The electron paramagnetic resonance spectra of partially purified cytochrome b_6f complex from spinach

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In addition to the signals exhibited by cytochrome *f* and a Rieske-type iron–sulfur cluster, cytochrome b_6f preparations exhibit a broad asymmetric peak near $g \sim 3.7$ due to cytochrome-*b*-563 components and a free radical signal which may be a bound semiquinone. Signals near $g \sim 6$ and $g \sim 2.9$ correspond at least in part to denatured cytochrome *b*-563; this suggests the possibility of strained bis-histine ligation in the native cytochrome. UHDBT but not antimycin A has a strong specific effect on the spectra of the complex.

Cytochrome b_6f *EPR spectra Semiquinone bis-Histidine ligation UHDBT (Spinach)*

1. INTRODUCTION

The cytochrome b_6f complex in chloroplasts is believed to be involved in electron transfer either in the cyclic pathway or between photosystems I and II [1]. Its function as a quinol oxidase/plastocyanin reductase is believed to be analogous to the functions of the mitochondrial and photosynthetic bacterial, cytochrome·bc_1 complex bacteria, which transfers electrons from quinol to *c*-type cytochromes [2,3].

Bacterial and mitochondrial bc_1 complex contain one *c*-type cytochrome (cyt. c_1), a (2 Fe 2 S)-type iron–sulfur cluster (Rieske's center) and two spectroscopically and thermodynamically distinct *b*-type cytochromes, cytochrome *b*-566 and *b*-562 [4–8]. The cytochrome b_6f complex has been reported to contain 1 mol. Rieske-type iron–sulfur cluster, and 2 cytochrome *b*-563 hemes/cytochrome-*f* [2,10]. Cytochrome *f* is a high potential electron carrier with a low field EPR peak near $g = 3.5$ and an α-band near 554 nm [3,9,10]; cytochrome *b*-563 is reduced at lower potentials [9,10].

Abbreviations: UHDBT, 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole; EPR, electron paramagnetic resonance

It had been identified with high spin-type EPR features near $g \sim 6.0$ [2,3,11,12], while others have recognized that insufficient spin concentration is accounted for by these features.

This report describes the EPR spectra of the cytochrome b_6f complex, including several previously undetected features. Some important analogies between the mitochondrial and chloroplast systems are extended.

2. MATERIALS AND METHODS

Reagents were purchased from Sigma Chemicals and Calbiochem and were of the highest available quality. UHDBT was the gift of Dr Bernard Trumpower. Fresh spinach was obtained locally.

Cytochrome b_6f complex was purified from spinach as in [2]. The final sucrose density gradient was omitted in order to obtain the highest possible concentration and yield of cytochrome. We obtained similar results with material prepared following [10] as truncated in [3], but found the method in [2] more satisfactory because the yield was higher.

Concentrations of cytochromes were determined optically using a Johnson Foundation DBS-3 dual wavelength scanning spectrophotometer. For EPR

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experiments, concentrations (of cytochrome *f*) of at least 100 μM were used. This necessitated use of high inhibitor concentrations in some cases; because of this, effects might be observed from relatively high K_d ligands.

EPR spectra were recorded on Varian E-109E and E-9 spectrometers. Low temperatures were achieved with an Air Products flowing helium cryostat. Difference spectra were obtained with a Nicolet signal averager.

3. RESULTS

Fig.1(a,b) show the EPR spectra of cytochrome b_6f complex reduced with ascorbate and oxidized with 500 μM potassium ferricyanide. The oxidized spectrum shows peaks centered around $g \sim 6.0$ due to high spin ferric heme, and $g \sim 4.3$ due to ferric high-spin, low-symmetry iron. A broad peak in the $g \sim 12$ region may be due to the formation of high spin heme dimers, and a peak near $g \sim 5.0$ may be due to intermediate spin heme or may be a feature of a rhombic high spin heme species centered slightly below $g \sim 6.0$. While these features are fairly intense, transition probability and anisotropy considerations place their concentrations well below those of other chemical species with less intense spectral features. In addition, the intensities of these features vary from preparation to preparation. We therefore feel that these features do not reflect the components of native b_6f complex.

