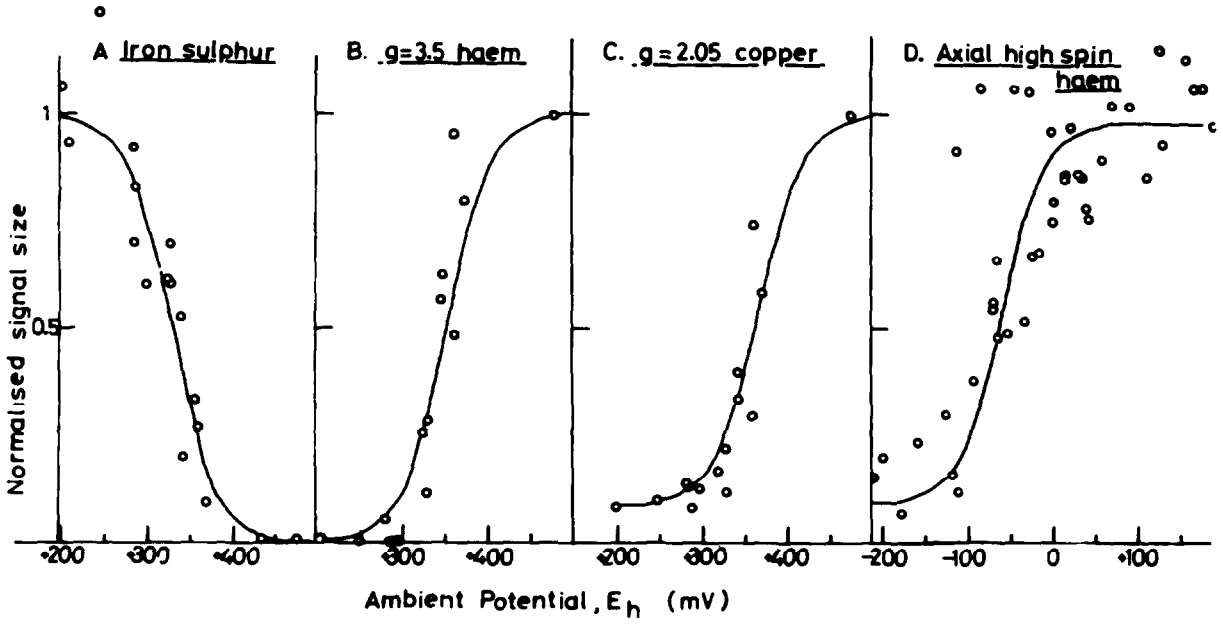

Fig.3. Redox titrations of features of the *bf* preparation EPR spectrum. Titrations were performed as in section 2. Cytochrome *f* was generally ~5 μM. The curves have been drawn through the normalized data points assuming $n = 1$ redox components.

