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THE ORIENTATION OF MEMBRANE BOUND RADICALS

AN EPR INVESTIGATION OF MAGNETICALLY ORDERED SPINACH CHLOROPLASTS

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Summary

The orientation of membrane-bound radicals in spinach chloroplasts is examined by electron paramagnetic resonance (EPR) spectroscopy of chloroplasts oriented by magnetic fields. Several of the membrane-bound radicals which possess *g*-tensor anisotropy display EPR signals with a marked dependence on the orientation of the membranes relative to the applied EPR field. The fraction of oxidized and reduced plastocyanin, *P*-700, iron-sulfur proteins A and B, and the X center, an early acceptor of Photosystem I, can be controlled by the light intensity during steady-state illumination and can be trapped by cooling. The X center can be photoreduced and trapped in the absence of strong reductants and high pH, conditions previously found necessary for its detection. These results confirm its role as an early electron acceptor in *P*-700 photo-oxidation. X is oriented with its smallest principal *g*-tensor axis (g_x) predominantly parallel to the normal to the thylakoid membrane, the same orientation as was found for an early electron acceptor based on time-resolved electron spin polarization studies. We propose that the X center is the first example of a high potential iron-sulfur protein which functions in electron transfer in its 'superreduced' state. We present evidence which suggests that iron-sulfur proteins A and B are 4Fe-4S clusters in an 8Fe-8S protein. Center B is oriented with g_y predominantly normal to the membrane plane. The spectra of center A and plastocyanin do not show significant changes with sample orientation. In the case of plastocyanin, this may indicate a lack of molecular orientation. The absence of an orientation effect for reduced center A is reconcilable with a 4Fe-4S geometry, provided that the electron obtained upon

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This article is dedicated to Professor John E. Willard on the occasion of his 70th birthday.

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HiPIP, high potential iron-sulfur protein; *P*-700, reaction center chlorophyll of Photosystem I; X, an early electron acceptor of Photosystem I.

reduction can be shared between any pair of Fe atoms in the center. Orientation of the 'Rieske' iron-sulfur protein is also observed. It has axial symmetry with $g_{\|}$ close to the plane of the membrane. A model is proposed for the organization of these proteins in the thylakoid membrane.

A new EPR signal was observed in oriented chloroplasts. This broad unresolved resonance displays a g value of 3.2 when the membrane normal is parallel to the field. It shifts to $g = 1.9$ when the membrane normal is perpendicular to the field. The signal is sensitive to illumination and to washing of the thylakoid membranes of broken chloroplasts. We suggest that there is a relation between this signal and the water-oxidizing enzyme system.

Introduction

Knowledge of the relative orientation of the cofactors which serve to transport electrons within photosynthetic membranes is valuable in mapping the path of electron transfer between acceptor and donor molecules. The overlap between the anisotropic orbitals of the acceptor and donor molecules is a function of this orientation. The overlap will affect the kinetics of the forward and back electron transfer process and thus will influence the efficiency of the overall charge separation involved in energy storage. These considerations are important in designing solar conversion schemes based on photosynthetic systems.

The technique of electron paramagnetic resonance (EPR) spectroscopy can yield orientation information when the paramagnetic center is immobilized, as in a membrane system. Studies of anisotropy in the hyperfine, dipolar or g tensors can reveal the direction of orientation relative to the membrane normal, provided that the membranes can be aligned in the spectrometer. We have aligned spinach chloroplasts using strong magnetic fields, as has been applied in studies of the dichroism of transient absorbance changes [1] and fluorescence polarization [2] of oriented membrane-bound chlorophyll.

This study was initiated to discover the orientation of the electron acceptors in Photosystem I. From time-resolved EPR studies, the orientation of the magnetic axes of an early electron acceptor was revealed by the anisotropy in the electron spin polarization of $P\text{-}700^+$, the oxidized reaction-center chlorophyll complex [3]. A species, designated X, which has properties characteristic of the primary acceptor, was detected by McIntosh and Bolton [4] and by Evans et al. [5,6] using EPR by illuminating chemically reduced Photosystem I particles. Ke et al. [7] reported that during the reductive titration of Photosystem I particles, the light-induced $P\text{-}700^+$ EPR signal becomes reversible with an E_m identical to that for reduction of the membrane-bound iron-sulfur center A (—530 mV). At potentials below —700 mV, this reversible signal is lost, reflecting the reduction of an earlier acceptor with a midpoint of approx. —730 mV. Under non-reducing conditions, chloroplasts which are strongly illuminated with continuous light at low temperature exhibit EPR signals that show an almost complete photo-oxidation of $P\text{-}700$ accompanied by the formation of reduced center A [8]. These changes reverse slowly in the dark below 77 K, during several hours. Detailed studies of Photosystem I particles using

optical detection show a light-induced *P*-700 signal that exhibits substantial reversibility depending on factors such as the temperature of the measurements, regardless of whether the sample was strongly illuminated and a donor was present during cooling [9]. The transition from an irreversible component at higher potentials to a reversible one between —480 mV and —666 mV has been reported from measurements at 15 K [10]. Transient optical studies on Photosystem I particles at room temperature reveal several kinetic components under conditions of increasing reduction, achieved either by chemical reduction or by illumination, and are interpreted as evidence for two acceptors preceding the two iron-sulfur centers A and B [11]. Photoreduction of iron-sulfur center B can be observed directly when center A is reduced prior to illumination [12].

