The EPR spectrum of cytochrome *b*-563 in the cytochrome *bf* complex from spinach

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Combined optical and EPR studies show that the two cytochromes *b*-563 in the isolated cytochrome *bf* complex from spinach chloroplasts both are in the low-spin state with g_z about 3.5. They have slightly different g_z-values and dissimilar reduction potentials.

Cytochrome b-*563* *Cytochrome* bf *complex* *EPR* *Cytochrome* *Chloroplast*

1. INTRODUCTION

EPR has greatly contributed to our understanding of the electron transport chains in photosynthetic systems. However, its use in the study of photosynthetic cytochromes in chloroplasts has been rather limited due to the relatively low EPR sensitivity for hemes. Only for cytochrome *f* and high-potential cytochrome *b*-559 have EPR signals been unambigously demonstrated [1]. From work on crude [2] and improved [3] preparations of the cytochrome *bf* complex it has been proposed that cytochrome *b*-563 gives rise to a high-spin signal, a suggestion that has been questioned on the basis of the low integrated intensity of this signal [4]. Here, where the cytochrome *bf* complex is studied at high concentration for improved signal to noise ratio, it is shown that the two cytochromes *b*-563 in the complex are both low-spin hemes with g_z-values of around 3.5.

2. MATERIALS AND METHODS

The cytochrome *bf* complex was prepared from spinach as in [5] and its activity was measured [3] in an assay mixture containing 40 μM plastoquinol-9 (a gift from Hoffman-La Roche through Roche AB, Stockholm) and 10 μM plastocyanin. The activity was found to be 21 μmol plastocyanin reduced/nmol cytochrome *f* per h.

Reductive titrations with sodium dithionite solutions were performed in a glass vessel flushed with argon and fitted with a combination Pt-Ag/AgCl electrode and a 2-mm optical cell [6]. As the titration proceeded, EPR samples were withdrawn and frozen. The titration mixture contained 60 μM cytochrome *bf* complex (measured as cytochrome *f*), 30 mM Tris–succinate (pH 6.5), 30 mM octylglycoside, 12.5 mM sodium cholate, 0.1% (w/v) soybean lecithin (Sigma Type II-S) and about 30% (w/v) sucrose. To this solution was added 20 μM each of *p*-benzoquinone, tetramethyl-*p*-phenylenediamine, tetramethyl-*p*-benzoquinone, 2,5-dihydroxy-*p*-benzoquinone, 2-hydroxy-1,4-naphthoquinone and 40 μM phenazine methosulfate. In one experiment 43% (v/v) glycerol was present in the titration mixture.

Room temperature optical spectra were recorded on a Beckman Acta MIV double beam spectrophotometer and low temperature optical spectra on a Johnson Research Foundation type DBS-1 dual wavelength scanning spectrophotometer. EPR spectra were recorded with a Varian E-9 spectrometer equipped with an Oxford Instruments

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ESR-9 helium flow cryostat. Concentrations of cytochromes were determined from EPR spectra as in [4].

3. RESULTS

Optical and EPR spectra in a reductive titration of the cytochrome *bf* complex, also containing the Rieske Fe-S center, are shown in fig.1,2. In the EPR spectrum of the oxidized sample (fig.2a) there are peaks due to high- and low-spin heme Fe(III) at $g = 5–6$ and 3.3–3.8, respectively, unidentified peaks at $g > 7$ and 4.5–5, a peak corresponding to a negligible amount of so-called rhombic Fe(III) at $g = 4.2$, and minor peaks around $g = 2$ due to a small fraction of reduced Rieske Fe-S protein and a Cu(II) impurity. At lower reduction potentials, about 160 mV, where cytochrome *f* is reduced (fig.1b), the narrow EPR peak at $g = 3.5$, previously assigned to this cytochrome [1,7], disappears and the signal of the reduced Rieske center appears ($g = 1.9–2.0$), with only small changes in the other signals. In particular, a broad low-spin heme component at $g = 3.5$ remains. At still lower potentials, about 100 mV, with very little further reduction of the cytochromes (fig.1c) most of the peaks at $g > 4.5$ vanish with insignificant reduction of the broad $g = 3.5$ peak. Finally, at full reduction of all cytochromes (fig.1d) all heme EPR signals disappear (fig.2d). No significant semiquinone radical signal was observed during the titration.

