Submitted to Biochimica et Biophysica Acta

LBL-7532
Preprint

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March 1978

Prepared for the U. S. Department of Energy
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THE ORIENTATION OF MEMBRANE BOUND RADICALS: AN EPR INVESTIGATION OF MAGNETICALLY ORDERED SPINACH CHLOROPLASTS

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

Abstract

The orientation of membrane-bound radicals in spinach chloroplasts is examined by 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 Zeeman field. The fraction of oxidized and reduced plastocyanin, P700, 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 P700 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. Ferredoxin center B is oriented with g_y predominantly normal to the membrane plane. The spectra of ferredoxin center A and plastocyanin do not show significant changes with sample orientation. The absence of an orientation effect for center A indicates that it is probably a 4Fe-4S center. In the case of plastocyanin, this suggests lack of molecular orientation. We present evidence which suggests that iron-sulfur proteins A and B are 4Fe-4S clusters in an 8Fe-8S ferredoxin. 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. Orientation of the "Rieske" iron-sulfur protein is also observed. It has axial symmetry with $g_{\parallel}$ 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 intact 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 in broken chloroplasts. We suggest that there is a relation between this signal and the water oxidizing enzyme system.

Abbreviations

CIDEP, chemically induced dynamic electron spin polarization; Fd_A, Fd_B, chloroplast membrane-bound ferredoxin A and B, respectively; Hepes, N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid; HIPIP, high potential iron-sulfur protein; P700, reaction center chlorophyll of Photosystem I; X, an early electron acceptor of Photosystem I.

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 anisotropically shaped orbitals on 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 so 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 an early electron acceptor was revealed by the anisotropy in the electron spin polarization of $P700^+$, 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 $P700^+$ EPR signal becomes reversible

at potentials below -700mV, reflecting the reduction of an early acceptor with a midpoint of approximately -730mV. Under non-reducing conditions, chloroplasts that are strongly illuminated with continuous light at low temperature exhibit EPR signals that show an almost complete photo-oxidation of P700 accompanied by the formation of a reduced iron-sulfur protein, ferredoxin center A (8). These changes reverse slowly below 77K, during several hours. Detailed studies of Photosystem I particles using optical detection show a light-induced P700 signal that exhibits substantial reversibility depending on factors such as the temperature of the measurements, 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 below about -480 mv has been reported from measurements at 15K (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 ferredoxin centers A and B (11). Photoreduction of ferredoxin center B can be observed directly when ferredoxin A is reduced prior to illumination (12).

We have examined the orientation dependence of the reduced X center, the ferredoxin 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 sec in a medium of 0.4M sucrose; .05M Hepes, pH 7.5; .01M NaCl; and 10^{-4}M EDTA (SHN buffer). This was followed by centrifugation and a second washing. All samples were used promptly and suspended to 50% in ethylene glycol and 1 x 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 3mm I.D. sample tube. The magnetic field produces chloroplast orientation in which the normal to the thylakoid membranes is parallel to the field direction (2). Reduced chloroplasts were prepared in a N_2 atmosphere and contained 0.01M sodium dithionite and 1.5 x 10^{-5}M dimethyltriquat (1,1'-trimethylene-4,4'-dimethyl-2,2'-bipyridilium bromide) at pH 10.1 in .05M glycine buffer. Ascorbate treated samples contained 1 x 10^{-3}M ascorbate/ascorbic acid and were suspended in SHN at pH 7.6. Untreated chloroplasts were suspended in SHN.

Photoreduction and trapping were carried out at 9 kG 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 220K 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 amde using a Varian E-3 or E-9 spectrometer at X band with 100khz magnetic field modulation. An Air Products Helitran cryostat provided temperatures below 77K. Integral area measurements were made using a planimeter. Temperature measurements below 77K were made with gold/chromel thermocouples. Light intensities were measured using a Quantrox 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 9kG during freezing is shown in Fig. 1 (top). A dark signal with axial symmetry is observed with $g_{\parallel} = 2.037$ and $g_{\perp} = 1.908$. The g peak decreases 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 unoriented chloroplasts. They attributed it to a high potential iron-sulfur protein (13).

Center A

An EPR signal which has been attributed to a reduced ferredoxin appears upon illumination of dark-adapted chloroplasts below 25K (8). This signal which is designated 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

It has been shown that 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 (14,15). 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 ∿220K. The spectrum of the photoreduced centers is identical to that induced by chemical reduction and is shown in the bottom trace of Fig. 2 for an oriented

sample.

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 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 (14) and of Cammack et al. (15) who also found this behavior 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 g_y of center B and is largest when parallel to the membrane normal. 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 photoreduced chloroplasts.

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 (4,5,6), except for g_y which is .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. 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 12K 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. Inspection of the EPR spectrum at low microwave power ($\leq$ 1 μW), of photolyzed samples without dithionite or mediators showed that $P700^+$ was not detectable, but a highly anisotropic signal II was observed (3). In all photolyzed samples a signal at g = 2.34 owing possibly to oxidized plastocyanin (see below) was observable.

All three principal g-tensor peaks of X^- display a marked dependence on sample orientation: the g_x peak is the largest when the normal to the thylakoid lies along the EPR magnetic field, while g_z and g_y lie perpendicular to that direction and predominantly in the membrane plane. The angle dependence of the g_x peak of X^- is given in Fig. 3. A peak is observed at 1.78 for all orientations, indicating imperfect ordering of the membranes or chloroplasts or both. This peak is a maximum when the direction of the alignment field and the 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 lower fields away from the 1.78 value found in randomly oriented samples. We have performed additional experiments at 21kG which confirm the general features of orientation observed at 9kG. They also show a significant increase in the extent of orientation. Thus the ratio $g_x(\perp)/g_x(\parallel)$ for the 1.89 peak of Fd_B increases from 1.2 at 9kG to 1.4 at 24kG. Also, the extent of alignment decreases for chloroplasts the longer they are stored following isolation, and is less for chloroplasts suspended in ethylene glycol than in aqueous buffer.

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

The relaxation properties of a paramagnetic center can provide information on the efficiency of coupling to lattice degrees of freedom or on the presence of low-lying excited spin states. The microwave power dependence of the g_x peak of X^- and of two of the peaks of centers A and B is shown in Fig. 4a. The data show that the X^- signal does not saturate so readily with increasing power as do those of centers A and B. This illustrates the very efficient relaxation properties possessed by X^- in comparison with those of the bound ferredoxins. This is also evident in the temperature dependence of the EPR of the X^- species shown in Fig. 4b. A sharp non-Curie dependence is evident, and no EPR signal is observed above about 25K. By contrast, the ferredoxin centers are observable almost to 77K, although these signals also decrease with increasing temperature more sharply than the Curie Law predicts.