We have examined the orientation of the reduced X center, the iron-sulfur centers A and B, the high potential 'Rieske' iron-sulfur protein [13] and plastocyanin relative to the plane of the thylakoid membrane. Features of a new EPR signal observable in oriented chloroplasts are presented and discussed in terms of a possible relation to the water-oxidizing enzyme system.

Materials and Methods

Chloroplasts were prepared from spinach leaves by grinding in a Waring blender for 10 s in a suspension medium of :0.4 M sucrose; 0.05 M Hepes, pH 7.5; 0.01 M NaCl; and $1 \cdot 10^{-4}$ M EDTA. This was followed by centrifugation and a second washing. All samples were used promptly and suspended to 50% in ethylene glycol and $1 \cdot 10^{-4}$ M EDTA prior to freezing. Magnetic field-oriented samples were frozen in the dark at 9 kG by addition of liquid nitrogen to the dewar containing the 3-mm internal diameter sample tube. The magnetic field produces chloroplast orientation in which the normal to the thylakoid membranes is parallel to the field direction [2]. Untreated chloroplasts were suspended in the medium used for grinding. Treated samples contained $1 \cdot 10^{-3}$ M ascorbate/ascorbic acid in addition to the suspension medium. Reduced chloroplasts were prepared in a N_2 atmosphere and contained 0.01 M sodium dithionite and $1.5 \cdot 10^{-5}$ M dimethyltriquat (1,1′-trimethylene-4,4′-dimethyl-2,2′-bipyridilium bromide) at pH 10.1 in 0.05 M glycine buffer.

Photoreduction and trapping at 9 kG was carried out by illumination of the samples with visible light from a tungsten source through a water filter, accompanied by gradual cooling of the sample in the magnet from room temperature to 220 K during several minutes (see figure legends for details). The duration and intensity of illumination were used to control the extent of photoreduction.

EPR measurements were made using a Varian E-3 or E-9 spectrometer at X band with 100 kHz magnetic field modulation. An Air Products Helitran cryostat provided temperatures below 77 K. Integral area measurements were made using a planimeter. Temperature measurements below 77 K were made with gold/chromel thermocouples. Light intensities were measured using a Quantronix model 504 Energy-Power Meter. Microwave powers were measured using a Hewlett Packard model 431C thermistor type meter. Magnetic field strength was measured using a Hall effect gaussmeter.

Results

g = 1.91 center

The EPR spectrum of dark-adapted frozen chloroplasts which were magnetically ordered at 9 kG during freezing is shown in Fig. 1 (top). A dark signal with axial symmetry is observed with $g_{\parallel} = 2.04$ and $g_{\perp} = 1.908$. The $g_{\perp}$ peak decreases in amplitude by 30% when the sample is rotated in the EPR field from a colinear to a perpendicular alignment relative to the membrane normal. The amplitude of the $g_{\parallel}$ peak does not appear to change; however, it is obscured by the strong $g = 2.00$ signals and the plastocyanin signal at 2.05, making intensity measurements difficult. This signal appears to be the same as that reported by Malkin and Aparicio using non-oriented chloroplasts [13]. They attributed it to a high potential iron-sulfur protein which functions between photosystems I and II [14].

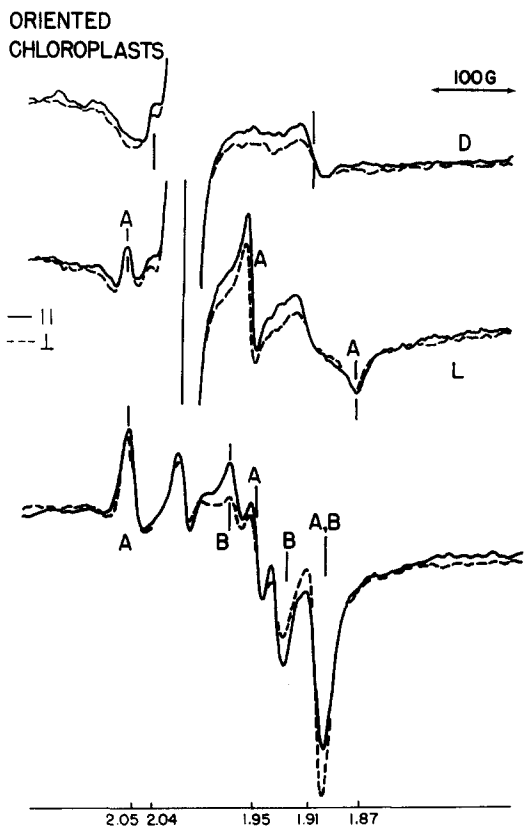


Fig. 1. EPR spectra of oriented chloroplasts; frozen untreated sample in 50% ethylene glycol in the dark (D, top trace), and following exposure to light at 12 K (L, middle trace). The parallel and perpendicular designations refer to the relative orientations of the membrane normal and the applied field. Bottom traces: chloroplasts reduced with dithionite and dimethyltriquat at pH 10 under anaerobic conditions. Peaks attributable to iron-sulfur proteins are designated A and B, respectively. Measurement temperature, 12 K; microwave power, 20 mW (top and middle), 1 mW (bottom); modulation amplitude, 10 G. The concentration of chloroplasts in the two samples is not the same, but is approx. 3 mg/ml total chlorophyll.

