On the Spatial Organization of Hemes and Chlorophyll in Cytochrome \( b_6f \)

A LINEAR AND CIRCULAR DICHROISM STUDY

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The organization of chromophores in the cytochrome \( b_6f \) from *Chlamydomonas reinhardtii* has been studied spectroscopically. Linear dichroism (LD) measurements, performed on the complex co-reconstituted into vesicles with photosynthetic reaction centers as an internal standard, allow the determination of the orientations of the chromophore with respect to the membrane plane. The orientations of the \( b_6- \) and \( b_1- \)hemes are comparable to those determined crystallographically on the cytochrome \( bc_1 \). The excitonic CD signal, resulting from the interaction between \( b \)-hemes, is similar to that reported for the cytochrome \( bc_1 \). LD and CD data are consistent with the differences between the \( b_6f \) and \( bc_1 \) leaving the orientation of the \( b \)-hemes unaffected. By contrast, the LD data yield a different orientation for the heme \( f \) as compared either to the heme \( c_1 \) in the crystallographic structures or to the heme \( f \) as studied by electron paramagnetic resonance. This difference could either result from incorrect assumptions regarding the orientations of the electronic transitions of the \( f \)-heme or may point to the possibility of a redox-dependent movement of cytochrome \( f \). The chlorophyll \( a \) was observed in a well defined orientation, further corroborating a specific binding site for it in the \( b_6f \) complex.

Cytochrome (Cyt)* \( b_6f \) is one of the three membrane protein complexes involved in electron transport in oxygenic photosynthesis. It receives electrons from plastoquinol reduced by the oxygen-producing photosystem II reaction centers and transfers them to plastocyanin, the electron donor to the NADP\(^+\) -reducing photosystem I reaction centers. Electron transfer through the Cyt \( b_6f \) complex is coupled to proton translocation across the membrane. Cyt \( b_6f \) therefore contributes to building up the electrochemical proton gradient that drives ATP synthesis. A homologous complex, Cyt \( bc_1 \), carries out an equivalent function in the respiratory chains of mitochondria and bacteria. The structure of mitochondrial Cyt \( bc_1 \) has been determined by x-ray diffraction (1–3), whereas only low resolution electron microscopy projection maps are available for Cyt \( b_6f \) (4, 5). The structure of two important extramembrane fragments of Cyt \( b_6f \), the catalytic domains of Cyt \( f \) and of the Rieske protein (9), has been established by x-ray diffraction.

Cyt \( b_6f \) resembles Cyt \( bc_1 \) in its general energy-transducing function and in some aspects of its subunit composition and properties (see Refs. 10–13), but it also exhibits structural and functional peculiarities. Functionally, the following differences may be noted: (i) the redox potential gap between the low potential \( (b \)-hemes) and high potential \( (Rieske \) protein, \( c_1/f \)-hemes) branches of the electron transfer pathway is significantly larger in the \( b_6f \) complex; (ii) the electron transfer steps leading to quinone reduction at the cytosolic quinone-binding site (Q\(_c\)) and the sensitivity of this site to inhibitors are different; and (iii) the mechanism that couples proton translocation to electron transport by Cyt \( bc_1 \), first formulated as Mitchell’s quinone \( (Q)\)-cycle hypothesis (14) and later modified (15, 16), appears to be complemented by active proton pumping in the case of Cyt \( b_6f \) (see Refs. 12, 13, 17, and 18). Structurally, the \( b_6f \) complex presents many differences with Cyt \( bc_1 \) (reviewed in Refs. 13 and 18) as follows: (i) the single protein that bears the two \( b \)-hemes in the \( bc_1 \) complex, apocytochrome \( b \), is split in the \( b_6f \) one into two subunits, apocytochrome \( b_a \) and subunit IV; (ii) the two heme-carrying histidine residues are separated by 13 residues in the fourth helix of apocytochrome \( b \) and by 14 residues in that of apocytochrome \( b_a \); (iii) the high potential cytochromes, Cyt \( f \) and Cyt \( c_1 \), are not homologous to each other and feature totally different folds and modes of heme ligation (2, 3, 7); (iv) with the exception of the third redox-active protein, the iron-sulfur Rieske protein, the other subunits in the two complexes (subunits PetG, PetL, and PetM in Cyt \( b_6f \); see Ref. 19 and references therein) appear unrelated; (v) the \( b_6f \) complex contains pigments, chlorophyll \( a \) (Chl\(_a\)), and \( \beta \)-carotene, which are not present in Cyt \( bc_1 \) (see Refs. 20–22 and references therein). All of these compositional and structural differences could entail discrepancies in the relative arrangement of the redox-active groups in the two types of complex, a matter about which nothing is known.

EPR or linear dichroism spectra recorded on oriented immobilized samples contain information about the orientation of probes that possess g-tensor anisotropy (paramagnetic transitions) or optical anisotropy (electronic absorbance transitions). Many EPR studies have been made on the Rieske protein of the Cyt \( bc_1 \) and \( b_6f \) complexes (24–28). The recent resolution of the crystallographic structure of mitochondrial Cyt \( bc_1 \) has made it possible to correlate the orientations of the \( g_a \), \( g_f \), and \( g_c \) axes

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‡ The abbreviations used are: Cyt, cytochrome; BChl, bacteriochlorophyll; BCHl, bacteriopheophytin; Chl, chlorophyll; IG, Hecameg (6-O-(6-heptylcarbamoyl)-methyl-a-n-glycoparapsirone); LD, linear dichroism; LDAO, lauryldimethylaminoxide; RC, reaction center; Q, quinone.