Plastocyanin

When chloroplasts are illuminated with weak light during cooling to 220K from room temperature, a large signal of axial symmetry is observed in addition to centers A and B. This is shown in Fig. 5. The principal g values associated with this axial center are $g_{\perp} = 2.05_5$ and $g_{\parallel} = 2.24$. This signal has been observed previously and was attributed to a copper-containing protein, plastocyanin (16,17). When observed at microwave powers below saturation, a symmetric signal of 7.5G linewidth at g = 2.003 appears. It has spectral features which indicate that it is due to oxidized reaction centers, probably $P700^+$. The signal II contribution is unobservable, presumably because it is too small. The two signals have different microwave saturation 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 (≤ 1 μW). The ratio of their integral areas is 1/.9 (axial center/symmetric signal) under non-saturating conditions. In oriented samples, the amplitude of the $g_{\perp}$ peak of the axial signal shows a 3% decrease when observed parallel to the membrane and a 5% reduction observed at 45° relative to the amplitude observed perpendicular to the membrane normal. An apparent hyperfine structure is partially resolved about $g_{\parallel}$. This hyperfine structure sharpens when the normal to the thylakoid membrane is parallel to the Zeeman field, as shown in Fig. 5 (lower curves). The hyperfine splitting is nonuniform, varying between 46-64G.

g = 3.2, 1.9 Signal

In oriented chloroplasts we often observe a highly anisotropic EPR signal that 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. 6. A 500G wide peak is observed with g = 1.9 when the Zeeman field is in 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 for orientations 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 12K, the signal is significantly larger as shown in Fig. 6 (lower curves).

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. The position of X^- relative to centers A and B cannot be inferred from our studies. This information could have been provided if an observable orientation dependent interaction existed between these paramagnetic centers. The data of Fig. 2 indicate that there is no change in the spectrum of reduced centers A and B upon reduction of X, indicating that no observable interaction exists between them. The orientation of $P700^+$ was not determined because it possesses predominantly isotropic magnetic features. It is clear from the existence of orientation effects for some of these paramagnetic species, that a preferred orientation of the acceptors is important in controlling the probability of electron transfer. 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.

g = 1.91 Center

The anisotropic center observed in dark adapted chloroplasts in Fig. 1 (top) is oriented in the membrane. Since $g_{\perp}$ is greatest along the membrane normal, this indicates that $g_{\parallel}$ lies close to the membrane plane. The $g_{\parallel}$ peak at 2.037 is masked by both the 2.00 signal and the 2.05 signal attributed to plastocyanin. These interfere with direct peak height measurements. The anisotropic g = 1.91 signal has been attributed to a high potential iron-sulfur center (13), and has been shown to function in electron transport between Photosystems I and II (18). Malkin and Aparicio have shown that this signal occurs in the reduced state, and that it has EPR and redox properties similar to that reported for the "Rieske" g = 1.90 iron-sulfur protein of mitochondrial Complex III (13).

Ferredoxin A and B

The results presented in Figs. 1 and 2 show that it is possible to photoreduce center B following reduction of center A and that these changes can be trapped upon cooling. The photoreduction of centers A and B in Photosystem I membrane fragments has been reported before at both cryogenic (12) and room temperatures (19). Various lines of evidence have shown that center A and center B are ferredoxin type iron-sulfur proteins (20,21). The reduction of center B produces marked changes in the spectrum of center A. The spectrum of the fully reduced iron-sulfur centers A and B cannot be interpreted in terms of two independent S = 1/2 centers. This has been discussed by Evans, Cammack, and Reeves (15) in relation to the redox behavior of centers A and B. Cammack and Evans (21) 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 (22) 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 g_x peak upon full reduction and the emergence of g_x for the fully reduced protein at a higher value (lower field). The peaks at 2.05, 1.96, 1.93 and 1.89 grow in upon full reduction. This behavior cannot be accounted for on the basis of two independent centers. A weak dipole or exchange coupling between the centers has been suggested as the cause of this additional splitting and the shift of g_x in the case of M. lactilyticus (22). A conformational change induced by addition of the second electron is also possible. Titrations of the 8Fe-8S clusters indicate two very close one electron-reduction midpoint potentials (23), a feature also found to be true for centers A and B (12,14). We believe that centers A and B are the 4Fe-4S centers in a 8Fe-8S cluster similar to those found in bacterial ferredoxins (24). The X-ray crystal structure of the 8Fe-8S protein from Peptococcus aerogenes shows two nearly identical 4Fe-4S units separated by 12Å. 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 (25). The presence of a magnetic coupling between the 4Fe4S 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 between 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 neighboring 4Fe-4S centers in a 8Fe-8S protein. The absence of an orientation effect for center A, which seems to be inconsistent with its being membrane bound, 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 (26). If all possible pairs of Fe atoms can share the additional electron in 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 it is oriented with g_x predominantly in the membrane plane and g_y along the normal. 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. According to the orientation found for g_x and g_y, the g_z peak should lie near the plane of the membrane and so be dependent on orientation. Our results suggest that this peak is a feature ascribable to center A only. Preliminary measurements show that the 2.05 peak remains independent of orientation up to 21kG. Also, 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 9kG. In reductive titrations of chloroplast membrane fragments the 2.05 peak appears to be formed upon two successive one-electron reductions at -535mV and -590mV (14,15). The increase in intensity of this peak upon reduction of center B (-590mV) may be a consequence of the coupling of the two centers 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 P700 from CIDEP studies (3). This indicates that centers A or B does not function as the initial electron acceptor for P700.

X^- Center

The data of Fig. 2 demonstrate that photoreduction of the X center occurs upon illumination of chloroplasts between 300K and 200K, and that non-physiological reductants are not necessary to accomplish this. X^- has been observed previously only in Photosystem I membrane fragments reduced with dithionite at high pH (4,5,6), conditions which conceivably might reduce the primary acceptor and lead to photoreduction of X in a non-physiological side reaction. Our data show that formation of X^-may be observed under conditions which are far less severe and so support the view that it is a normal photochemical step in Photosystem I.

The successive photoreduction of iron-sulfur centers A and B and X with increasing light intensity demonstrates that the rate-limiting electron transfer step follows transfer to the bound iron-sulfur centers. We did not detect any reduction of X using dithionite as reductant and 1,3 dimethyl-triquat as mediator down to -600mV.. Ke et al. estimate an E_m of -730mV for the reduction of X from electrochemical titrations (7).

The large g anisotropy and orientation of the X^- center within the thylakoid membrane correlates with the results found for an early electron acceptor of P700 from chemically induced dynamic electron spin polarization (CIDEP) of the $P700^+$ radical (3). These experiments indicate that a radical pair, $P700^+X^-$, is formed in less than 2 μsec in unreduced chloroplasts following photo-oxidation of P700. The 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 $P700^+$ reduction kinetics observed optically. One of these is presumably X, although it is not clear whether X is the first or second acceptor that they infer from the kinetic observations.

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).

Cammack has recently made EPR measurements of iron-starved Nostoc which show a considerable decrease in the signal due to X^-, suggesting that X may contain iron. However, he indicates that it might alternatively be due to a decreased synthesis of reaction centers at the low iron level (28). Malkin et al. have isolated a bound iron-sulfur protein from chloroplast membranes which contains 4 atoms each of Fe and labile S in a protein of molecular weight 8000 (29). They suggested it may be the primary acceptor of Photosystem I, but were unable to provide evidence for it.

