Orientation of membrane-bound cytochromes in chloroplasts, detected by low-temperature EPR spectroscopy

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1. INTRODUCTION

There are 4 types of membrane-bound cytochromes in chloroplasts; 1 *c*-type cytochrome (cytochrome *f*) and 3 *b*-type cytochromes (known by their α-band absorption maxima as cytochrome b_{563} (also known as b_6), b_{559} (LP) and b_{559} (HP), where LP and HP refer to low and high potential forms, respectively). Only within the last few years have the EPR characteristics of chloroplast cytochromes been determined [1–3].

Here, we report EPR spectroscopy of oriented chloroplasts to elucidate the orientation of the magnetic axes of the cytochromes. In [4] we reported new EPR signals at g 2.3 and g 2.15 that were highly oriented in chloroplast thylakoid membranes. Here, we report that these signals are due to the low and high potential forms of cytochrome b_{559}, respectively. In addition, we find that cytochrome *f* is highly oriented in the membrane, contrary to [5].

This work has also been reported in [6,7].

2. MATERIAL AND METHODS

Broken spinach chloroplasts were prepared and oriented by partial dehydration as in [4]. The orientation which is obtained is along a single dimension, normal to the plane of the membranes. There is no preferential orientation in the plane of the membrane. For samples in which 2,3-dichloro-5,6-dicyano-1,4-benzoquinone and potassium ferricyanide were used, these oxidants were added prior to partial dehydration at 1 mM and 10 mM, respectively.

X-band (9.2 GHz) EPR spectra were obtained at cryogenic temperatures as in [4].

3. RESULTS AND DISCUSSION

The iron atom of the heme group, to a first approximation, is centered in a distorted octahedron with the corners of the octahedron occupied by ligands of which 4 are the nitrogen atoms of the porphyrin ring. In general, this symmetry restricts the possible spin states of a single cytochrome to either high spin ($s = 5/2$) or low spin ($s = 1/2$). All reports on plant cytochromes have been interpreted in terms of these spin states. By measuring the orientation of the magnetic axes of cytochromes and using the relationship between magnetic and molecular axes for high and low spin cases, the orientation of the heme plane in the membrane can be determined. All of the EPR signals thought to arise from oxidized cytochromes show marked angular dependence, as discussed below.

3.1. *High potential cytochrome* b_{559}

Unique to chloroplast cytochromes, the high potential form of cytochrome b_{559} is photooxidizable at cryogenic temperatures [8,9]. The only reports of the EPR detection of cytochrome b_{559} (HP), on the basis of low temperature photooxidation, were made by monitoring the g 3.1 signal [1,5].

Fig.1 displays the results of an experiment which

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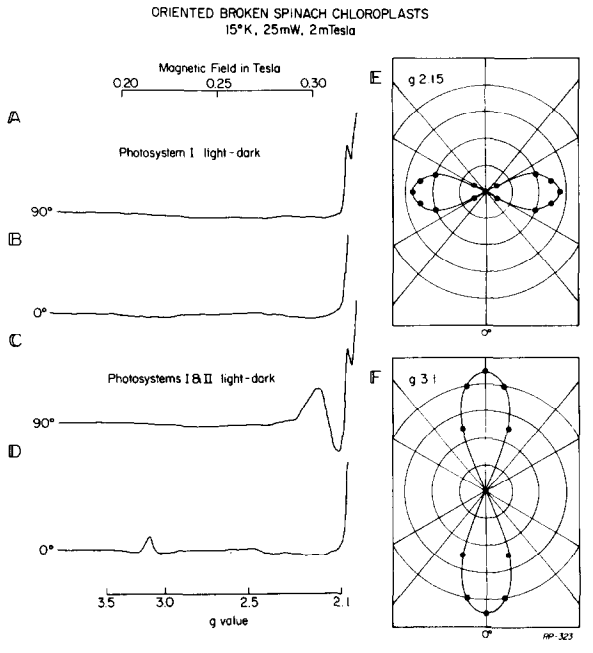