Center A

An EPR signal which has been attributed to a reduced ferredoxin appears upon illumination of dark-adapted untreated chloroplasts below 25 K [8]. This signal, which arises from center A, is observable in oriented chloroplasts, as shown in Fig. 1 (middle). The g-tensor has rhombic symmetry with principal values $g_x = 1.87$, $g_y = 1.95$, $g_z = 2.05$. The signal shows no dependence on alignment of the chloroplasts in the magnetic field. It forms irreversibly in the light.

Center B

The chemical reduction of a second center, designated B and also considered to be a ferredoxin, occurs at more negative potentials than required for reduction of center A [15,16]. The spectrum of fully reduced centers A and B is shown in the lower trace in Fig. 1. Center B can also be photoreduced and trapped in oriented chloroplasts free of added reductants by illumination during cooling from room temperature to approx. 220 K. The spectrum of the photoreduced centers is indistinguishable from that induced by chemical reduction when observed under identical instrumental conditions. The chemically reduced samples do not orient so well as the photoreduced samples, presumably because of chloroplast swelling at the higher pH. A qualitatively similar spectrum is shown in the bottom trace of Fig. 2, where instrumental conditions were optimized for detection of the X center (high microwave power and modulation amplitude).

The reduction of center B induces a change in the spectrum of center A. The most prominent change is the disappearance of the 1.87 peak of center A, accompanied by the appearance of the 1.89 peak of center B. The 1.93 peak also appears upon partial reduction of center B, while the 1.96 peak appears upon further reduction. The 2.05 peak, which appears initially upon reduction of center A, continues to grow during reduction of center B. This agrees with the results of Ke et al. [15] and of Cammack et al. [16] who also found this behaviour for the 2.05 peak. The peaks associated with center B at $g_x = 1.89$ and $g_y = 1.93$ are orientation dependent, with the x-axis predominantly in the membrane plane and the y-axis predominantly normal to the plane. The 1.96 peak also depends on orientation. It appears to be associated with the g_y component of center B and its amplitude is largest measured parallel to the membrane normal. The amplitudes of the 2.05 and 1.95 peaks of center A remain independent of orientation following reduction of center B. The same orientation effects are observed in chemically reduced or in photoreduced chloroplasts.

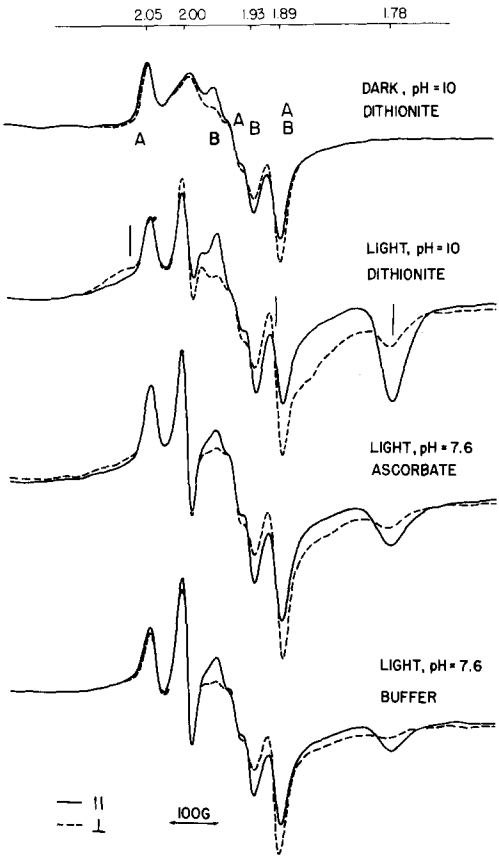
We have performed additional experiments on samples oriented at 21 kG which confirm the general features of orientation observed at 9 kG. They also show a significant increase in the extent of orientation. Thus, the ratio of amplitudes $A_\perp (g_i)/A_\parallel (g_i)$ for the 1.89 peak of center B increases from 1.2 at 9 kG to 1.5 at 21 kG, while the inverse of the ratio for the 1.93 peak increases from 1.8 to 2.5. Also, the extent of alignment decreases for chloroplasts the longer they are stored at 0°C following isolation, and is less for chloroplasts suspended in ethylene glycol than in aqueous buffer alone.