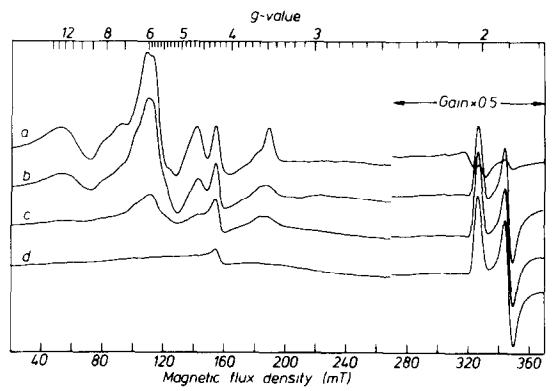

Fig.2. EPR spectra of the cytochrome *bf* complex. Traces a–d correspond to the oxidation states in fig.1. EPR conditions were: Temperature, 15 K, microwave power, 20 mW, microwave frequency, 9.23 GHz, and modulation, 3.2 mT.

Fig.3 shows that the disappearance of the broad $g = 3.5$ peak is proportional to the appearance of the absorption due to reduced cytochromes *b*-563, whereas there is no correlation between the high-spin heme EPR signal to the cytochrome *b*-563 absorption. There is also a small shift to higher *g*-value from $g = 3.52$ to $g = 3.57$ as reduction proceeds (fig.4).

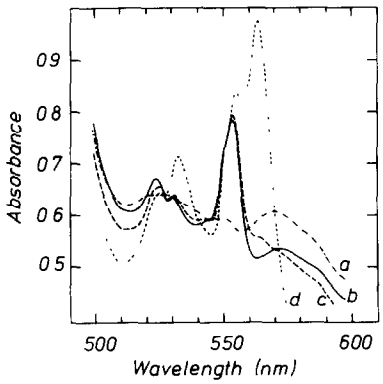

Fig.1. Optical spectra of the cytochrome *bf* complex in a reductive titration. In: a (-·-·-) the complex is oxidized; b (——) cytochrome *f* is reduced; c (– – –) a small fraction of cytochrome *b*-563 is also reduced; d (·····) both cytochromes are fully reduced.

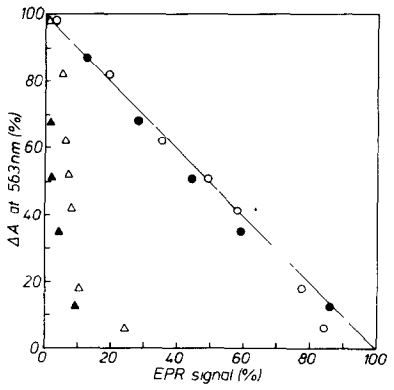

Fig.3. Change in the absorbance at 563 nm *vs* the intensity of the EPR peak at $g = 6$ (△, no glycerol; ▲, 43% glycerol) and the broad peak at $g = 3.5$ (○, no glycerol; ●, 43% glycerol). In all samples cytochrome *f* was fully reduced.

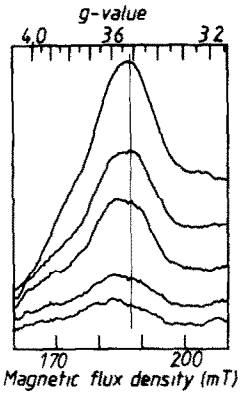

Fig.4. Change in shape of the broad $g = 3.5$ EPR peak in a reductive titration. The spectra have no contribution from cytochrome *f* which was reduced already in the top spectrum. The spectrum of the fully reduced sample has been subtracted from all spectra shown.