Fig.1. Light-minus-dark spectra of oriented broken spinach chloroplast membranes. Spectra were recorded at 10° intervals in the dark, chloroplasts were illuminated with far-red light (5×10^4 ergs/cm^2 . s) in the spectrometer cavity, only activating photosystem I, and another series of spectra were recorded at the same angles. Broad-band illumination (2×10^5 ergs/cm^2 . s), to excite both photosystems was then applied and another series of spectra recorded. The spectra represent light-minus-dark difference spectra and show that the g 3.1 and g 2.15 signals are generated only when photosystem II is activated (C,D). EPR conditions: microwave power, 25 mW; modulation, 2 m Tesla; temp., 15 K. The signal near g 2.05 is due to photoinduced iron–sulfur centers A and B (see [4]). (E,F) are polar plots of the amplitude of the g 2.15 and g 3.1 difference spectra obtained with broad-band red light. Radial scale is in arbitrary units. 0° represents the angle when the applied magnetic field is parallel to the membrane plane.

shows that both g 2.15 and g 3.1 signals are photooxidized at low temperature (15 K) only by photosystem II light. Fig.1a,b show the light-minus-dark difference spectra or oriented chloroplasts illuminated at 15 K with photosystem I light (≥ 730 nm). The signal intensity of signal I was monitored to assure that photosystem I was activated by this light. Fig.1c,d show the results when the long wavelength filter was removed and the chloroplasts were exposed to broad-band red light. Highly oriented, light-induced signals at g 3.1 and g 2.15 were detected (fig.1e,f; see fig.5 in [4]). These signals are assigned to the g_z (g 3.1) and g_y (g 2.15) axes of cytochrome b_{559} (HP). This is in agreement with an earlier report that a low temperature light-induced EPR signal at g 2.12 might be due to a cytochrome [10].

High potential cytochrome b_{559} is a low-spin heme, so the g_y axis is in the plane of the heme [11]. Therefore, the heme of cytochrome b_{559} (HP) is oriented perpendicular to the membrane plane. This result agrees with [5] and is consistent with the optical study which found the heme plane of cytochrome b_{559} (HP) to make an angle $>35°$ from the membrane plane [12].

3.2. *Cytochrome* f

The g_z axis of cytochrome f is the only g-value for this center to be reported to date. Cytochrome f is reduced in dark-adapted chloroplasts, however upon the addition of potassium ferricyanide it is

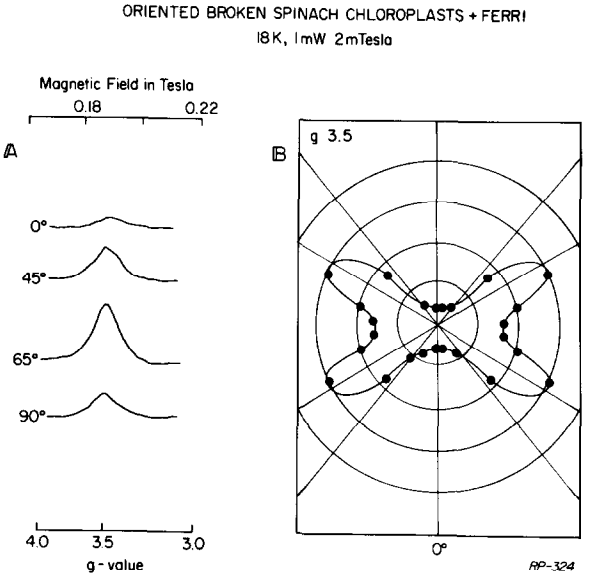

Fig.2. The g 3.5 signal obtained when chloroplasts were incubated with 10 mM $K_3Fe(CN)_6$. The signal due to residual ferricyanide has been subtracted from these spectra. EPR conditions: microwave power, 1 mW; modulation, 2 m Tesla; temp., 15 K.

oxidized and gives rise to an EPR signal at g 3.5 [5]. This signal is highly oriented (dichroic ratio = 4:1) with the g_z 3.5 signal intensity maximum at ~60° with respect to the membrane plane (see fig.2). Since cytochrome f is a low-spin heme, the heme plane is therefore oriented 30° from the membrane plane. These results are in apparent disagreement with [5] reporting that cytochrome f was poorly oriented in the membrane. However, they only measured spectra at 0° and 90°, and these angles will not represent the true dichroic ratio for this cytochrome (fig.2b).