X center

High intensity illumination of chloroplasts which are either strongly reduced with dithionite, mildly reduced by ascorbate or untreated except in buffer produces another paramagnetic center of rhombic symmetry, as seen in Fig. 2. We find the principal g values of this center in oriented chloroplasts to be g_x = 1.78, g_y = 1.90 and g_z = 2.09, based on the difference between spectra from samples reduced with dithionite in the dark and in the light (upper two sets in Fig. 2). These agree with the g values reported for the X^- center in unoriented chloroplasts [4—6], except for g_y which is 0.02 units larger. The spectra reported here display a signal/noise ratio superior to that reported in the earlier observations of X^-. The extent of formation of this signal, hereafter designated X^-, is greatest with dithionite, less with ascorbate, and least in buffer alone. The more negative the ambient electrochemical potential of the sample, the larger is the X^- signal, indicating that it forms by photoreduction. All spectra are recorded in the dark; the light-induced changes are trapped irreversibly at the low temperature. The top spectrum of Fig. 2 shows that, under anaerobic conditions, dithionite plus the mediator dimethyltriquat at pH 10 do not reduce X in a sample cooled in the dark. Illumination of this frozen sample at 12 K with red light produces a reversible X^- spectrum which has the same g-tensor values as the trapped X^- spectrum. The lower two spectra show that X^- is formed by photoreduction in the absence of a strong reductant capable of directly reducing the centers A and B. $P\text{-}700^+$ was not detectable in the EPR spectrum at low microwave power ($\leqslant 1$ μW) of illuminated samples without dithionite or mediators, but a highly anisotropic signal II was observed [3]. In all illuminated samples an inflection positioned at g = 2.34 owing to oxidized plastocyanin was observable at low power (see below).

All three principal g tensor peaks of X^- display a marked dependence on sample orientation: the g_x peak is larger when the normal to the thylakoid lies along the EPR magnetic field, while the g_z and g_y components lie perpendicular to that direction and predominantly in the membrane plane. The angle dependence of the amplitude of the g_x peak of X^- is shown in Fig. 3. There is less than a 5-G shift in the position of this peak to lower field upon rotation of the sample. The absence of an appreciable shift in position indicates imperfect ordering of the membranes, the chloroplasts or the paramagnetic centers. This peak is a maximum when the direction of the alignment field and the applied EPR Zeeman field are parallel or antiparallel. The decrease in intensity at g_x is accompanied by an increase in intensity at g_y and g_z. The observations indicate that g_x lies predominantly parallel to the alignment axis and therefore predominantly colinear with the membrane normal. An orientation of g_x off-axis from the membrane normal would result in a shift of this peak to higher g values away from the 1.78 value found in randomly oriented samples, since the effective g is orientation dependent.

The full linewidth at half maximum of the g_x peak at 12 K decreases from 81 G in nonoriented samples to 59 G in samples oriented with g_x along the applied field. This indicates that the intrinsic linewidth of absorption for a single orientation is narrower than 59 G.



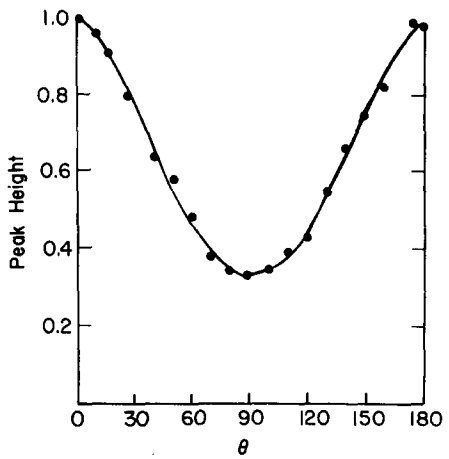


Fig. 2. EPR spectra of oriented chloroplasts observed in the dark following trapping of X^- by illumination during cooling from room temperature to 220 K. The headings on each spectrum indicate the treatment given during trapping. Dimethyltriquat is present in the anaerobic dithionite reduced samples. Temperature, 12 K; total chlorophyll concentration 5 mg/ml in each sample; microwave power 200 mW; modulation amplitude, 32 G; illumination intensity, $2 \cdot 10^3$ J · m^{-2} · s^{-1} in the visible region. A and B designate features due to iron-sulfur centers A and B.

Fig. 3. The orientation anisotropy of the $g_x = 1.78$ peak height of X^- measured under the same conditions as in Fig. 2. θ is the angle between the direction of the orienting field and the direction of the EPR applied field.

Plastocyanin

When chloroplasts are illuminated with weak light during cooling to 220 K from room temperature, a large signal of axial symmetry is observed at 15 K in addition to centers A and B. This is shown in Fig. 4. The principal g values associated with this axial center are $g_\perp = 2.05$ and $g_\parallel = 2.24$. This signal has been observed previously and was attributed to the copper-containing protein plastocyanin in its oxidized form [17,18]. When observed at microwave powers below saturation, a gaussian signal of 7.5 G linewidth at $g = 2.003$ appears. It has spectral features which indicate that it is due to oxidized reaction centers, probably $P\text{-}700^+$. The signal II contribution is unobservable, presumably

