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EPR SIGNALS AND ORIENTATION OF CYTOCHROMES IN THE SPINACH CHLOROPLAST THYLAKOID MEMBRANE

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The cytochromes in spinach chloroplasts were studied using EPR spectroscopy. In addition to the low-spin heme signals previously assigned, cytochrome *f* (g_z 3.51), high-potential cytochrome *b*-559 (g_z 3.08) and cytochrome *b*-559 converted to a low-potential form (g_z 2.94), a high-spin heme signal was induced by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). However, this signal cannot be due to cytochrome *b*-563 in its native form. The orientation of the cytochromes in the thylakoid membrane was studied in magnetically oriented chloroplasts. Cytochrome *b*-559 in the native state and in the low-potential form was found to have its heme plane perpendicular to the membrane plane. The orientation was the same for cytochrome *b*-559 oxidized by low-temperature illumination, which suggests that also the reduced heme is oriented perpendicular to the membrane.

Introduction

In the electron-transport system of chloroplasts of higher plants, four different cytochromes have been identified by optical methods, cytochromes *f*, *b*-563, and high- and low-potential forms of cytochrome *b*-559. The cytochromes of photosynthetic bacteria have been studied in some detail by EPR [1,2], whereas the plant cytochromes only recently were detected by EPR [3–5], because of the relatively low sensitivity of this technique for cytochromes. Cytochrome *f* and the high- and low-potential forms of cytochrome *b*-559 were identified [3] as low-spin hemes with g_z 3.5, 3.1 and 2.9, respectively. Cytochrome *b*-563 was reported to be high spin [5].

This report describes EPR signals of low-spin cytochromes in chloroplast. High-spin signals in untreated chloroplasts and induced by quinone-type oxidants cannot be associated with any cytochrome in its native form. The orientation of the cytochromes has been studied in magnetically oriented thylakoid membranes. The heme plane of cytochrome *b*-559 is found to be oriented perpendicular to the membrane plane under all conditions whereas cytochrome *f* has an oblique orientation.

Materials and Methods

Preparation. Broken spinach chloroplasts were prepared in the following way. Fresh spinach leaves were ground in a Waring Commercial Blendor for two periods of 5 s in a grinding medium consisting of 350 mM sucrose, 50 mM Tricine-KOH, pH 7.5, 10 mM NaCl and 0.5 mM EDTA (medium I). The homogenate was strained through four layers of 20 mesh nylon cloth and centrifuged at 3000 × *g* for 2 min. The pellet was then suspended in a medium

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; Tricine, *N*-tris(hydroxymethyl)methylglycine; PS, photosystem.

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consisting of 50 mM Tricine-KOH, pH 7.5, 10 mM NaCl and 0.2 mM EDTA (medium IIa). The suspension was centrifuged at 4000 × *g* for 5 min and the chloroplast fragments were resuspended in the same medium to a chlorophyll concentration of 7–10 mg/ml as determined by the method of Arnon [6].

For orientation studies the chloroplasts were prepared as follows. The leaves were ground, strained and centrifuged as above. The pellet was then suspended in a medium consisting of 350 mM sucrose, 100 mM Tricine-KOH, pH 7.5, 20 mM NaCl and 0.2 mM EDTA (medium IIb). After centrifugation at 4000 × *g* for 3 min, the chloroplasts were resuspended in the same medium and ethylene glycol was added (25%, v/v) to a final chlorophyll concentration of 7–10 mg/ml.

Samples oxidized with ferricyanide were prepared by adding 10 mM $K_3Fe(CN)_6$ to medium IIa in which chloroplast fragments were suspended and kept at 0°C for 30 min. The samples were then washed and centrifuged twice in medium IIa and the fragments were resuspended in the same medium.

The Tris-washed samples were prepared by resuspending the chloroplast fragments in 0.8 M Tris-HCl, pH 8.0. The suspension was kept on ice for 30 min, centrifuged and resuspended in the same medium. EDTA to a concentration of 0.5 mM was added.

Oxidation by DDQ and 1,4-benzoquinone was obtained by adding the chemicals to the chloroplasts in EPR tubes to a final concentration of 5 mM of each.