The extremely negative potential of the X/X^- couple ($E_m \sim -730$mV, ref. 7) is another feature which distinguishes this electron carrier protein from the ferredoxin centers (typically -400mV for 2Fe2S, 4Fe4S, 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. 30), 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 (31). A "three-state" hypothesis has been proposed by Carter et al. (32) to explain these differences: that the non-magnetic forms, reduced HIPIP and oxidized ferredoxin, represent an equivalent redox state, C, of the 4Fe4S 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 correspond to the first example of a HiPIP type protein which normally functions in the "superreduced" state. 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 (+350mV) 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.

The X^- center has very efficient spin relaxation properties as shown in Fig. 4a, a feature which is not so pronounced for centers A and B. There is some evidence for the occurrence of low-lying excited spin states which may be thermally occupied even below 18K. This is suggested from the behavior of the g_x peak of X^-, which is shown in Fig. 4b. This peak decreases sharply with increasing temperature, even though there is no concomitant increase in linewidth, at least below 18K. This behavior is well known for multinuclear transition metal complexes which are coupled by weak antiferromagnetic exchange interactions (33). It is also typical of high spin Fe(II) in distorted tetrahedral symmetry where the splitting between successive spin states induced by spinorbit coupling has been observed to be 10 - 15 cm^{-1} in a number of inorganic host lattices (34), in reduced rubredoxin (35), and its synthetic analogs (36). However, the g anisotropy of X^- is not typical of an isolated Fe(II).

We have made calculations of the temperature dependence of the EPR for model 2Fe-2S and 4Fe-4S centers in their reduced (paramagnetic) oxidation

state. For such systems low-lying excited spin states occur as a result of exchange coupling between Fe atoms (37,38,39). The temperature dependence of the EPR intensity does not follow that predicted for either of these models if the exchange interaction between all pairs of Fe atoms is assumed to be equal. An example of similar calculations is given in the article by Blum et al. who investigated the EPR of oxidation Chromatium HiPIP (38). Their data could not be accounted for in full by the assumption of an equivalent exchange interaction. We have made calculations for reduced 4Fe-4S clusters in which the iron atoms are considered to couple in pairs (Fe^{2+} - Fe^{2+} and Fe^{2+} -Fe^{3+} with exchange energies of different magnitude. These pairs then couple together by exchange also. This lifts the degeneracy of the spin states described by the total spin $S = S_1 + S_2 + S_3 + S_4$, and provides a large number of low-lying states which are thermally accessible but unobservable by EPR. The sharp temperature dependence we observe for X^- is readily accounted for by such a model and supports the identification of X as a 4Fe-4S protein. We are currently planning experiments to extend our temperature measurements, given in Fig. 4, below 10K. These are necessary to provide an unambiguous evaluation of the above model.

There is ample evidence that 4Fe-4S proteins may possess non-equivalent Fe atoms. The existence of nonequivalent pairs of Fe atoms has been demonstrated in the 4Fe-4S protein from B. stearothermophilus from Mössbauer studies (26). Also, non-equivalent Fe atoms were deduced from the EPR spectrum of reduced 4Fe-4S centers from Bacillus polymyxa and Clostridium acid-urici from the g-value shifts in the presence of an electric field (40).

Sequence of Electron Acceptors

Using the assumption that the redox potential of an acceptor is a suitable marker of its kinetic sequence in an electron transport series, a possible

sequence in Photosystem I would be $X \rightarrow Fd_B \rightarrow Fd_A$. This assumption is unverified and it neglects the possibility of a nonserial electron flow in which X^- transfers electrons directly to Fd_A. We have reported kinetic EPR experiments which show that X is an earlier electron acceptor than ferredoxin centers A and B (3).

We have no evidence that electron transfer from X occurs directly to Fd_B or Fd_A. There may be an intermediate acceptor or acceptors prior to the bound ferredoxins which is unobservable by EPR and hence undetected in the kind of experiment reported here. In this regard, the two unidentified acceptors reported by Sauer et al., which have more negative redox potentials than the bound ferredoxins, may correspond to earlier acceptor species (11). One of these is presumable X. The existence of another acceptor molecule which precedes X is suggested by an analysis of the electron spin polarization of $P700^+$ (41). This may correspond to the other acceptor suggested by the results of the optical experiments of Sauer et al. Recently Arnon, Tsujimoto, and Hiyama reported that the bound ferredoxin centers A and B in Nostoc muscorum may have different electron donors (19). Unfortunately, they chose the 1.87 peak of Fd_A as a marker of its reduction. This peak, which disappears upon full reduction of centers A and B, does not signal the disappearance of the reduced center A, as we and others have shown. Their intrepretation is therefore based on an incorrect interpretation of the EPR spectra. The kinetic sequence of electron transfer to the bound ferredoxins remains an unsolved issue.

Nelson and Notsani treated photosystem I particles with detergents and separated 3 polypeptides that are essential for the observation of the bound ferredoxin centers A and B (43). They still observed optically reversible light-induced P700 changes in the fragments which were missing these three subunits, suggesting to them that the "very primary" acceptor was situated in their P700 fragment.

The three subunits which dissociate may be X,Fd_B, and Fd_A. The "very primary" electron acceptor which they propose to be present in the P700 fragment may correspond to the A_1 acceptor species inferred in the optical studies of Sauer et al. (11) and from EPR studies (41). Alternatively, X may be associated with the P700 fraction, since evidence for its assoication with the three dissociated subunits was not reported.

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 (41) studies is given in Fig. 7. The observed g tensor orientations are also depicted. As suggested by 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 (44).

Plastocyanin

The plastocyanin EPR signal (Fig. 5) shows small but reproducible changes depending on membrane orientation. The evidence for a preferred orientation is not convincing, and suggests little net orientation. The hyperfine structure, which is partially resolved along $g_{\parallel}$, sharpens when the membrane normal and the Zeeman field are parallel; however, $g_{\perp}$ also increases slightly for this orientation. The lack of a substantial orientation effect may be a consequence of a binding site at the membrane surface with a non-rigid geometry. It also suggests that 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 (47).

The photochemical trapping of oxidized plastocyanin in chloroplasts is accompanied by the formation of fully reduced ferredoxin centers A and B as shown in Fig. 5. At low microwave power a g = 2.00 signal with 7.5 G linewidth, due presumably to $P700^+$, is detected in nearly equal amount to the plastocyanin (integrated areas). The state of the system appears to be one in which two electrons, one each from P700 and plastocyanin, have been transferred to ferredoxin centers A and B. These results indicate that one plastocyanin is oxidized for each P700 reduced. Results from other studies have been interpreted as evidence that plastocyanin is the primary donor to P700 (16,11). We have no evidence which bears on this. If plastocyanin is located near the inner membrane surface as suggested above, then presumably P700 is also located close to that side. A schematic representation of the possible location of plastocyanin relative to the other Photosystem I components is shown in Fig. 7.

