The EPR spectrum and orientation of cytochrome *b*-563 in the chloroplast thylakoid membrane

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The EPR spectrum of cytochrome *b*-563 in spinach chloroplasts shows that both hemes of the cytochrome are in a low-spin state with $g_z = 3.5$. The orientation of the two heme planes is found to be perpendicular to the thylakoid membrane plane in magnetically aligned chloroplasts. This may be relevant for the function of cytochrome *b*-563, e.g., in electron transport coupled to proton translocation.

Cytochrome b-*563* *Cytochrome* *Heme orientation* *EPR* *Chloroplast*

1. INTRODUCTION

The use of EPR in the study of cytochromes in the thylakoid membrane of chloroplasts has been rather limited due to the relative low EPR sensitivity for hemes. For high- and low-potential cytochrome *b*-559 and cytochrome *f*, EPR signals have previously been demonstrated, but no EPR signal from cytochrome *b*-563 was assigned [1,2]. It was proposed, however, that cytochrome *b*-563 is in a high-spin state with an EPR signal at $g = 6$ from studies on a partially [3] or highly [4,5] purified cytochrome *bf* complex, on sub-chloroplast particles [6], and on chloroplasts [7–9]. For chloroplasts this was questioned due to the low integrated intensity of the high-spin signal [1,2], and with a highly purified cytochrome *bf* complex [10,11] it was shown that cytochrome *b*-563 is low-spin with $g_z = 3.5$.

Here it is shown that in chloroplasts cytochrome *b*-563 is also low-spin. Furthermore, like cytochrome *b*-559 [2] but unlike cytochrome *f* [2], the orientations of its heme planes are found to be perpendicular to the membrane.

Abbreviations: E_{m7}, midpoint reduction potential at pH 7 relative to a standard hydrogen electrode; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)

2. MATERIALS AND METHODS

Broken spinach chloroplasts were prepared as follows. Fresh leaves were ground in a Waring Commercial Blendor in a medium consisting of 400 mM sorbitol, 50 mM Pipes-NaOH, pH 7.0, 10 mM NaCl, and 1 mM EDTA (medium I). After centrifugation, the chloroplasts were washed and centrifuged twice in 50 mM Pipes-NaOH, pH 7.0, 10 mM NaCl, and 1 mM EDTA (medium II). Finally, the chloroplasts were washed once in a medium consisting of 20 mM Pipes-NaOH, pH 7.0, 15 mM NaCl, and 5 mM $MgCl_2$ (medium III), centrifuged, and resuspended in the same medium. Reductive titrations with sodium dithionite were performed in weak light in a glass vessel flushed with argon and fitted with a combination Pt-Ag/AgCl electrode. The titration mixture contained chloroplasts in medium III with a chlorophyll concentration around 7 mg/ml with 20 μM each of *p*-benzoquinone, tetramethyl-*p*-phenylenediamine, tetramethyl-*p*-benzoquinone, 2,5-dihydroxy-*p*-benzoquinone, 2-hydroxy-1,4-naphthoquinone and phenazine metosulfonate as mediators. As the titration proceeded, EPR samples were withdrawn, centrifuged at 3500 × *g* for 10 min in sealed EPR tubes to increase the concentration of the chloroplast suspension, and frozen.

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For orientation studies the chloroplasts were prepared as above with the following exceptions. To medium II and III 350 mM sorbitol was added and after the last resuspension 25% (v/v) ethylene glycol was added. The magnetic field alignment of the thylakoid membranes was performed as in [2].

EPR spectra were recorded with a Varian E-9 spectrometer equipped with an Oxford Instruments ESR-9 helium flow cryostat. Concentrations of cytochromes and photosystem I reaction centers were determined from EPR spectra as in [2]. The EPR spectra of the chloroplast samples have been corrected for the presence of rhombic iron at $g = 4.3$ by subtraction of a simulated isotropic $g = 4.3$ signal to obtain a straight baseline.