Orientation of thylakoid membranes was achieved by exposing the chloroplast samples for 2–3 min to a magnetic flux density of 1.4 T before cooling in the magnetic field to liquid nitrogen temperature. The degree of orientation was judged from a sample reduced by dithionite and frozen in intense white light [7].

EPR spectroscopy. EPR measurements were made at 9.25 GHz with a Varian E-9 spectrometer equipped with an Oxford Instruments ESR-9 helium flow cryostat. Spectra were recorded with the samples kept in darkness at about 20 K at a nonsaturating microwave power of 25 mW and a modulation amplitude of 3.2 mT. The spectrometer was connected on-line to a Nova minicomputer [7].

Integration and simulation of EPR signals. EPR measurements can be used for determination of the concentration of paramagnetic species through comparison with a known standard. The total intensity of a rhombic powder spectrum can be calculated from the area under the g_z 'absorption peak' in the first-derivative spectrum [8]. This area is related to the intensity of the signal through a proportionality factor which is a function of the three *g* values, g_z, g_y and g_x. For chloroplast samples in general only one or two *g* values can be located, expecially if g_z is greater than 3.3. Based on the theoretical prediction that the sum of the squares of the three *g* values should be near 16, the proportionality factor can be rearranged into an approximative form, a function of g_z exclusively [9].

The concentration of high-spin heme, which has a strong EPR signal around *g* 6, can be determined through comparison of the double integral over a certain part of the spectrum with a known standard [10]. For chloroplast samples, there is some difficulty in integrating the *g* 6 signal directly because of overlapping signals from other paramagnetic species. Quantification is in this case better made by simulation of high-spin signals and comparison with a known standard.

Cytochrome *c* and myoglobin were used as standard samples for the determination of absolute concentrations of low- and high-spin heme, respectively. Concentrations relative to that of the PS I reaction center were obtained from a comparison with the Fe-S signals from samples, fully reduced by illumination with strong light in the presence of dithionite (cf. Ref. 7).

Results and Discussion

The high-spin heme EPR signals

The peaks in the region *g* 5.4–6.6 (Fig. 1) are conceivably due to high-spin heme Fe^{3+}. In untreated (Fig. 1a) or ferricyanide-treated chloroplasts (Fig. 1b), the amount of high-spin heme was found small and was estimated to be less than 0.1 Fe^{3+} per PS I reacion center.

If DDQ ($E_{m,7} > +550$ mV) is added to chloroplasts a relatively large high-spin signal is observed (Fig. 1d) with a shape very similar to that reported by Rich et al. [5]. The intensity increases with the

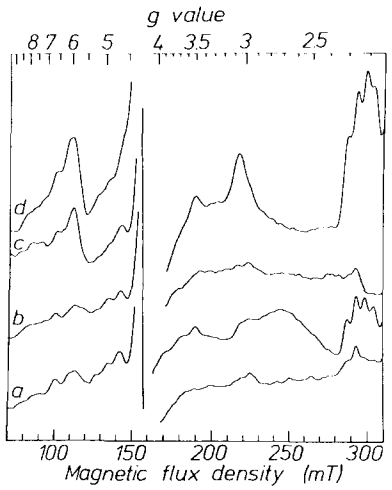

Fig. 1. EPR spectra of dark-adapted chloroplast fragments after different treatments. Chlorophyll concentration, 10 mg/ml. Additions: (a) none, (b) ferricyanide (see Materials and Methods), (c) 5 mM 1,4-benzoquinone, (d) 5 mM DDQ.

DDQ concentration (Fig. 2), amounting to 0.6 Fe^{3+} per PS I reaction center at a 1000-fold excess of DDQ. Concomitantly, the g 4.3 iron signal increases. Benzoquinone ($E_{m,7} = 260$ mV) also induces a signal of the same kind (Fig. 1c). This signal has been associated with cytochrome b-563 [5]. However, this cytochrome should be oxidized already in the untreated chloroplasts. Note, that

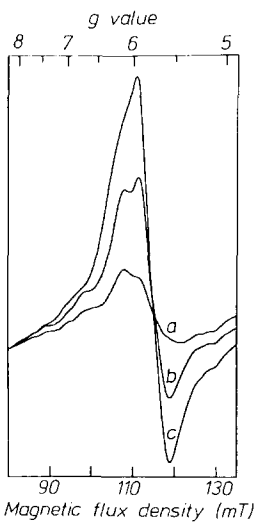

Fig. 2. High-spin heme EPR signals from broken chloroplasts at three different DDQ concentrations. (a) 1 mM, (b) 5 mM and (c) 25 mM. Chlorophyll concentration, 8 mg/ml.