3.3. *Low potential cytochrome* b_{559}

The low potential form of cytochrome b_{559} has been detected in a low-spin ferric state [1–3]. It has been assigned EPR signals at g_z 2.9 and g_y 2.26, as these signals are present in particles in which the only cytochrome present is the low potential form of cytochrome b_{559} [13].

In [4] we observed, in dark-adapted chloroplasts, an oriented EPR signal at g 2.3 which was oriented 90° with respect to the membrane plane (fig.1,2 in [4]). It now seems clear that this signal represents the g_y axis of the low potential form of cytochrome b_{559} [1] and its orientation is consistent with the orientation of the corresponding g_z axis [5]. The heme plane of cytochrome b_{559} (LP) is therefore oriented perpendicular to the membrane plane.

3.4. *Cytochrome* b_{563} (b_6)

The EPR characteristics of cytochrome b_6 are controversial; its function and optical spectra suggest a low spin heme, but there have been several reports in which cytochrome b_6 has been assigned as a high-spin heme with EPR signals in the g 6 region [2,3,14]. In [3] high-spin signals in b–f particles titrated with E_m = 0 mV, which is the value expected for cytochrome b_6. However, in [1] signals in the g 6 region were <20% of the concentration of the reaction center, P700, although the concentration of cytochrome b_6 would be expected to be twice that of P700. Therefore, the specific nature of these high-spin signals remains unclear.

In dark-adapted chloroplasts, cytochrome b_6 is oxidized and there are 2 signals in the g 6 region (g 6.2 and g 7.2) oriented ~50° from the membrane plane (fig.3). If the g 7.2 is due to a high-spin heme, a corresponding component near g 4.8 would be expected which we did not detect, but this may be lost in the g 4.3 signal attributed to rhombic iron. When the oxidant, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, was added to chloroplasts [3], much more intense EPR signals were detected split about g 6 at g 5.95 and 6.26, both oriented ~50° from the membrane plane (fig.4). It is interesting to note that all these signals are oriented at the same angle, 50°, suggesting that they might originate from the same center. The splitting about