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 Fig. 8. The successive photoreduction of ferredoxin 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 cylic path involving Photosystem I is a slower process than reduction of $P700^+$ by plastocyanin, thus accounting for trapping of plastocyanin in its oxidized state.

g = 3.2, 1.9 EPR Signal

A new light- sensitive EPR signal associated with intact spinach chloroplasts (Fig. 6) can be observed in oriented samples. 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 recently reported a g = 3 signal in chloroplasts (46). They demonstrated that it shows period four oscillations and is sensitive to Tris washing, suggesting that it may be a marker of the water oxidizing enzyme system. The signal presumably arises from a transition metal-containing site; however, an identification on the basis of the essentially featureless spectrum cannot be made with confidence. Paramagnetic resonance for Fe(II) in predominantly cubic symmetry has been shown to give rise to g values near 3.4 (47). Feher et al. (48) have observed a broad EPR signal at g = 3.8 in membrane fragments of reaction centers of Rhodopseudomonas spheroides R-26 in which Fe is partially replaced with Mn(II). Unfortunately, the similarity in g values is not a sufficiently distinct marker for an unambiguous assignment, but it does suggest plausible candidates. The efficient spin relaxation which we observe indicates that two or more coupled transition ions may be involved. The signal is not observed in broken chloroplasts which are aged following isolation or repeatedly washed, conditions which are known to destroy the O_2 evolving capacity. It therefore

seems probable that it may be a direct or indirect monitor of the water oxidizing enzyme system.

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. Information about the organization of the proteins is important in determining how they interact and the role of the membrane in stabliizing the separated charges.

Acknowledgements

This work was supported by the Division of Biomedical and Environmental Research of the U.S. Department of Energy. We thank Dr. H. Frank for assistance with measurements performed at 21kG

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Figure Legends

Fig. 1. EPR spectra of oriented chloroplasts frozen in buffer; in the dark D, (top trace) following exposure to light at very low temperature, L (middle trace). The parallel and perpendicular designations refer to the relative orientations of the membrane normal and the Zeeman field. Bottom traces; oriented chloroplasts reduced with dithionite at pH 10 and dimethyltriquat under anaerobic conditions. Peaks attributable to ferredoxin species 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 typically 3 mg/ml total chlorophyll.

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 dithionite reduced samples, which were also prepared anaerobically. Temperature, 12 K; total chlorophyll concentration 5 mg/ml in all samples; microwave power: 200 mW; modulation amplitude, 32 G; illumination intensity, $2 \times 10^3 Jm^{-2}s^{-1}$ in the visible region. A and B designate features due to ferredoxin centers A and B.

Fig. 3. The orientional anisotropy of the $g_x = 1.78$ peak height of X^-, measurement at the same conditions as in fig. 2. θ is the angle between the direction of the orienting field and the direction of the Zeeman field.

Fig. 4.a The dependence of EPR peak heights on microwave power for selected peaks attributed to reduced ferredoxins A and B and X^-. Samples are not oriented. X^- is measured in chemically reduced samples which were illuminated during cooling, while the ferredoxin are measured in chemically reduced samples or photoreduced samples. The behavior of the ferredoxin peaks is independent of X^- photoreduction. Temperature, 12 K, modulation amplitude, 32 G.

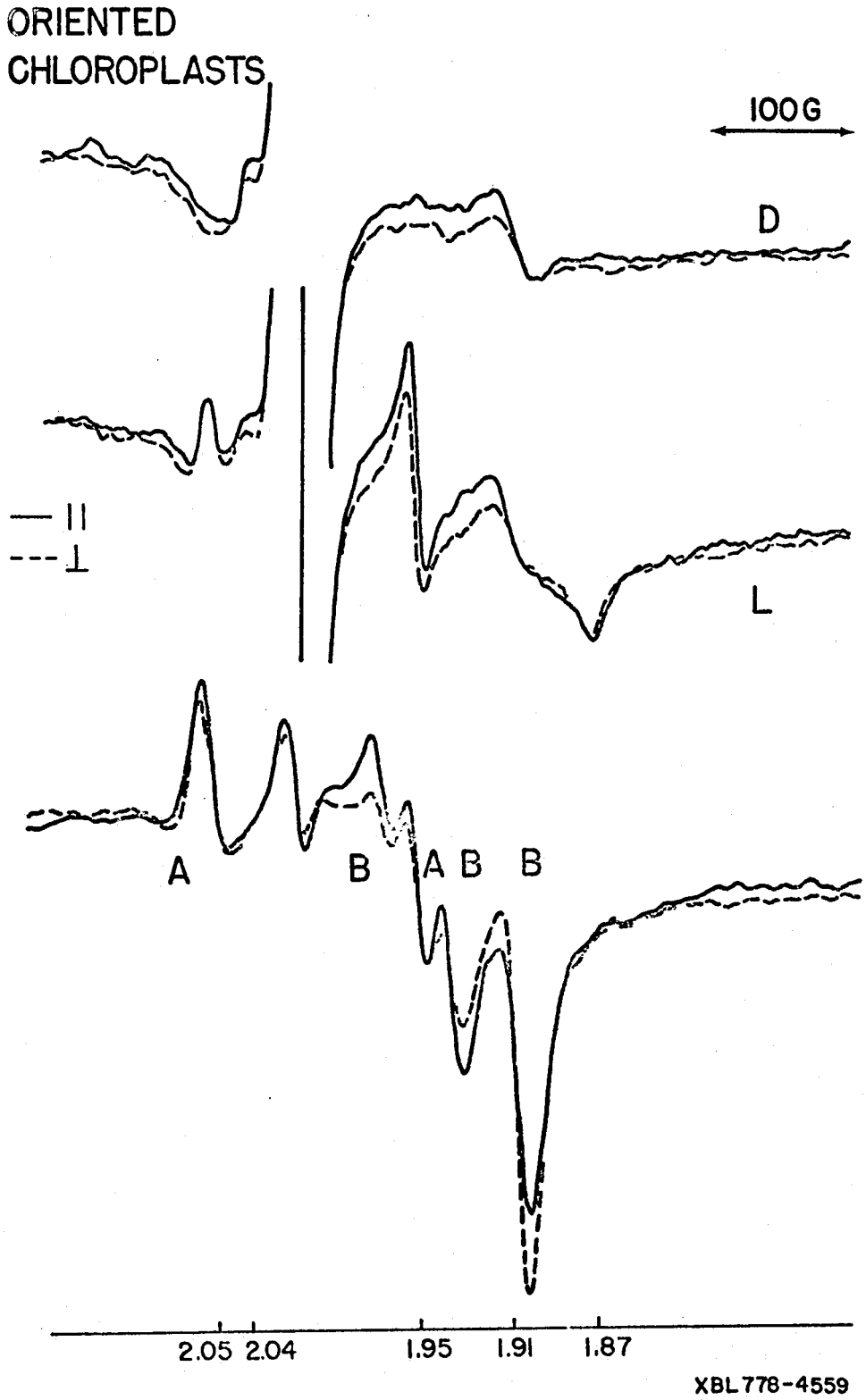
Fig. 4.b The temperature dependence of the linewidth (▲) and area (●) under the derivative peak of g_x for X^-. ΔH is the full width at half height of g_x in a sample oriented with the membrane normal and the Zeeman field parallel. The area x temperature product is plotted to remove the Curie temperature dependence. The sample is the same as in Fig. 2. (photoreduced + dithionite). Modulation amplitude 32G, microwave power, 5.0 mW.

Fig. 5. The EPR spectrum of chloroplasts in buffer observed in the dark. Samples were illuminated at low intensity during cooling from room temperature to 220 K. Light intensity during trapping, $5 \times 10^2 Jm^{-2}s^{-1}$; total chlorophyll concentration, 3 mg/ml; Upper trace, unoriented 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).