3. RESULTS

Fig.1 shows the EPR spectra of chloroplasts at different reduction potentials in the g_z region of low-spin heme. The right-most peak at $g = 2.9$–3.0 represents low-potential cytochrome *b*-559 [1,2]. The integrated intensity of the left-hand peak, $g = 3.5$, was around 1.8 per photosystem I reaction

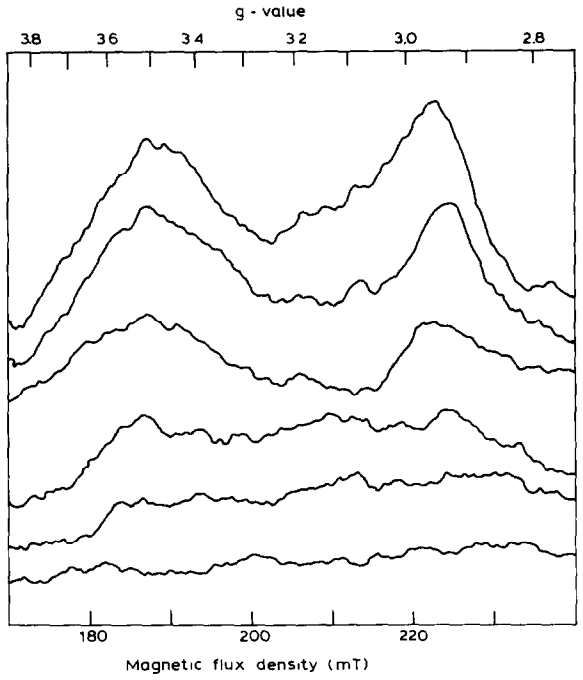

Fig.1. EPR spectra of chloroplasts in a reductive titration. The spectrum of a simulated isotropic $g = 4.3$ signal has been subtracted from all spectra. EPR conditions were: temperature, 10 K, microwave frequency, 9.22 GHz, microwave power, 20 mW; modulation, 3.2 mT. Chlorophyll concentration was 12 mg/ml.

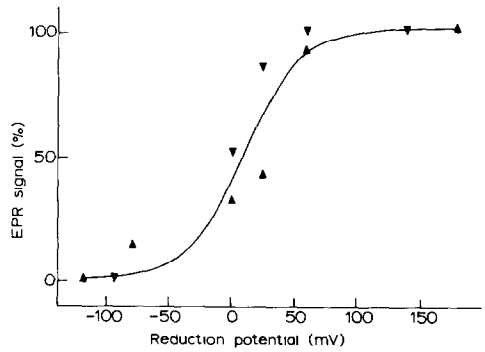

Fig.2. Reductive titration of two different chloroplast preparations (▲, ▼). The ordinate shows the integrated EPR intensity under the $g = 3.5$ peak and the abscissa the reduction potential at pH 7 relative to the standard hydrogen electrode. The curve represents the theoretical Nernst behavior with $n = 1$ and $E_{m7} = +5$ mV.

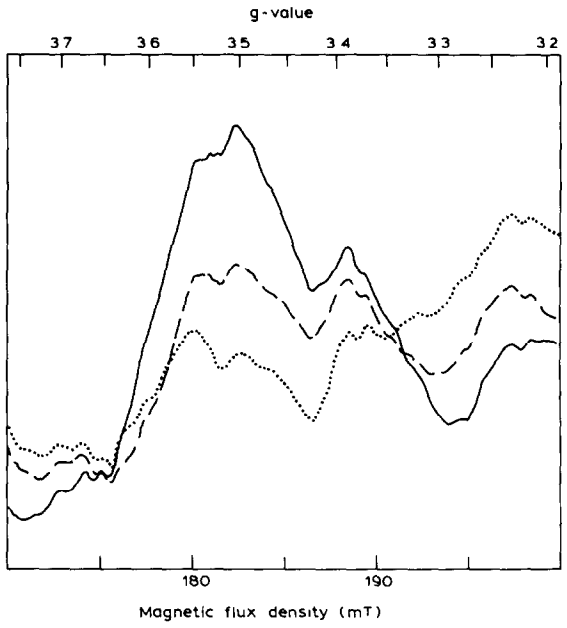

Fig.3. EPR signal of cytochrome *b*-563 in aligned chloroplasts. The angle between the spectrometer field and the membrane normal was 0 (···), 45 (– – –), and 90° (——). EPR conditions were: temperature, 8 K, microwave frequency, 9.22 GHz, microwave power, 80 mW; modulation, 3.2 mT. Chlorophyll concentration was 9 mg/ml.