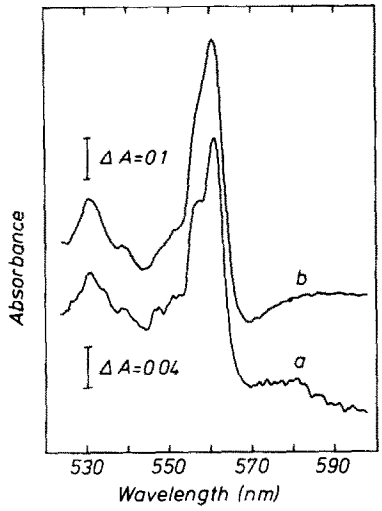

Fig.5. Low temperature optical absorption spectra in a reductive titration. Spectrum a represents the difference between a spectrum with cytochrome *b*-563 reduced to about 35% and a reference with only cytochrome *f* reduced. Spectrum b shows the difference between the fully reduced sample and the same reference. The samples, with 43% glycerol added to the medium, were contained in EPR tubes and spectra were recorded at 80 K with an optical bandwidth of 1.2 nm. Due to different light-scattering properties of the samples, the amplitudes are not directly comparable.

A similar reductive titration of the cytochrome *bf* complex was made with a medium containing 43% glycerol, which permitted low-temperature optical absorption and EPR spectra to be recorded from the same sample. During the initial stages of the reduction of cytochrome *b*-563, the α-band is split into two peaks with maxima at 561 and 557 nm (fig.5a), whereas at full reduction, the low wavelength peak is discernible only as a weak shoulder (fig.5b). The development of the split α-band occurs simultaneously with the shift in g-value of the $g = 3.5$ peak (fig.4) in the EPR spectrum, although the shift is less pronounced in the glycerol-containing medium (not shown).

The amount of the Rieske center was determined at non-saturating EPR conditions from the $g = 2.0$ peak, that of cytochrome *f* from the narrow $g = 3.5$ peak in a difference spectrum between fig.2a and b and that of low-spin heme with the broad peak at $g = 3.5$ from a difference spectrum between fig.2b and d. The relative concentrations were 1 : 1 : 1 : 8. From the optical spectra in fig.1 the ratio of cytochrome *b*-563 to *f* was 1.8.

4. DISCUSSION

In the initial phase of the reductive titration of the cytochrome *bf* complex, cytochrome *f* and the Rieske Fe-S center were reduced (fig.1,2). This is in agreement with the known reduction potential of these acceptors. In the potential region 200–100 mV all the EPR signals with g-values larger than 4.2 disappear, but only very small optical changes typical of heme reduction are seen during this phase of the experiment (fig.1–3). Thus, these signals, although with a considerable amplitude, do not correspond to a significant fraction of the cytochromes, and, in particular, they cannot represent cytochrome *b*-563 in the isolated complex. From quantitative estimates this conclusion was earlier reached for the high-spin signal at $g = 6$ [1,4], but now it can be extended to all signals other than those of low-spin heme type. In fact, the close correspondence (fig.3) between the optical changes at 563 nm and intensity of the broad EPR peak at $g = 3.5$ identifies cytochrome *b*-563 as a low-spin heme. Also, the relative concentration of the cytochrome, determined from EPR spectra and based on this assignment, to those of

cytochrome *f* and the Rieske center, is in agreement with [3,5].

The change in shape of the cytochrome *b*-563 EPR peak as reduction occurs (fig.4) indicates that the two cytochromes *b*-563 are slightly different with dissimilar reduction potentials. The band-shape changes in the low-temperature optical spectra, recorded during the reduction of cytochrome *b*-563 (fig.5), provide additional evidence of two separate spectroscopic and potentiometric forms of cytochrome *b*-563 in the cytochrome *bf* complex. This is in agreement with a recent report that the cytochromes *b*-563 have different potentials and low-temperature optical spectra [8].

The present findings accentuate the similarities between the cytochrome *bf* complex and the bc_1-fragment of mitochondria [9,10]. Both have a Rieske Fe-S center, and cytochrome *f* with $g = 3.5$ corresponds to cytochrome c_1 with $g = 3.35$ and 3.49 in beef heart and yeast, respectively. The *b* cytochromes are in all cases low-spin, with a high-potential form at $g = 3.5$–3.6 and a low-potential form with $g = 3.6$–3.8. Functionally these two complexes may, therefore, be closely related.

ACKNOWLEDGEMENT

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