Fig. 6. 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 intensify during trapping $2x10^3 Jm^{-2}s^{-1}$ in the visible region.

Fig. 7, 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 in the membrane of these and other Photosystem I components is suggested. PCy is plastocyanin ; A is an early electron acceptor.

Fig. 8. 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 77K or below at $5x10^2 Jm^{-2}s^{-1}$ 3) illuminated during cooling from room temperature to 220K at $5x10^2 Jm^{-2}s^{-1}$, stored at 77K prior to observation; 4) same as 3 except an intensity of $2x10^2 Jm^{-2}s^{-1}$. The presence of ascorbate or dithionite plus mediators increased the yield of X^- in 4.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XBL 778-4559

Fig. 1

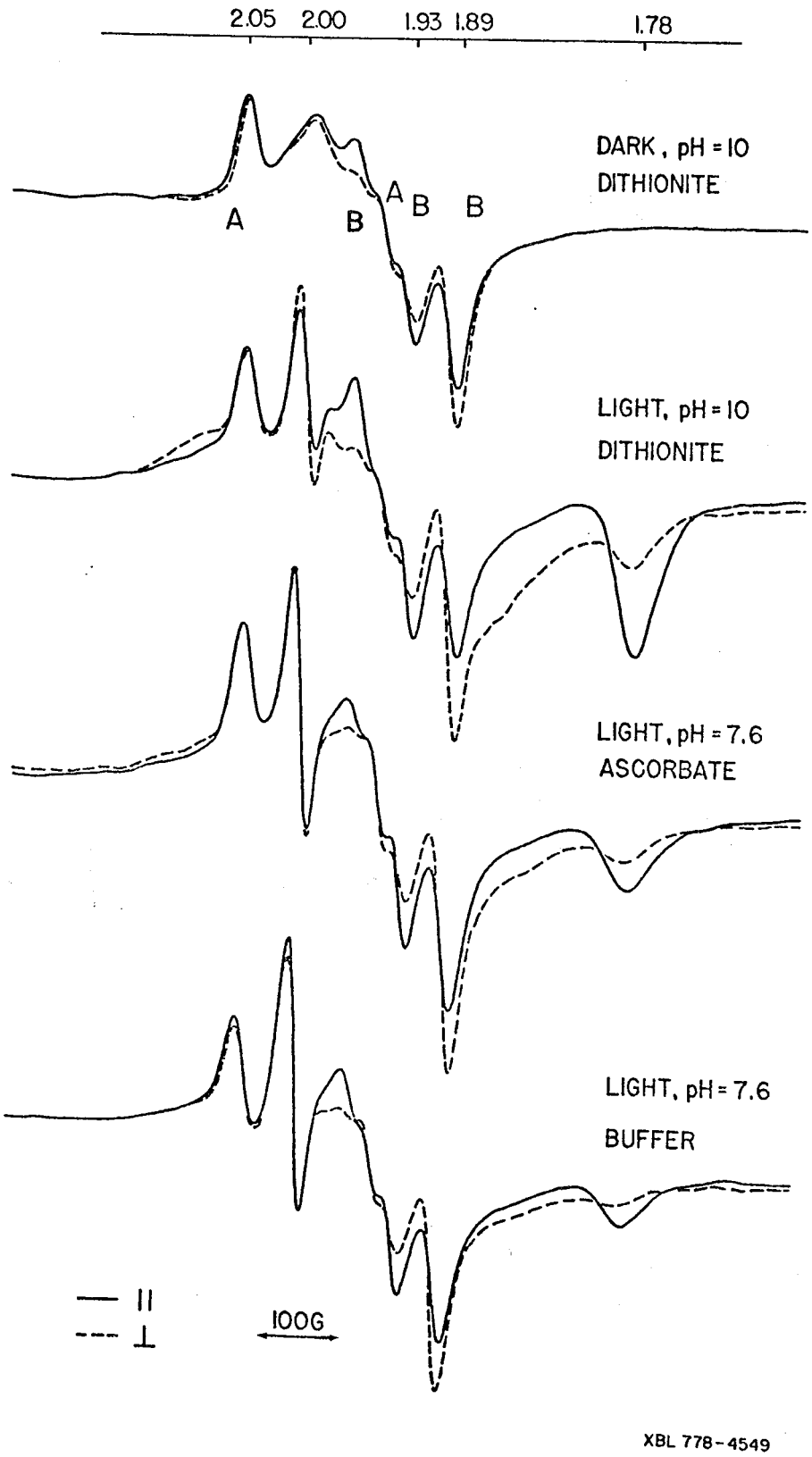
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Fig. 2