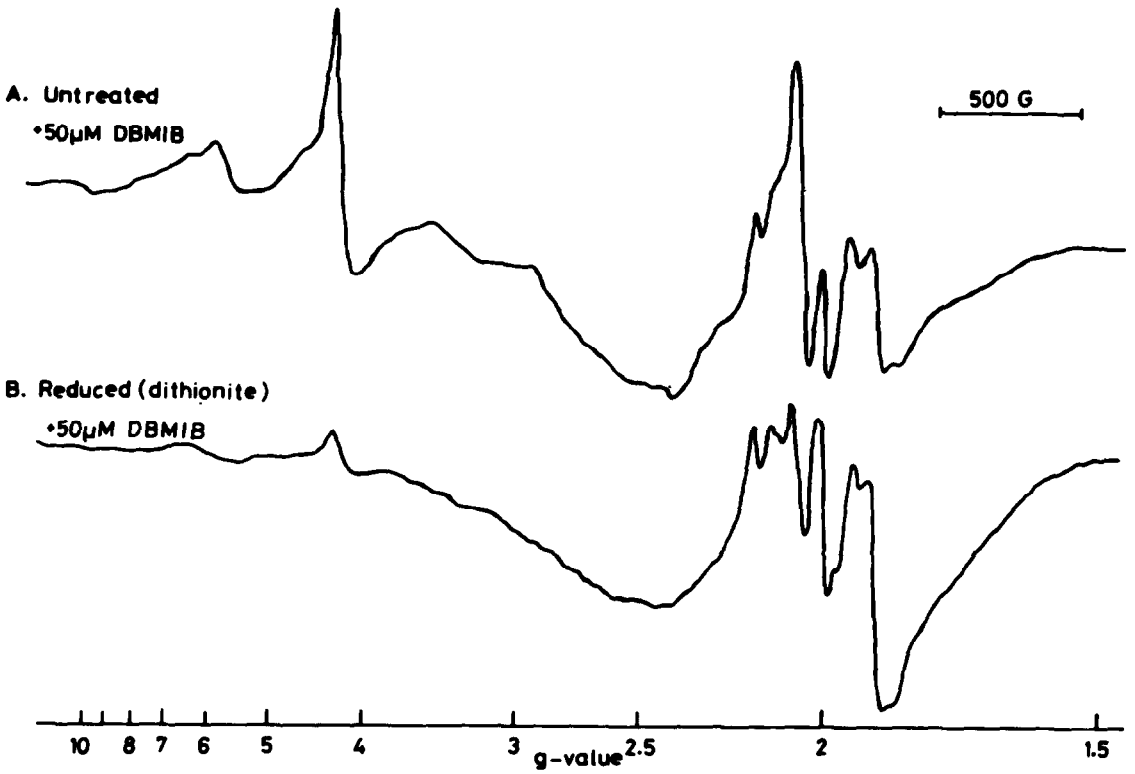

Fig.4. The effects of DBMIB on the features of the *bf* preparation EPR spectrum. Identical samples of a *bf* preparation in 100 mM MES at pH 6 (cytochrome *f* ~6 μM) were dark-adapted for 30 min at 4°C before being frozen in liquid nitrogen. The top trace represents a sample which had been treated with 50 μM DBMIB 10 min before freezing and the bottom trace represents a sample which had been treated with 50 μM DBMIB and a few crystals of solid sodium dithionite 10 min before freezing. EPR conditions were as for fig.1.

added to an aerobic dark-adapted *bf* preparation, the cytochrome *f* and plastocyanin signals diminished in size considerably and the Rieske centre signal increased, i.e., all three of these components became at least partially reduced. This result is in agreement with the findings in [20]. We have noted that solutions of DBMIB (yellow in the oxidized form) are rapidly photoreduced by sunlight (to the colourless reduced form) and hence the finding that DBMIB may act as a reductant is not surprising. The result is consistent with our notions of the mechanism of electron transfer from quinols in model and biological systems [16].

We were unable to detect any effect DBMIB on the EPR spectrum of the Rieske centre (cf. fig.2C with 4B), and neither did any new signals appear. This is in marked contrast to the results in [20] where a dramatic change of the Rieske spectrum on addition of DBMIB was reported.

Antimycin A (20 μg/ml) had no effect on the EPR spectra of any of the five components which were observable.

4. Conclusions

1. In catalytically active cytochrome *bf* preparations, the following components may be detected by EPR: (a) cytochrome *f* as $g_z = 3.5$; (b) the Rieske centre as $g_y = 1.89$; (c) plastocyanin with $g_\perp = 2.05$; (d) tentatively identified, cytochrome *b*-563 with the high-spin haem signal around $g = 6$; (e) cytochrome *b*-559 with $g_z = 2.95$.
2. A catalytic amount of photosystem I is still present and this may be used to photooxidize cytochrome *f*, the Rieske centre and plastocyanin.
3. No binding sites for either DBMIB or antimycin A could be discerned.

Acknowledgement

The authors would like to thank the Science Research Council for financial support.

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