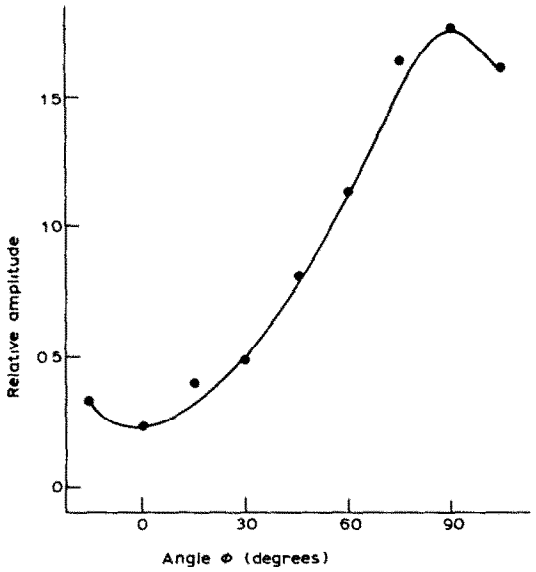

Fig.4. Plot of the amplitudes of the $g_z = 3.5$ peak vs the angle ϕ between the magnetic field and the membrane normal. The amplitudes have been normalized with respect to the amplitude of the peak from a non-aligned sample.

center. There is no contribution to the spectra from cytochrome *f* (E_{m7} = 385 mV [12]) which was already reduced in the top spectrum. Fig.2 shows that the reduction of the $g = 3.5$ species is a one-electron reaction with an E_{m7} around +5 mV.

Figs 3 and 4 illustrate the orientation dependence of the broad $g = 3.5$ signal. The peak has its highest and lowest amplitude when the membrane normal is perpendicular and parallel to the magnetic field, respectively.

4. DISCUSSION

Here it is shown that both hemes of cytochrome *b*-563 are in a low-spin state in chloroplasts with a g_z value around 3.5 in agreement with the isolated cytochrome *bf* complex [11]. The amount of low-spin cytochrome *b*-563, 1.8 per photosystem I, also agrees with published data from optical studies of chloroplasts [13] and the isolated cytochrome *bf* complex [5,11]. The midpoint potential (just above 0 mV, see fig.2) of the $g_z = 3.5$ component indicates that this represents neither cytochrome *f* (E_{m7} = 385 mV [12]) nor low-potential cytochrome *b*-559 ($E_{m7} \geqslant +20$ mV [14]) but titrates in agreement with previously published potentials of cytochrome *b*-563 (E_{m7} = 0 mV [15] or +5 mV [16]), although lower potentials have been reported, e.g. [14] (recent review [17]).

The sloping baseline caused by the large $g = 4.3$ signal and the low signal intensity from low-spin hemes with broad lines and $g_z \geqslant 3.5$ make such heme signals difficult to detect. This may have been the reason why so many EPR investigators did not recognize the cytochrome *b*-563 signal but instead assigned the high-spin $g = 6$ signal to this cytochrome. However, the high-spin signal in chloroplasts is too small to represent a significant fraction of cytochrome *b*-563 [1,2].

The aligned chloroplasts show the strongest g_z peak of cytochrome *b*-563 when the membrane planes are parallel to the magnetic field. Thus, in analogy with studies on other membrane-bound cytochromes [2,18], the heme planes are oriented perpendicular to the membrane plane. In this analysis the contribution of the cytochrome *bf* complex situated in the margins of the grana stacks has been neglected. This is justified by the small surface area of such margins. Furthermore, the high degree of orientation shows that most of the cytochrome *b*-563 is oriented in the same direction.

The obtained orientation is also supported by recent theoretical predictions from the amino acid sequence [19]. In an early study on cytochrome *b*-563 in chloroplasts (assigning high-spin $g = 6$ signal to cytochrome *b*-563) the angle was estimated to be 50° [7]. This discrepancy is probably due to an altered orientation of the heme in a denatured cytochrome.

The perpendicular orientation of spinach chloroplast cytochrome *b*-563 is the same as that of the hemes of cytochrome *b* of pigeon breast mitochondria [18] and may be relevant for the function of the two hemes of cytochrome *b*-563 in electron transport coupled to proton translocation across the membrane in a Q-type [20–22] or *b*-type [23] mechanism (recent discussion [24]).

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