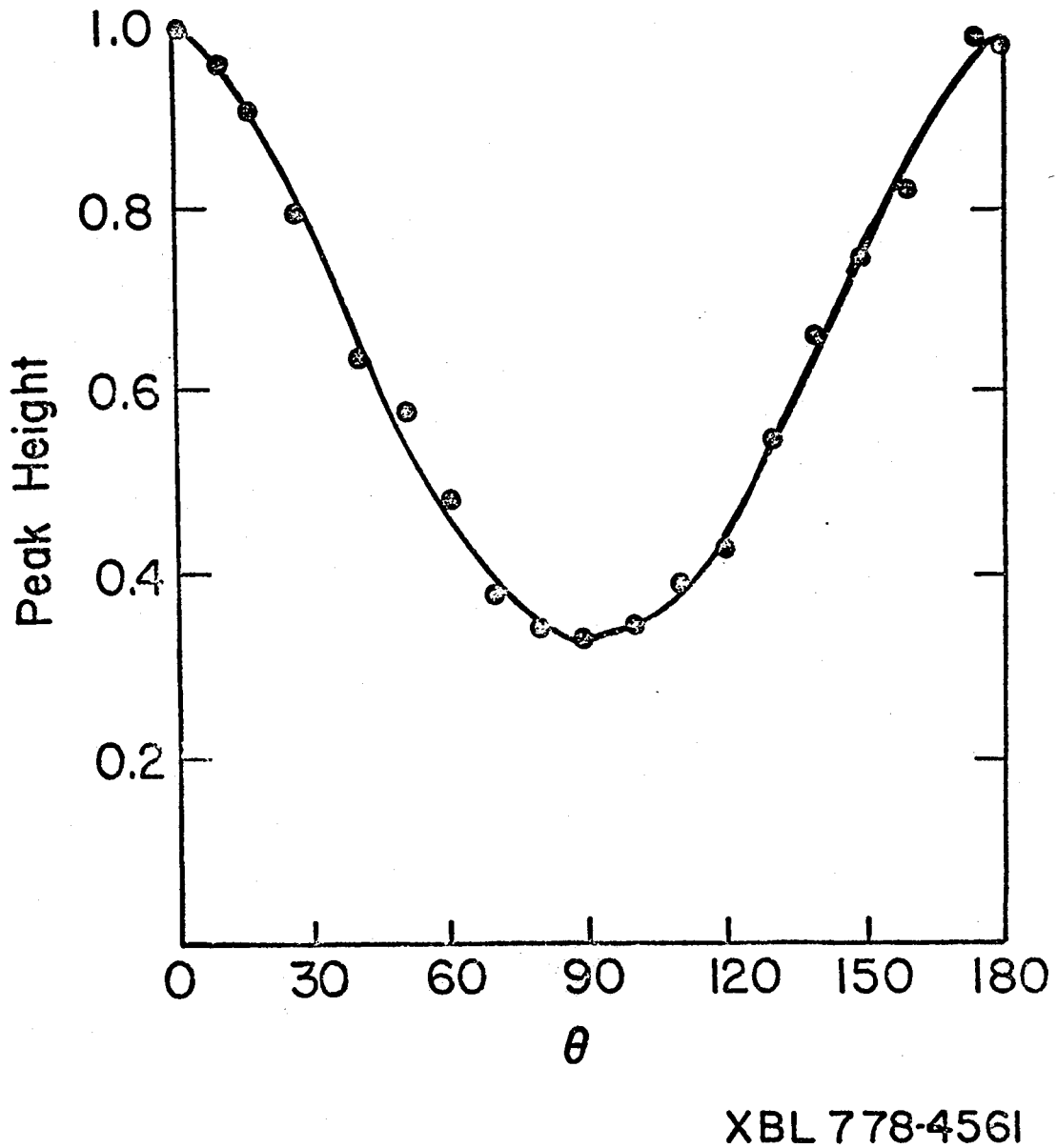

Fig. 3

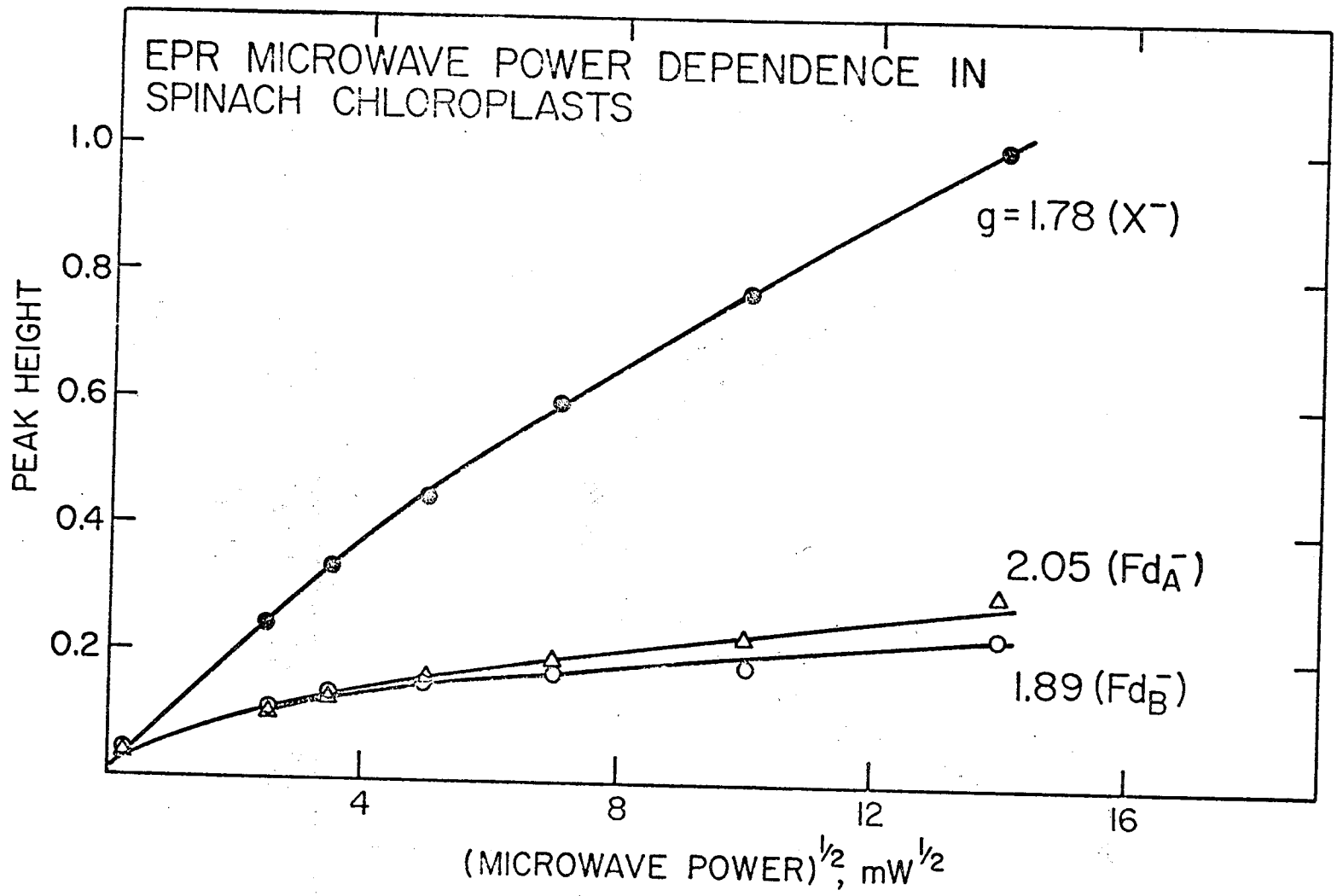

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Fig. 4a

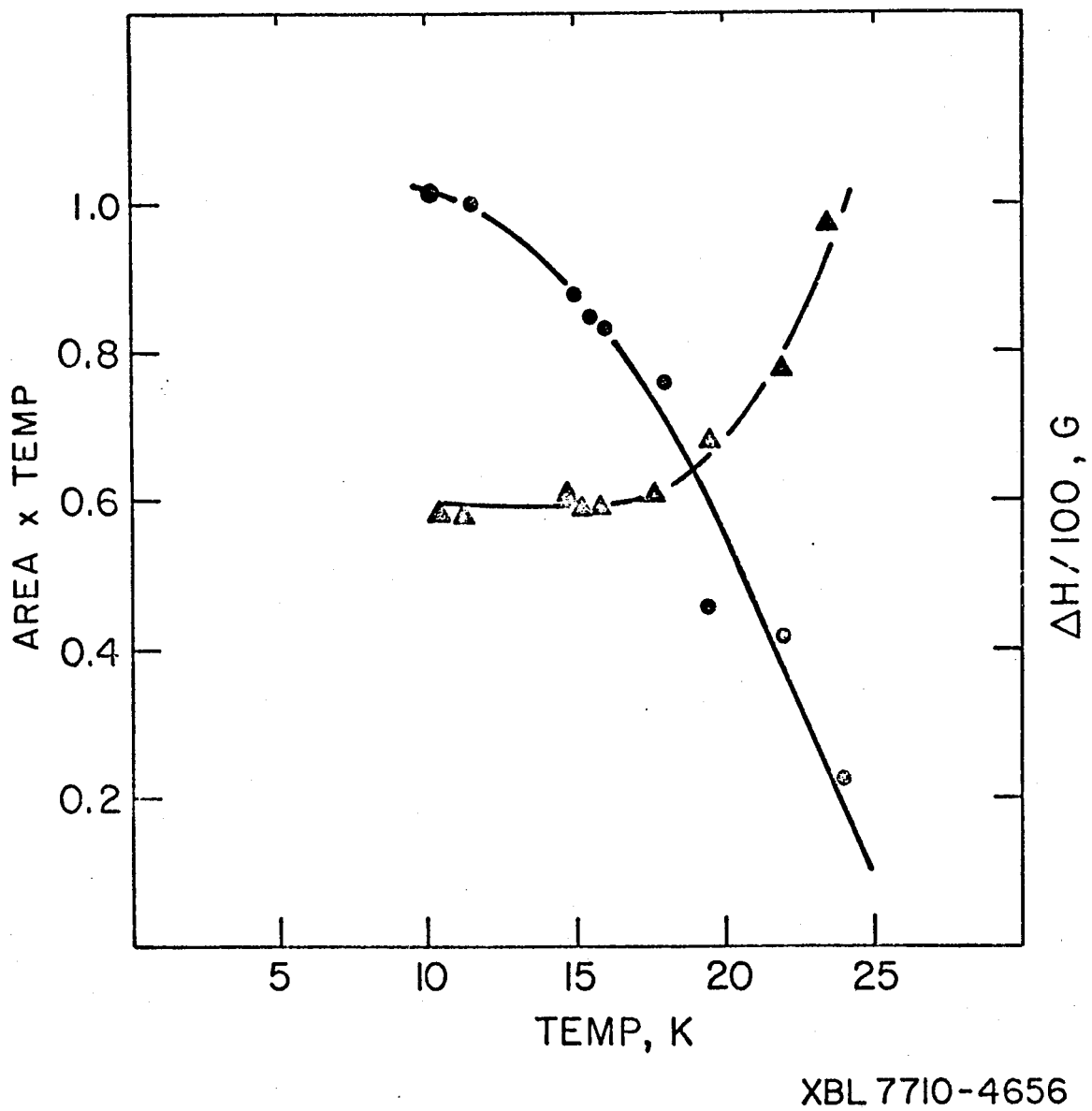