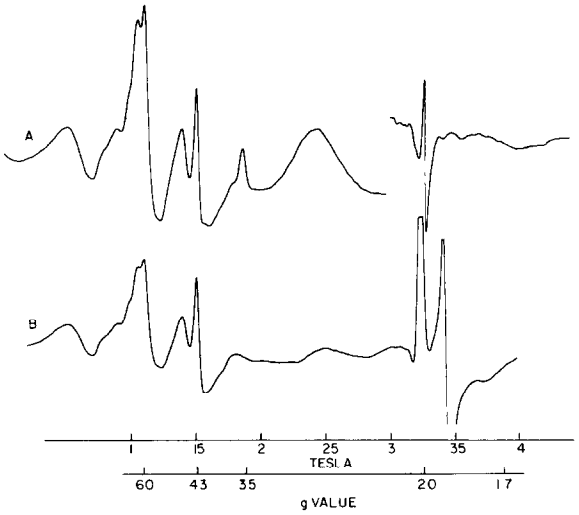

Fig.1. EPR spectra of cytochrome b_6f complex. Conditions were: sample temp., 13 K; microwave power, 20 mW; modulation amplitude, 20 G; scan time, 4 min; time constant, 0.25 s. The scan range was 0–0.4 T at about 3-times the gain. The samples were about 100 μM in cytochrome *f* (A): cytochrome *f* and Rieske's center were oxidized with 500 μM ferricyanide which gives rise to the observed $g \sim 2.6$ signal; (B) cytochrome *f* and Rieske's center were reduced with 1 mM ascorbate.

At $g = 3.5$ in the oxidized spectrum, a sharp peak due to cytochrome *f* is clearly visible. On the low field side of the peak a shoulder can be seen. Reduction of cytochrome *f* with ascorbate reveals a broad asymmetric peak with a maximum of $g \sim 3.7$. We assign this peak to cytochrome *b*-563, which resembles other low spin *b* cytochromes by optical criteria and had been identified with the high spin signals above.

The spectrum of the oxidized complex also includes a small feature above $g = 2.0$ probably due to Cu^{2+} or cubic high spin Fe^{3+} of adventitious origin. This signal was attributed to plastocyanin [3], which is a reasonable assignment. The sharp, heavily saturated free radical at $g \sim 2.00$ is probably due to a bound semiquinone. It is quenched by ascorbate or dithionite reduction and diminished on prolonged incubation with 1 mM ferricyanide. The intense peaks visible near $g \sim 2.02$, 1.9 and 1.77 in the ascorbate reduced spectrum are due to the Rieske-type Fe–S cluster.

The spectrum of the oxidized complex in the region between $g \sim 3.0$ and 4.0 is shown in fig.2a. The sharp peak is the low field feature of cytochrome *f*; the shoulder is contributed by cytochrome *b*-563. The low field trough is the negative excursion of the $g \sim 4.3$ feature seen in fig.1. The tail of the broad peak contributed by ferricyanide ($g \sim 2.6$) is visible at high field.

Fig.2b shows the EPR spectrum of the complex as isolated. It differs from fig.2a in that cytochrome *f* is 80% reduced. Reduction with 1 mM ascorbate (fig.2c) completely reduces cytochrome *f*. The remaining peak, at $g \sim 3.7$, is due to cytochrome *b*-563. The asymmetry of the peak is evident, with the high field side much broader than the low field side.

Addition of 500 μM antimycin A to ascorbate-reduced b_6f complex had a slight effect on the spectrum of cytochrome *b*-563 (fig.2d). The slight apparent narrowing of the peak is much less pro-

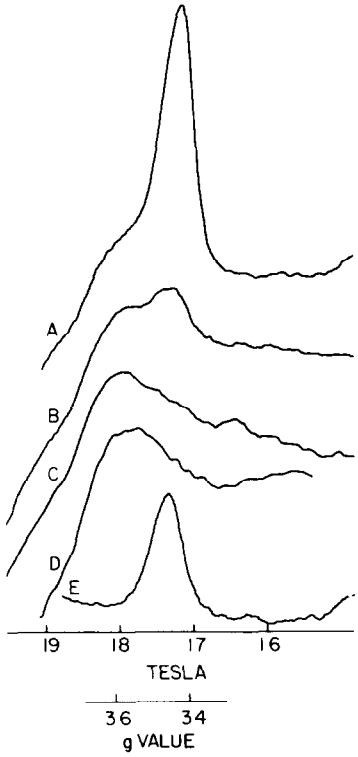

Fig.2. EPR spectra of the region between $g = 3$ and $g = 4$. Conditions were as in fig.1, except that the field was scanned from 0.13–0.23 T, and the gain was increased by a factor of 5: (a) as in fig.1(A); (b) b_6f complex as isolated; (c) as in fig.1(B); (d) as in fig. 1(B), with the addition of 0.5 mM antimycin A; (e) (a) minus (c).