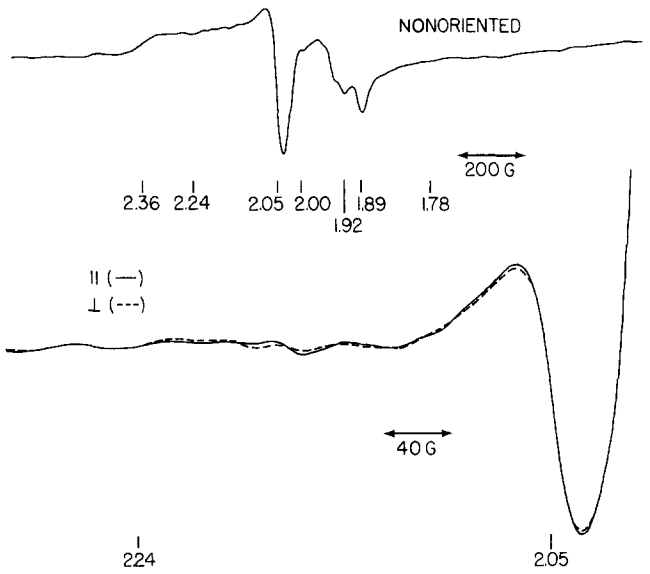


Fig. 4. The EPR spectrum of untreated chloroplasts in 50% ethylene glycol observed in the dark. Samples were illuminated at low intensity during trapping, $5 \cdot 10^2$ J · m^{-2} · s^{-1}; total chlorophyll concentration, 3 mg/ml; upper trace, nonoriented sample; lower trace, oriented sample and an expanded scale in $g = 2.05$ region. Temperature, 15 K; microwave power, 50 mW (upper trace), 10 mW (lower trace); modulation amplitude, 32 G (upper), 16 G (lower).

because it is too small. The two signals have different microwave behavior, which allows the symmetric $g = 2.003$ signal to be observed with little interference from the axial center by use of very low powers ($\leqslant 1$ μW). The ratio of the doubly integrated areas (plastocyanin$^+$/*P*-700$^+$) was estimated using a planimeter and an assumed lineshape for each. The absorption lineshape of plastocyanin is approximately triangular while that of *P*-700 is gaussian. The area ratio was 1.1 from two measurements. There was no appreciable orientation effect observed for plastocyanin in samples oriented at 9 kG.

g = 3.2, 1.9 signal

In oriented chloroplasts we often observe a highly anisotropic EPR signal which is not observable in unoriented samples, presumably because the signal is too broad. Its appearance is highly variable, and the conditions which insure its appearance have not been fully determined. Repeated washing of broken chloroplasts in the suspending medium results in the absence of this EPR signal. The spectrum observed in reduced chloroplasts which are oriented and illuminated during cooling is shown in Fig. 5. At 12 K a 500-G wide peak is observed with $g = 1.9$ when the EPR field is parallel to the plane of the membrane, and this shifts to $g = 3.2$ when it is normal to the membrane plane. The g value takes on intermediate values of orientation between these limits. The signal does not saturate readily with microwave power, and it displays a sharp decrease with increasing temperature. When observed in the dark following exposure to light at 12 K, the signal is significantly larger, as shown in Fig. 5 (lower curves).

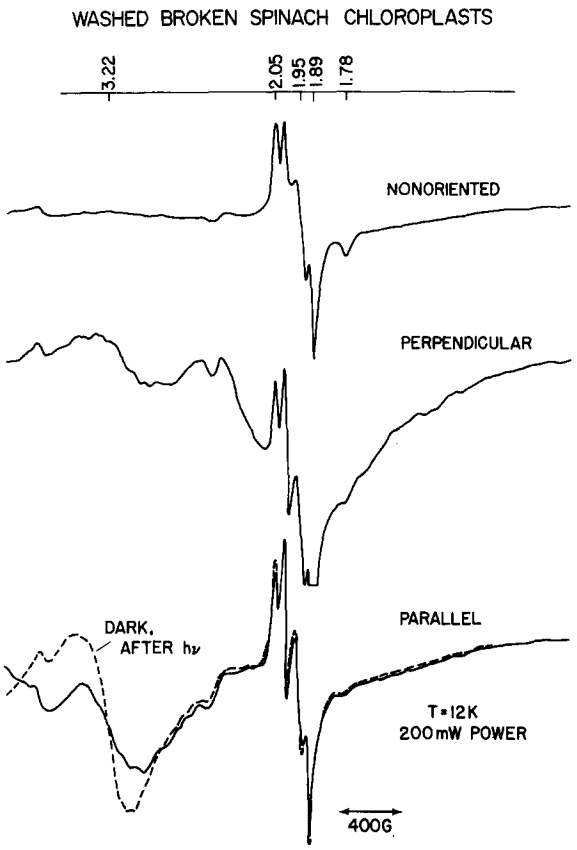


Fig. 5. EPR spectra of reduced spinach chloroplasts observed in the dark. The samples were illuminated during cooling in the presence of dithionite and dimethyltriquat at pH 10. The spectra for the middle and lower traces were recorded for oriented samples. Illumination intensity during trapping $2 \cdot 10^3$ J · m^{-2} · s^{-1} in the visible region.

Discussion

We have determined the absolute orientation relative to the thylakoid membrane of the *g*-tensors of several membrane-bound cofactors involved in electron transport. The relation between the principal axis system of the *g*-tensor and the molecular axis system is presently unknown for the iron-sulfur proteins. This information is potentially available from single crystal EPR studies, but has not yet been reported. In the sections which follow, we discuss features of the orientation of the electron transfer proteins and provide evidence concerning the character of X and centers A and B.