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Fig. 4b

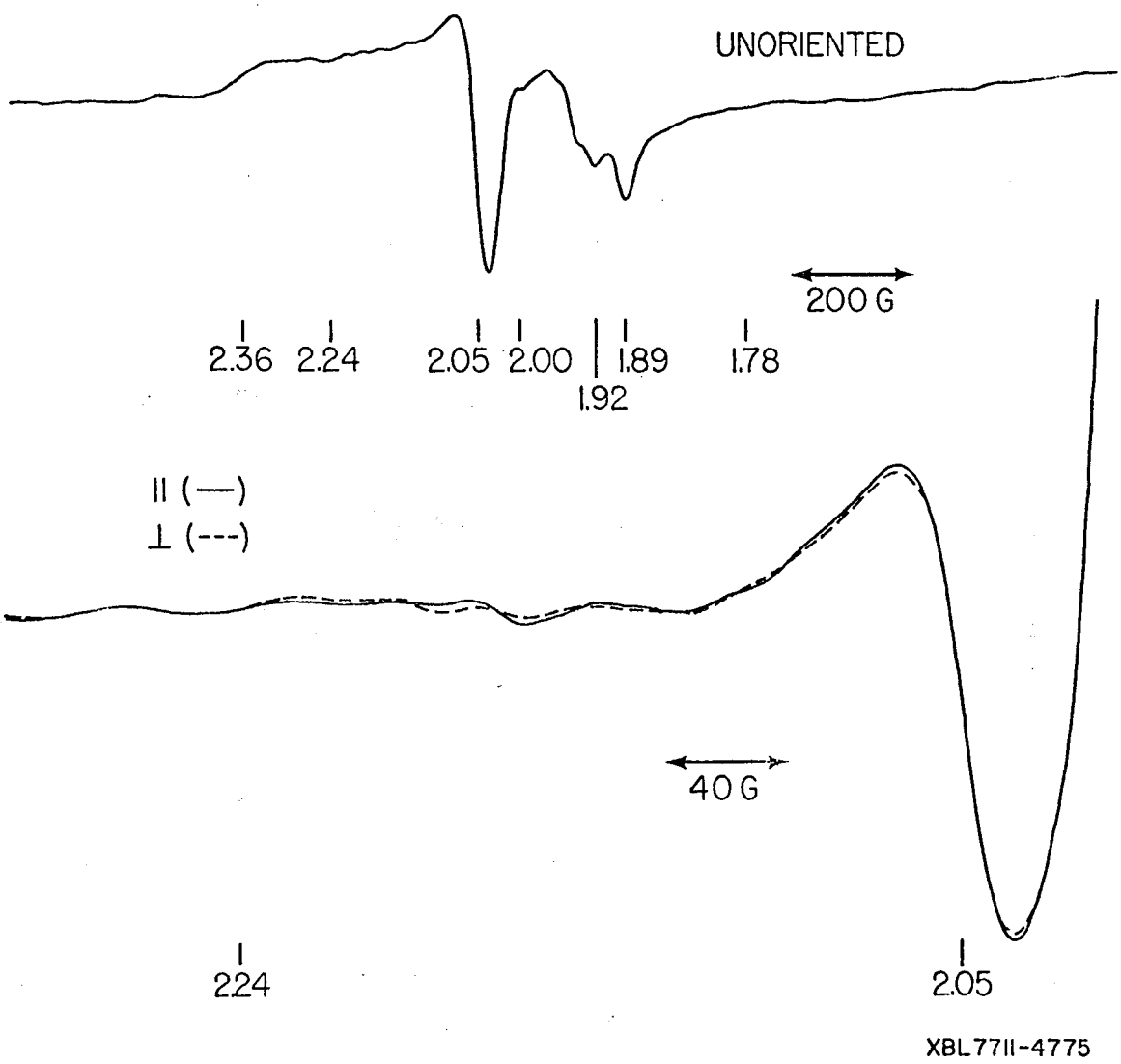
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Fig. 5