the ferricyanide-treated sample (Fig. 1b), which is at a potential high enough to show some cytochrome f oxidized, also shows little high-spin signal. Thus, the DDQ-induced signal cannot represent the 'native cytochrome' b-563. It also follows that even if the quantification of these signals is difficult, the high-spin signal observed with no addition (Fig. 1a) cannot represent a significant fraction of cytochrome b-563. On the other hand, it cannot be excluded that quinones alter the heme environment, so that cytochrome b-563 gives a signal in the g 6 region. An interaction between quinones and cytochrome b-563 is indeed supported by recent studies of the electron-transport system functioning between the two photosystems [11].

Of the other peaks at g values larger than 4.5, the features at g 4.7 and 6.7 are the most prominent. They may be due to high-spin heme with large rhombicity, but no quantitative estimate has been made. It is possible that they are associated with cytochrome b-563 in its native form.

The low-spin heme EPR signals

In the region g 2.8–3.6, various peaks, associated with low-spin heme, appear in the EPR spectra of chloroplasts (Fig. 1). The cytochrome b-559 signal induced by light at low temperature has in addition to the previously reported g_z peak at 3.08 [3] a g_y peak at 2.16. Oxidation with DDQ gave signals (Fig. 1d) from cytochrome f and high-potential cytochrome b-559 as previously reported.

The determinations of low-spin heme concentrations are uncertain, since all g values are not known and the signal-to-noise ratio is relatively poor. On the whole, the ratio of the amounts estimated for cytochrome f and high-potential cytochrome b-559 are consistent with the prevailing view of one cytochrome f and two high-potential cytochromes b-559 per PS I reaction center, whereas the absolute concentrations tend to be lower by a factor of 1.5–2. Low-temperature photooxidation of high-potential cytochrome b-559 always yielded less than one cytochrome oxidized per PS I center, in part due to incomplete photooxidation of the optically very dense sample.

The orientation of the cytochromes

Magnetic field-induced orientation of thylakoid

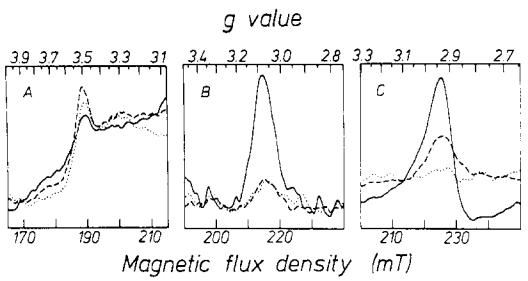

Fig. 3. Low-spin EPR signals from oriented chloroplasts. Treatments: (a) DCMU + methyl viologen, illuminated during freezing, (b) no addition, difference spectrum, light-minus-dark at 20 K, (c) Tris washed. The angle between the spectrometer field and the membrane normal was 0 (· · · · ·), 45 (- - - -) and 90° (———)

membranes, described in Ref. 12, has earlier been used in combination with EPR to study the orientation and interaction of iron-sulfur centers in chloroplasts [7,13] and in combination with linear dichroism to study the orientation of high-potential cytochromes [14]. A preliminary report of the orientation studies with EPR, described in this work, has already been presented [15].

Fig. 3 illustrates the orientation studies of the low-spin signals. Fig. 3A shows the g 3.51 signal from cytochrome *f*, Fig. 3B shows the g 3.08 signal of high-potential cytochrome *b*-559, and the peak in Fig. 3C comes from cytochrome *b*-559 converted to a low-potential form. The signals all show an orientation dependence, consistent with the view that the cytochromes are membrane bound with a fixed geometrical relationship relative to other electron carriers to facilitate electron transfer. Table I gives a list of g values and the angles between the spectrometer field and the membrane normal, where a particular peak has its maximal amplitude.