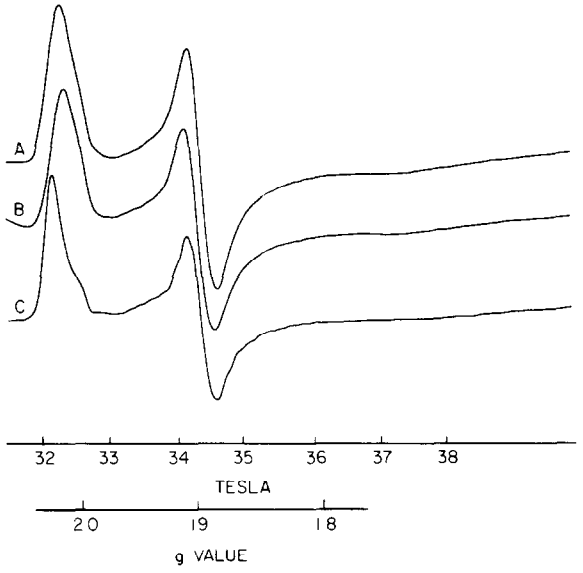

Fig.3. EPR spectrum of the $g = 2.0$ region in ascorbate-reduced b_6f complex. Conditions as in fig.1, except microwave power, 10 mW; scan, from 0.25–0.35 T: (a) as in fig.1(B); (b) as in fig.2(D); (c) as in (a) with the addition of 0.5 mM UHDBT.

nounced than the analogous effect of antimycin on mitochondrial cytochromes *b*. It may reflect an effect on a small portion of the cytochromes or a weak, nonspecific effect at this high concentration.

Fig.2e shows the difference spectrum corresponding to fig.2(a) minus (c). This is the low field peak of ferric cytochrome *f*. Of interest is the sharp, nearly symmetrical lineshape.

The spectra of the Rieske iron–sulfur cluster in ascorbate reduced samples is shown in fig.3A–C. The lineshape is similar but not identical to the corresponding species in mitochondrial preparations; in particular the trough at $g \sim 1.77$ is broader in the b_6f preparation than in cytochrome bc_1 complex. High (500 μM) antimycin levels lead to a slight shift in the position of the lines at $g \sim 2.02$ (upfield) and $g \sim 1.90$ (downfield); again these appear to be non-specific effects. Similar levels of UHDBT lead to a pronounced shift in the position of the $g \sim 2.02$ peak and a sharpening of the two low field features, as observed in mitochondrial, bacterial and chloroplast systems [13–15]. We observed a previously unreported shift to higher field of the $g \sim 1.77$ feature, which simultaneously broadened on addition of UHDBT. This response is the opposite of the effect seen in mitochondrial and chloroplast system. UHDBT had no observable effect on the spectra of the *b*-cytochromes, and diminished but did not completely quench the free radical observed in oxidized samples. We interpret these results to indicate that UHDBT has a pronounced, specific effect on the Rieske center while antimycin has a weak, non-specific effect at high concentrations, possibly mediated through another component in the complex.

Previous workers reported contamination of the preparation by cytochrome *b*-559, with an EPR peak near $g \sim 2.95$. We sometimes observe a peak in this region. Stepwise addition of Triton X-100 up to 10% leads to an increase in the intensity of this peak and a smaller decrease in the $g \sim 3.7$ species. This is expected for interconversion of the

two species, since the crystal field of the $g \sim 3.7$ species must be nearly axial and the transition probability much lower than for the $g \sim 2.9$ species. The latter species appears to be a denatured form of cytochrome *b*-563, although it may also contain some denatured cytochrome *b*-559. Partial denaturation of mitochondrial *b*-cytochromes yields similar species; these closely resemble bis-imidazole model complexes and suggest that the native cytochromes *b*-559 and *b*-563 have bis-histidine ligation. The spectral changes may result from changes in the heme site ligand geometry due to relaxation of strain imposed by the native protein conformation [16].

4. DISCUSSION

The above results show that cytochrome *b*-563 is low spin by EPR criteria. This is consistent with the optical spectrum which is typical of low spin *b*-cytochromes. The position of the peak, near $g \sim 3.7$, is comparable to the mitochondrial *b*-cytochrome peak positions near $g \sim 3.78$ (*b*-566) and 3.45 (*b*-562). The asymmetric lineshape, sharper on the low field side, is reminiscent of cytochrome *b*-566. However, the width of the feature is much wider, and in fact covers the spectral regions in which the two mitochondrial *b*-cytochrome peaks are observed.

The *b*-563 peak most closely resembles the spectrum of cytochrome *b*-562 and cytochrome *b*-566 in lipid-extracted succinate cytochrome *c* reductase (unpublished). In that preparation, the *b*-cytochrome peaks are broadened so that only a single asymmetric peak is observed. This suggests that there are two spectroscopically distinct forms of cytochrome *b*-563, and that the cytochrome b_6f complex may be substantially modified during purification, although it is not completely impossible that the native cytochrome *b*-563 is merely conformationally more flexible than other cytochromes. While these spectra are typical of our preparations, we have sometimes observed a double-peaked line in this region, although the components are never clearly resolved as in mitochondrial succinate–cytochrome *c* reductase.