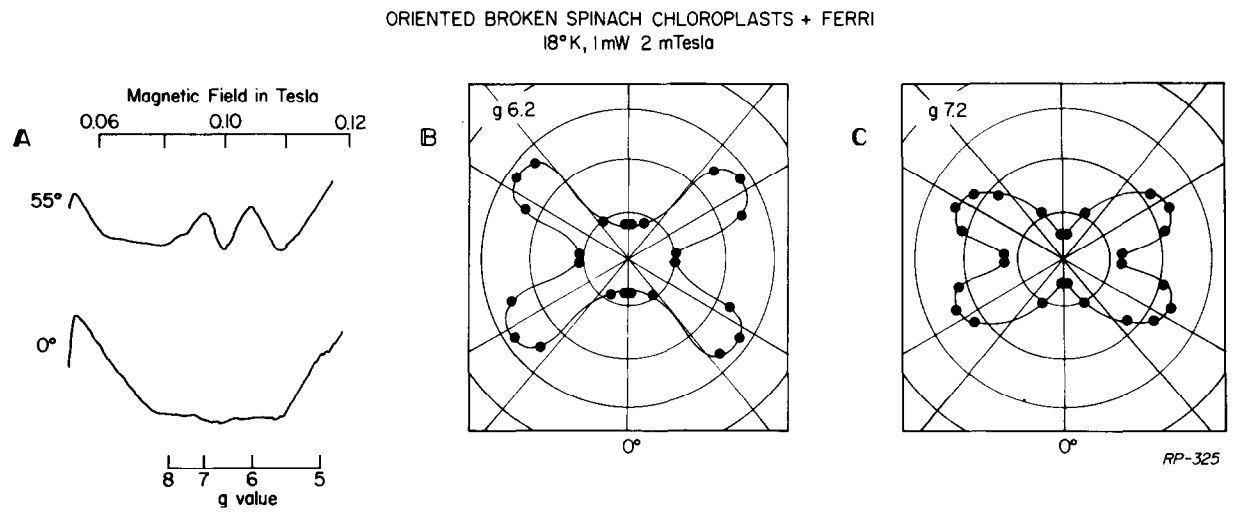

Fig.3. The g 6.2 and g 7.2 signals obtained from dark-adapted and ferricyanide-treated chloroplasts. EPR conditions as in fig.2.

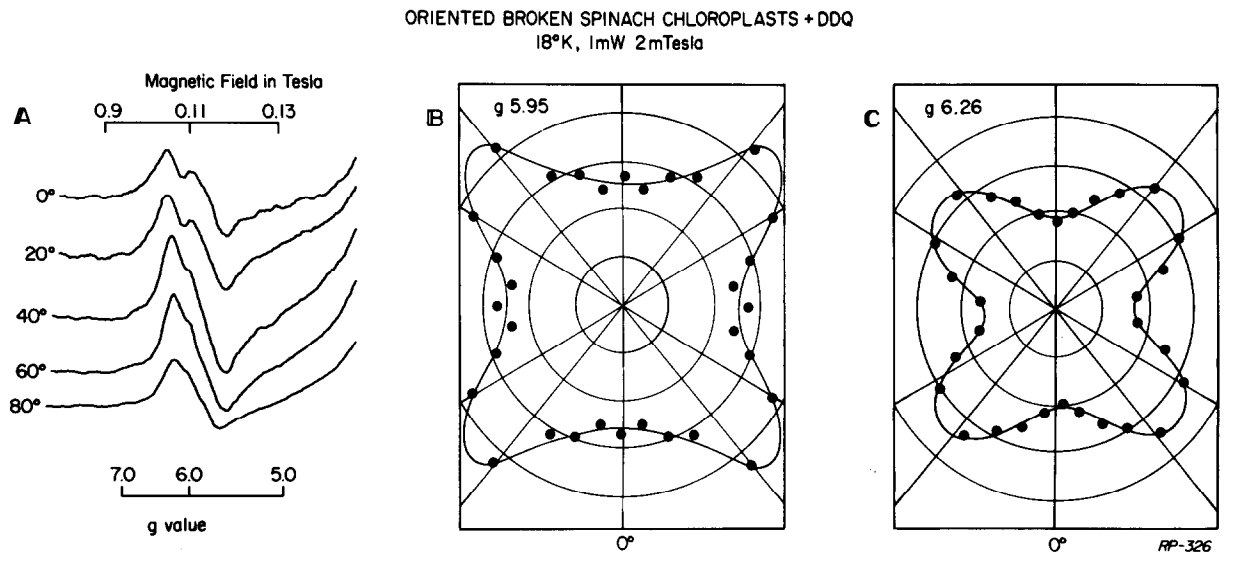

Fig.4. The signals induced in the g 6.0 region by incubating chloroplasts with 1 mM 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. EPR conditions as in fig.2.

g 6, after the addition of the high potential quinone, is characteristic of a high-spin heme with rhombic distortion. For a high-spin heme, the g 6 axes are in the plane of the heme [15]. If these high-spin signals do represent cytochrome b_6, then the heme plane of this cytochrome is oriented 50° from the membrane plane, but this assignment for the orientation of cytochrome b_6 will have to be rather tentative until the EPR characteristics of this cytochrome, with and without adventitious quinones, are better understood.

In summary, we conclude that both high and low potential cytochromes b_{559} have their heme planes oriented perpendicular to the membrane plane. Cytochrome f has its heme plane at 30° to the membrane. If we tentatively assign the high spin signal to cytochrome b_6, this cytochrome has its heme plane oriented 50° to the membrane plane. This orientation of cytochrome f (and b_6) is the first report of membrane bound cytochromes oriented at angles other than 0° or 90°.

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