TABLE I

g VALUES AND ORIENTATION OF CYTOCHROMES

The peaks have their maximal amplitude at the angles ϕ between the magnetic field and the membrane normal (cf. Figs. 3 and 4). n.d., not detected.

		y	z
Cytochrome *b*-559 (low-potential)	g	2.26	2.94
	ϕ	0	90
Cytochrome *b*-559 (high-potential)	g	2.16	3.08
	ϕ	0	90
Cytochrome *f*	g	n.d.	3.51
	ϕ	–	25

The high-potential cytochrome *b*-559 shows the most clear orietation effect. The g_z peak gives the strongest signal when the membrane normal is perpendicular to the spectrometer field (Figs 3B and 4) and the g_y peak has its maximum at 0° (not shown). Oxidation with chemicals (DDQ) (not shown) and by light at low temperature (Fig. 3B) gives the same orientation dependence. Since no reorientation of the heme plane is likely at low temperature, this result indicates that oxidation induces no change in the heme orientation. Furthermore, the two high-potential cytochromes *b*-559 per reaction center are oriented in the same way. Since the g_z peak arises from molecules with the heme plane perpendicular to the spectrometer field [16], the heme plane of cytochrome *b*-559 is predominantly perpendicular to the membrane. The results of this EPR study are consistent with the result obtained by Vermeglio et al. [14], who, from measurements of linear dichroism, concluded that the angle between the heme plane of high-potential cytochromes *b*-559 and the membrane plane was greater than 35°.

The cytochrome *b*-559 converted to a low-potential form by Tris shows essentially the

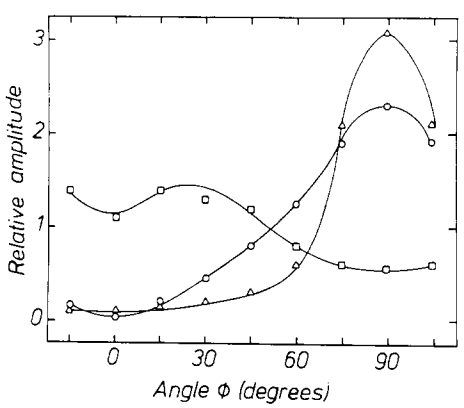

Fig 4. Plot of the amplitudes of the g_z peaks vs. the angle ϕ between the magnetic field and the membrane normal. The amplitudes have been normalized with respect to the amplitudes of the peaks in unoriented samples. g_z 2.94 (○), g_z 3.08 (△), g_z 3.51 (□).

same orientation dependence (Figs. 3C and 4), in both g_z and g_y, as the high-potential form. The somewhat higher amplitude of the g_z peak at 45° for the low-potential form could be due to a greater variation of the orientation in the membrane induced by the treatment.

The g_z peak of cytochrome *f* (Figs. 3A and 4) has its lowest amplitude when the membrane normal is perpendicular to the spectrometer field and has its maximum around 25° in agreement with previous results [17]. This shows that cytochrome *f* has an oblique orientation with respect to the membrane. No significant difference is observed between chemically or light-oxidized cytochrome *f*. The rather small angular variation may, however, suggest that the degree of orientation is smaller for cytochrome *f* than for cytochrome *b*-559.

The location of the cytochromes in the membrane system may affect the observed orientation. Location in the partition region of the grana will give a higher degree of order than location in the stroma lamellae or at the edge of the thylakoids. The high degree of orientation of cytochrome *b*-559 indicates that it is located in the partition region while the small degree of orientation of cytochrome *f* could be due to a location in the PS I-enriched region in the stroma lamellae or the edge of thylakoids.

The DDQ-induced high-spin signal also shows an orientation dependence (not shown). Although the signal may represent a cytochrome in a nonnative state, it is still oriented in a specific way in the membrane.

Significance of the heme orientation

The perpendicular orientation of cytochrome *b*-559 is the same as those found for the cytochromes of cytochrome *c* oxidase in mitochondria [16]. This may be important for the function of these redox components in the electron flow across the membrane. This would support the suggestion in Ref. 18 that high-potential cytochrome *b*-559 takes part in electron transport across the photosynthetic membrane in a cyclic flow around PS II. Presumably, electrons are also translocated through the membrane by the redox chain between the two photosystems. However, it has been proposed that this translocation is coupled to the proton transport through the membrane and that it is mediated by quinones [11]. Thus, in contrast to cytochrome *b*-559, cytochrome *f* might be involved in a lateral transport, consistent with the oblique orientation of its heme plane.

Acknowledgements

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