Iron-sulfur centers A and B

The orientation behavior of the EPR signals from iron-sulfur centers A and B is most easily interpreted in terms of neighboring 4Fe-4S centers in a single 8Fe-8S protein. Evidence for an interaction between centers A and B has been reported previously in relation to their redox behavior [16]. Cammack and

Evans [19] concluded that bound iron-sulfur centers in Photosystem I membrane fragments denatured with dimethylsulfoxide show EPR spectra typical of 4Fe-4S centers. A spin-spin interaction between two 4Fe-4S centers in an 8Fe-8S cluster of *Micrococcus lactilyticus* has been reported [20] to show EPR features upon reduction of both centers which are qualitatively the same as those for centers A and B shown in Fig. 1. Most notable is the loss of the high field peak $g_x = 1.87$ upon full reduction and the emergence of g_x for the fully reduced protein at $g_x = 1.89$. The peaks at 2.05, 1.96, 1.93 and 1.89 grow in upon full reduction while the 1.87 peak disappears. The appreciably lower anisotropy of the amplitude of the 1.89 peak, compared to the 1.96 and 1.93 peaks, at both 9 kG and 21 kG indicates that the 1.87 peak of center A reappears at 1.89 upon reduction of center B. The absence of an orientation effect for center A is responsible for the lower anisotropy at 1.89. The net absorption at 1.89 appears to be due to both A and B, which agrees with the tritration behavior displayed by this peak [15,16]. This behavior cannot be accounted for on the basis of two independent centers. Titrations of the 8Fe-8S clusters indicate two very close, one electron-reduction midpoint potentials [21], a feature also found to be true for centers A and B [12,15]. We believe that centers A and B are the 4Fe-4S centers in an 8Fe-8S protein similar to those found in bacterial ferredoxins [22]. The X-ray crystal structure of the 8Fe-8S protein from *Peptococcus aerogenes* shows two nearly identical 4Fe-4S units separated by 12 Å. Each is a distorted cube with the Fe and S atoms at alternate corners, so that any pair of Fe atoms in a unit is separated by a diagonal along the face of the cube [23]. The presence of a magnetic coupling between the 4Fe-4S units suggests a path for electron transfer in the 8Fe-8S protein which may be relevant to its biological function. One possibility is that the branching point betwen noncyclic and cyclic (Photosystem I) electron flow occurs at ferredoxins A and B.

The orientation effects observed for centers A and B can be reconciled with their assignment as neighbouring 4Fe-4S centers in an 8Fe-8S protein. The absence of an orientation effect for center A may arise because the electron obtained upon reduction can be shared between any two Fe atoms along a cube diagonal of the 4Fe-4S center A. In the reduced state of a 4Fe-4S center protein from *Bacillus stearothermophilus* the additional electron is shared between a pair of Fe atoms along a cube diagonal. It is not fully delocalized over all four Fe atoms or localized on one [24]. The presence of non-equivalent Fe atoms was also deduced from Stark effect measurements on 4Fe-4S clusters from *Bacillus polymyxa* and *Clostridium acid-urici* [25]. If all possible pairs of Fe atoms can share the additional electron in reduced center A, then no preferred orientation of the *g*-tensor should be detected even though the protein may be oriented to a high degree.

The orientation effects observed for center B indicate that the electron delocalization in the reduced state is different from that found in center A. Center B is oriented with g_x predominantly in the membrane plane and g_y along the normal. Evidently electron delocalization occurs either between a specific Fe pair or among all four Fe atoms, with the resulting *g* anisotropy a consequence of the low symmetry of the entire cluster of Fe atoms. We estimate that the maximum angle the *y*-axis of the *g*-tensor of center B may make

with the membrane normal is 14° (see next section). A puzzling observation is that the amplitude of the 2.05 peak, which others have attributed to both centers A and B, does not change significantly with orientation for samples oriented at 9 kG and 21 kG. According to the orientation found for g_x and g_y of center B, the g_z peak should lie near the plane of the membrane and so be dependent on orientation. The absence of an orientation effect for the 2.05 peak suggests that this peak is a feature ascribable to center A only. At 21 kG, a broad anisotropic peak appears at 2.00 which is most intense in the membrane plane. It appears to be the g_z peak of center B, which evidently is too broad to observe in samples oriented at 9 kG. The increase in intensity of the 2.05 peak observed during titration of center B [15,16] may be a consequence of the coupling of centers B and A in the doubly reduced state, rather than a result of center B absorbing at 2.05 as well. This interpretation is more consistent with our observations.

The orientation of centers A and B is not consistent with that found for an early electron acceptor for *P*-700 from electron spin polarization studies [3]. This indicates that centers A or B do not function as the initial electron acceptor for *P*-700.

X center

The data of Fig. 2 demonstrate that photoreduction of the X center occurs upon illumination of chloroplasts between 300 K and 200 K, and support the view that the photoreduction of X is a normal step in the Photosystem I reactions.