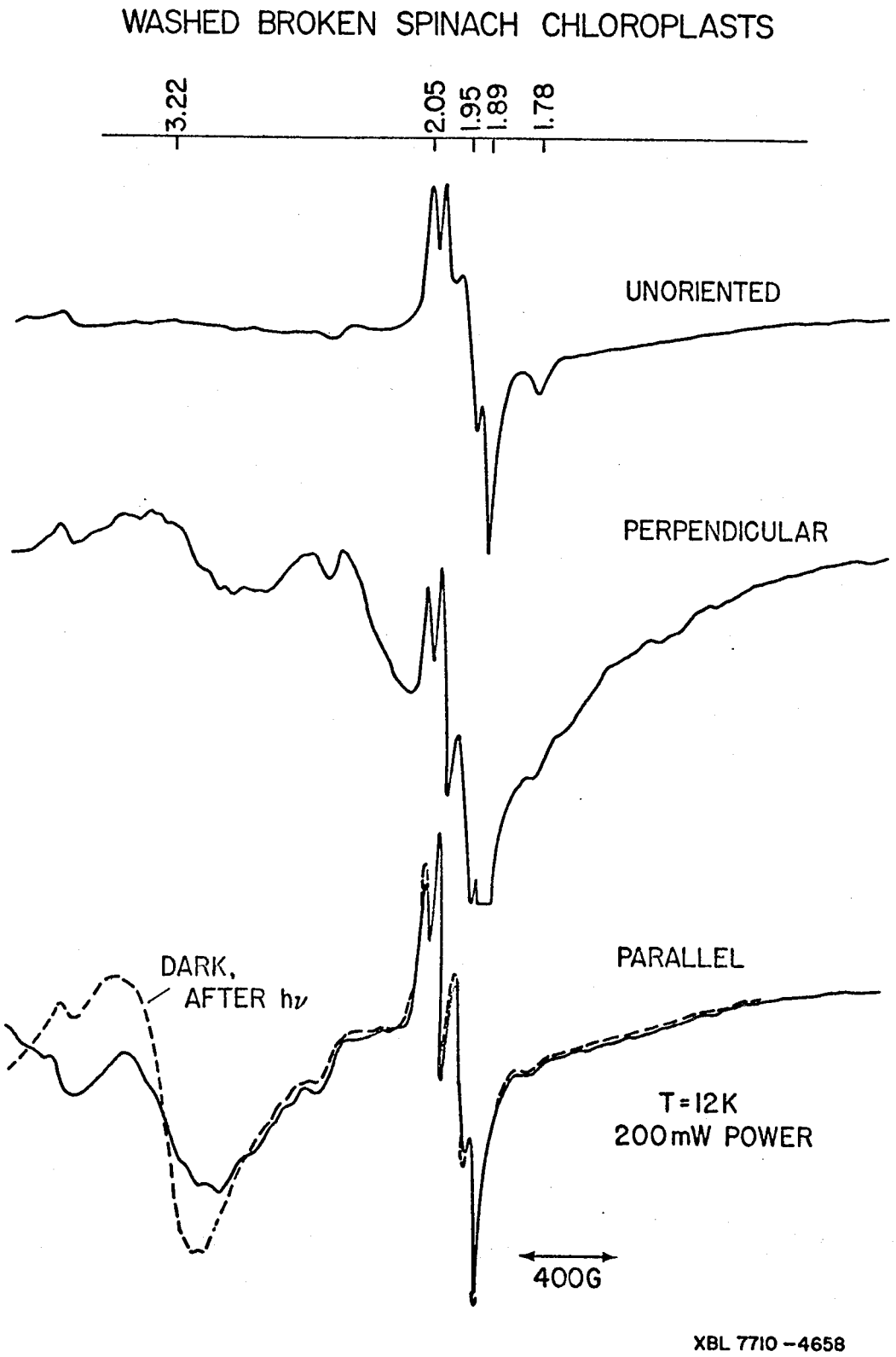
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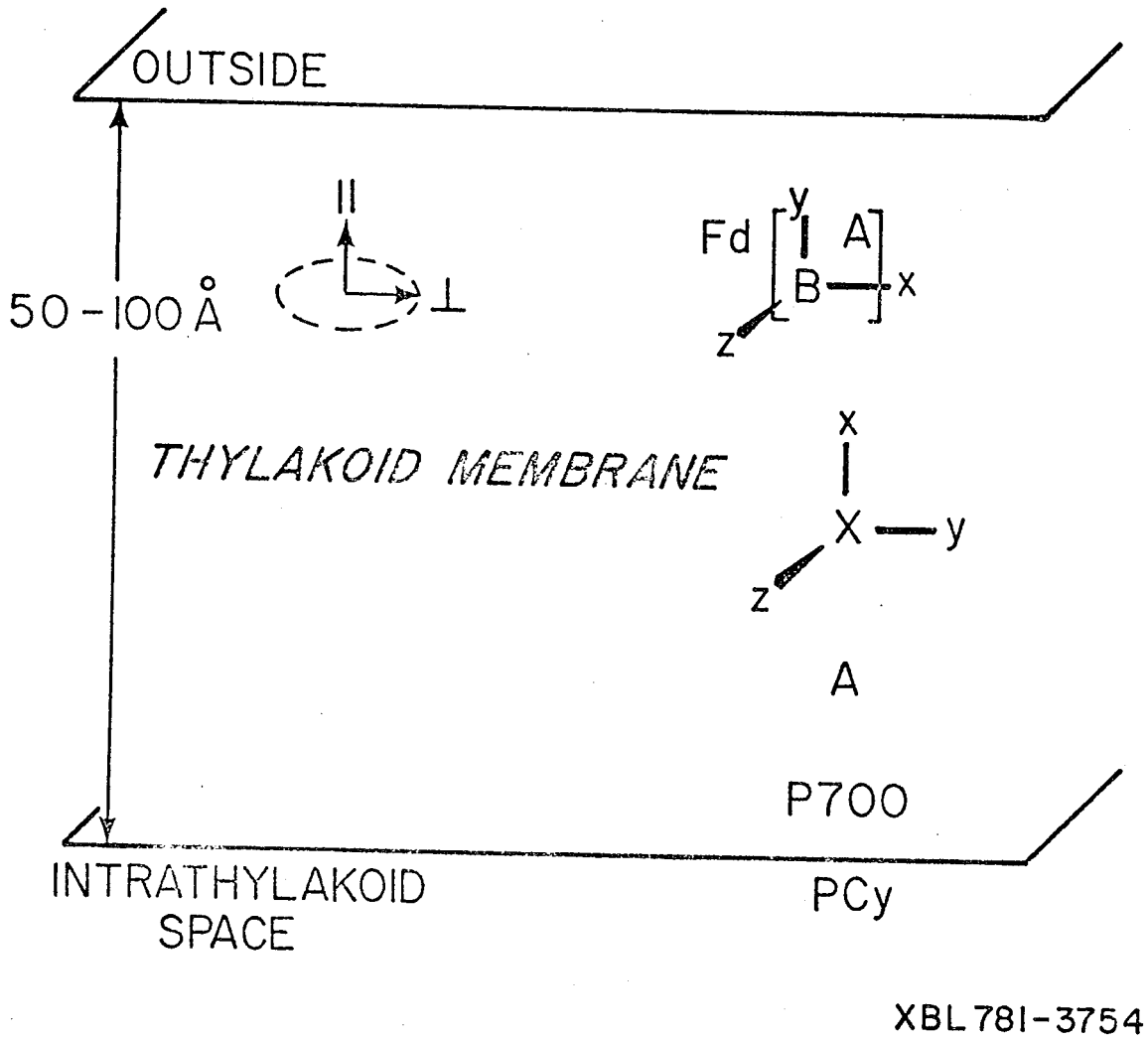
Fig. 6



XBL 781-3754

Fig. 7

PHOTOCHEMICAL TRAPPING IN CHLOROPLASTS

1)	PC	P700	X	Fd_B	Fd_A	frozen in the dark
2)	PC	$P700^+$	X	Fd_B	Fd_A^-	dark while cooling, hν at ≤ 77K
3)	PC^+	$P700^+$	X	Fd_B^-	Fd_A^-	hν while cooling
4)	PC^+	P700	X^-	Fd_B^-	Fd_A^-	intense hν, cooling or intense hν + reductant, cooling
		$P700^+$	X^-			not trapped

Fig. 8