In [17], cytochrome *b*-563 titrated potentiometrically as two components in a modified version of their cytochrome b_6f preparation. Cytochrome *b*-563 titrated as a single component at -70 to -90 mV in [3,7,10]. Our optical titrations gave two major components with midpoint potentials of -50 and $+70$ at pH 7.3. While these results suggest that 'cytochrome *b*-563' consists of two distinct cytochromes, several factors cloud the issue. The EPR-monitored titrations in [3] measured the high spin heme component, which probably does not correspond to native cytochrome *b*-563. In addition, a variable amount of $g \sim 2.9$ species is observed corresponding to denatured *b* cytochrome. Both these components will show up in an optically monitored titration; however, the EPR spectrum of cytochrome *b*-563 tends to support the two-component hypothesis.

However, cytochrome *f* exhibits a singularly narrow peak at $g \sim 3.5$. The nearly symmetrical resonance was relatively insensitive to denaturation by moderate concentrations of Triton X-100 (2–3%) which increased the $g \sim 2.9$ signal. The broad intermediate *g*-value of cytochrome *f* was detected near $g \sim 1.7$. Unfortunately, for $g \sim 3.5$ the position of this intermediate feature is relatively insensitive to crystal field. The value of the tetragonal crystal field component could be anywhere between $\sim 2.5\ \lambda$ (the spin orbit coupling constant) and $4.0\ \lambda$ even assuming that the orbital reduction factor $K \sim 1.0$.

The free radical signal observed in samples after the addition of ferricyanide may correspond to bound semiquinone species analogous to those observed in the mitochondrial bc_1 complex [18–22]. The cytochrome b_6f complex is clearly partially reduced as isolated. Addition of a roughly stoichiometric amount of ferricyanide might be expected to produce either turnover conditions or a related mixed valence steady state. Under similar conditions both antimycin-sensitive and antimycin-insensitive radicals can be observed in the bc_1 complex. Additional experimentation is needed to prove that the radical is a semiquinone species and to determine its properties.

These observations strengthen and extend the analogy between the chloroplast and mitochondrial cytochrome '*bc*' type complexes. Clearly, the analogy is imperfect since the b_6f complex has fewer subunits and lacks antimycin sensitivity. It is unclear whether the differences are inherent or merely reflect increased fragility in the cytochrome b_6f complex.

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REFERENCES

[1] Bouges-Bocquet, B. (1980) FEBS Lett. 117, 54–58.
[2] Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 591–599.
[3] Puch, P.R., Neathcole, P., Evans, M.C.W. and Bendall, D.S. (1980) FEBS Lett. 116, 51–56.
[4] Rieske, J.S. (1976) Biochim. Biophys. Acta 456, 195–247.
[5] Malkin, R. and Aparcio, P.J. (1975) Biochem. Biophys. Res. Commun. 63, 1157–1160.
[6] Whitmarsh, J. and Cramer, W.A. (1979) Proc. Natl. Acad. Sci. USA 76, 4417–4420.
[7] Prince, R.C., Lindsay, J.G. and Dutton, P.L. (1975) FEBS Lett. 51, 108–111.
[8] Wilson, D.F. and Dutton, P.L. (1970) Biochem. Biophys. Res. Commun. 39, 59–64.
[9] Rich, P.R. and Bendall, D.S. (1980) Biochim. Biophys. Acta 591, 153–161.
[10] Nelson, N. and Neumann, J. (1972) J. Biol. Chem. 247, 1817–1824.
[11] Nugent, J.H.A. and Evans, M.C.W. (1980) FEBS Lett. 112, 1–4.
[12] Warden, J.T. (1977) Biophys. J. 17, 197a.
[13] Bowyer, J.R., Dutton, P.L., Prince, R.C. and Crofts, A.R. (1980) Biochim. Biophys. Acta 592, 445–460.
[14] Bowyer, J.R. and Trumpower, B.L. (1980) FEBS Lett. 115, 171–174.
[15] Hurt, E., Hauska, G. and Malkin, R. (1981) FEBS Lett. 134, 1–5.
[16] Salerno, J.C., Kelley, S.E. and Ohnishi, T. (1982) Biophys. J. 37, 401a.
[17] Hurt, E. and Hauska, G. (1982) J. Bioenerget. Biomembr. 14, 405–424.
[18] Siedow, J.N., Power, S., De la Rosa, F.F. and Palmer, G. (1978) J. Biol. Chem. 253, 2392–2399.
[19] Ohnishi, T. and Trumpower, B. (1980) J. Biol. Chem. 255, 3278–3284.
[20] Salerno, J.C. and Ohnishi, T. (1978) in: Frontiers of Biological Energetics (Dutton, P.L. et al. eds) vol.1, pp.191–200, Academic Press, New York.
[21] Konstantinov, A. and Ruuge, E.K. (1977) FEBS Lett. 81, 137–141.
[22] DeVries, S., Berden, J.A. and Slater, E.C. (1982) in: Function of Quinones in Energy Conserving Systems (Trumpower, B. ed) pp.235–246, Academic Press, New York.