We can estimate the maximum allowable tilt of the x-axis of the g-tensor for X^- away from a parallel alignment with the membrane normal. Combining all sources of disorder into a single wobble angle, β, between the x-axis and the normal, we can calculate the maximum value of β that will not produce a detectable shift of the g_x peak from 1.78. This is limited by $\Delta H/2$, the half-width of the g_x peak. From the expression for the g value, $g^2 = g_\parallel^2 \cos^2\beta + g_\perp^2 \sin^2\beta$, for which $g_\parallel = g_x$ and $g_\perp^2 = \frac{1}{2}(g_y^2 + g_z^2)$, we find that $\beta \leqslant 14°$ for the experimentally measured linewidth $\Delta H/2 = 30$ G. A similar estimate can be made for the g_y peak of center B. These estimates ignore the distribution of orientations of the chloroplasts, the membranes, and the paramagnetic centers.

The large g anisotropy and orientation of the X^- center within the thylakoid membrane correlate with the results found for an early electron acceptor of *P*-700 from chemically induced dynamic electron spin polarization of the *P*-700$^+$ radical [3]. These experiments indicate that a radical pair is formed in less than 2 μs in nonreduced chloroplasts following photooxidation of *P*-700. The electron acceptor has the same orientation and large anisotropy as found for trapped X^-. The acceptor implied from the spin polarization experiments and the X^- center are probably the same. Recently Sauer et al. [11] reported evidence that two electron acceptors precede ferredoxins A and B on the basis of *P*-700$^+$ reduction kinetics observed optically. One of these is presumably X, although it is not clear whether X is the first or the second acceptor that they infer from the kinetic observations. The existence of another acceptor molecule which precedes X is suggested by an analysis of the electron spin polarization of *P*-700$^+$ [26]. This may correspond to the other acceptor inferred from the

results of the optical experiments of Sauer et al. [11]. Evidently this acceptor has a potential more negative than X (—730 mV, ref. 7) and so is not trapped in its reduced state by our technique.

There is little known about the identity or molecular structure of the X^- center. Its average g value is 1.92. This is significantly smaller than 1.95 which is the value observed for ferredoxin centers A and B. The ferredoxins belong to a large class of iron-sulfur proteins having $\langle g \rangle = 1.95$ [27].

Malkin et al. [28] have isolated a bound iron-sulfur protein from chloroplast membranes which contains four atoms each of Fe and labile S in the protein of molecular weight 8000. They suggested that it may be the primary acceptor of Photosystem I, but were unable to provide evidence for that assignment.

The extremely negative potential of the X/X^- couple is another feature which distinguishes this electron carrier protein from the ferredoxin centers (typically —400 mV for 2Fe-2S, 4Fe-4S and 8Fe-8S centers. The one exception is the 'superreduced' form of the 4Fe-4S high potential iron-sulfur protein (HiPIP) of *Chromatium* ($E_m < -640$ mV in 70% dimethyl sulfoxide, ref. 29), which under normal circumstances is paramagnetic in its oxidized form and nonmagnetic in its reduced form ($E_m = +350$mV). The corresponding 'superoxidized' form of the ferredoxins has also been observed [30]. A 'three-state' hypothesis has been proposed by Carter et al. [31] to explain these differences: that the non-magnetic forms, reduced HiPIP and oxidized ferredoxin, represent an equivalent redox state, C, of the 4Fe-4S center. The 4Fe-4S center in HiPIP can undergo oxidation to the paramagnetic C^+ state, while the 4Fe-4S ferredoxins normally undergo reduction to the paramagnetic C^- state. No evidence has been found for the C^- HiPIP state under physiological conditions. The redox properties of the X center suggest that it may corrrespond to the first example of a HiPIP type protein which normally functions in the 'superreduced' state. Recent electron spin echo studies of oxidized HiPIP and reduced 4Fe-4S ferredoxin proteins have shown that, although they share a common 4Fe-4S core, the protein environment of the two is distinctly different [25]. Solvent exchangable protons are present near the core in the ferredoxins but not HiPIP, and this may be the origin of their different redox behavior. If this hypothesis is correct, there should also exist an EPR signal for the oxidized form (C^+) of the X^+ center with a midpoint potential near that of HiPIP (+350 mV) and an orientation similar to that of the X^- center. Such a signal has not been reported; however, if the strong relaxation behavior evident in X^- is also a feature of its oxidized paramagnetic counterpart, then it might easily go undetected.

One proposal for the organization of the electron transfer proteins in the thylakoid membrane inferred from this work and which is in agreement with results from optical [11] and spin polarization [3,26] studies is given in Fig. 6. The observed g-tensor orientations are also depicted. As suggested in this figure, one reason for the participation of several proteins in the electron transfer process is that it allows the separated charges to span the entire width of the membrane and so create oxidizing and reducing sites on opposite sides of the membrane. The thickness of the membrane is estimated to be between 50 Å and 100 Å, the lower limit being typical of lipid bilayers and the upper referring to estimates from native thylakoids [32].

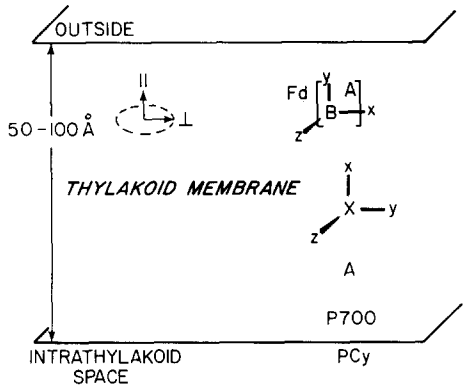


Fig. 6. The orientation of the principal g-axes of X and ferredoxin center B, Fd (B), relative to the thylakoid membrane is shown. A possible arrangement, relative to the membrane, of these and other Photosystem I components is suggested. PCy is plastocyanin; A is an early electron acceptor.

Plastocyanin

The plastocyanin EPR signal (Fig. 4) shows neglible dependence on membrane orientation. The lack of a substantial orientation effect may be the consequence of a binding site at the membrane surface with a non-rigid geometry. Alternatively, a large fraction of the plastocyanin may not be membrane bound at all. Because plastocyanin is retained in broken chloroplasts which are washed repeatedly, a location within the intra-thylakoid space is implied. This location is consistent with the observation that sonication of chloroplasts, a treatment which breaks the thylakoid membrane, also releases plastocyanin [33].

The trapping of photo-oxidized plastocyanin in untreated chloroplasts is accompanied by the formation of fully reduced iron-sulfur centers A and B, as shown in Fig. 4. At low microwave power, a $g = 2.00$ signal with 7.5-G linewidth, due presumably to $P\text{-}700^+$, is detected in amount nearly equal to the plastocyanin. The steady-state of the system under low intensity illumination appears to be one in which P-700 and plastocyanin are oxidized, while iron-sulfur centers A and B are photoreduced. The integrated areas indicate a 1 : 1 ratio of photo-oxidizable plastocyanin/P-700. If plastocyanin is located near the inner membrane surface above, then presumably P-700 is located nearby. A schematic representation of the possible location of plastocyanin relative to the other Photosystem I components is shown in Fig. 6.

The various redox states which can be prepared either by chemical control of the ambient redox level or by steady-state illumination are summarized in Table I. The successive photoreduction of iron-sulfur centers A and B and the X center can be accomplished by appropriate control of the illumination intensity and terminal electron acceptors. The more negative the redox potential of the species, the higher the light intensity needed to insure its photoreduction. At low light flux the rate-limiting step in the reduction of oxidized plastocyanin by Photosystem II or by an endogeneous cyclic path involving Photosystem I is a slower process than reduction of $P\text{-}700^+$ by plastocyanin, thus accounting for trapping of plastocyanin in its oxidized state.

TABLE I

PHOTOCHEMICAL TRAPPING IN CHLOROPLASTS

The trapped redox states of Photosystem I electron carriers achieved by dark incubation or by illumination. Headings indicate the treatment received. Illumination conditions: (1) frozen and observed in the dark; (2) frozen in the dark, illuminated at 77 K or below at $5 \cdot 10^2$ J · m^{-2} · s^{-1}; (3) illuminated during cooling from room temperature to 220 K at $5 \cdot 10^2$ J · m^{-2} · s^{-1}, stored at 77 K prior to observation at 15 K; (4) same as 3 except an intensity of $2 \cdot 10^3$ J · m^{-2} · s^{-1}. The presence of ascorbate or dithionite plus mediators increases the yield of X^- in 4. PCy = plastocyanin.

1	PCy	*P*-700	X	Fd_B	Fd_A	frozen in the dark
2	PCy	*P*-700$^+$	X	Fd_B	Fd_A^-	dark while cooling, $h\nu$ at $\leqslant$77 K
3	PCy$^+$	*P*-700$^+$	X	Fd_B^-	Fd_A^-	$h\nu$ while cooling
4	PCy$^+$	*P*-700	X^-	Fd_B^-	Fd_A^-	intense $h\nu$, cooling or intense $h\nu$ + reductant, cooling
		P-700$^+$	X^-			not trapped

g = 3.2, 1.9 EPR signal

A new light-sensitive EPR signal associated with intact spinach chloroplasts can be observed in oriented samples (Fig. 5). The absence of this signal in unoriented samples appears to be due to the large *g* anisotropy, large linewidth and sensitive dependence on temperature, which make detection difficult. The orientation behavior indicates that the species is membrane bound, and the sensitivity to illumination implicates an association with the light reactions. The sharp decrease of the signal with increasing temperature and the absence of power saturation show that this paramagnetic center is efficiently relaxed. The spectrum shows no evidence of a resolved hyperfine or fine structure. Slabas and Evans [34] recently reported a $g = 3$ signal in chloroplasts. They demonstrated that it appears and disappears on successive flash illumination and is sensitive to Tris washing, suggesting that it may be a marker of the water-oxidizing enzyme system. In support of this assignment, we do not observe this signal in broken chloroplasts which are aged following isolation or repeatedly washed, conditions which are known to destroy the O_2-evolving capacity.

Concluding remarks

We have demonstrated that orientation effects can be observed for some of the electron transfer components in oriented photosynthetic membranes using EPR spectroscopy. The presence or lack of a preferred orientation may be related to the binding properties of the protein in the membrane and how neighboring proteins interact.

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