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with the geometry of the iron-sulfur cluster. Similarly, EPR studies on a crystal of oxidized Cyt c (29) established that the g∥ and g⊥ axes of the tensor lie along the two N-Fe-N axes, and the g∥ axis is normal to the plane. In principle, this information could be used to try and determine by EPR the orientation of the c∥- and b-hemes in the bf-bc complexes. In practice, however, the low amplitude and overlap of the EPR signals render a precise study difficult (26, 30). Linear dichroism (LD) constitutes an alternative method. The a, b, and γ optical absorbance bands of Cyt c are known to correspond to electronic transitions lying in the plane of the heme (31), a conclusion tentatively extended to other hemes (32). Each of these bands corresponds, in many but not all cases (33), to the overlap of two transitions. The directions of the two transitions in the plane of the heme are not known with certainty but have been proposed to lie along the X and Y Fe-pyrrrole ring directions (34, 35). Bacteriochlorophylls (BChl) and chlorophylls (Chl) lack the high degree of symmetry of porphyrins, and the absorbance bands associated with different directions in the plane of the chlorin ring are therefore overall distinct (32, 36).

In the present work, we have applied LD to studying the orientation of the hemes and of Chl a in the Cyt bf f complex from the freshwater alga *Chlamydomonas reinhardtii*. This study has been complemented by measuring the circular dichroism (CD) signal that results from excitonic interaction between the b-hemes, whose intensity depends on their distance and relative arrangement in space. Altogether, these studies on a crystal of oxidized Cyt c (29) established that the g∥ and g⊥ axes of the tensor lie along the two N-Fe-N axes, and the g∥ axis is normal to the plane. In principle, this information could be used to try and determine by EPR the orientation of the c∥- and b-hemes in the bf-bc complexes. In practice, however, the low amplitude and overlap of the EPR signals render a precise study difficult (26, 30). Linear dichroism (LD) constitutes an alternative method. The a, b, and γ optical absorbance bands of Cyt c are known to correspond to electronic transitions lying in the plane of the heme (31), a conclusion tentatively extended to other hemes (32). Each of these bands corresponds, in many but not all cases (33), to the overlap of two transitions. The directions of the two transitions in the plane of the heme are not known with certainty but have been proposed to lie along the X and Y Fe-pyrrrole ring directions (34, 35). Bacteriochlorophylls (BChl) and chlorophylls (Chl) lack the high degree of symmetry of porphyrins, and the absorbance bands associated with different directions in the plane of the chlorin ring are therefore overall distinct (32, 36).

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**EXPERIMENTAL PROCEDURES**

**Purification of Cyt bf Complex and Photosynthetic Reaction Center—** C. *reinhardtii* Cyt bf f was purified as described previously (19). Cyt bf f concentrations were determined by absorbance measurements performed on a Hewlett-Packard 8543 spectrophotometer following reduction with either ascorbate or dithionite, taking the molar extinction coefficients presented in this paper refer to normal to the heme plane as defined by the gel compression axis, and α a constant that is a function both of the statistical orientation parameter of the sample in the polyacrylamide gel and of the characteristics of the spectrophotometer.

When the two transitions X and Y are energetically distinct the angles α and β for each transition can be determined. Assuming X and Y transitions to be orthogonal to each other and to lie in the plane of the tetrapyrrrole ring, the tilt α of the normal with respect to the gel compression axis is given by Equation 2.

\[
\alpha = \arcsin \left( \frac{D}{A_{\text{normal}}} \right)
\]

where \(\alpha\) is the angle between the X and Y transitions.

A fitting program (Sigma Plot, Jandel Scientific) was used to simultaneously fit LD and absorption (from dichroism and redox titration experiments) spectra. The fitting procedure held, for a heme, the wavelength positions of the X and Y transitions constant in the LD and absorption spectra and held the \(A_y/A_x\) areas ratio constant in all absorption spectra and between two LD spectra, but allowed these ratios to vary between absorption and LD spectra. Gaussian line shapes were used.

There are two sources of experimental errors, measurements and analysis. The laboratory-built spectrophotometer used yields measurements (recorded twice and averaged) whose noise level does not exceed 1% of the signal. The main source of errors therefore arises from the fitting procedure. For a given heme, we allowed a maximum 2% of variation in the respective contributions of the X and Y transitions to independent spectra. Global fits were constrained to respect a maximum 3% of deviation of fitted versus experimental values. From Equation 1, we can evaluate the propagation of errors, \(\Delta LD\) and \(\Delta A\), on the orientation of a single transition, \(\Delta \beta\), as shown in Equation 4.

\[
\Delta \beta = \arcsin \left[ \frac{1/3}{2} \sqrt{2 \Delta LD / A_{\text{normal}}} \right] + 1/2
\]

The value of \(\Delta \beta\) depends on that of \(\Delta LD\), but in the worst case and considering the largest error, the margin of error is \(\pm 2.5^\circ\). Most results presented in this paper refer to normal to the heme plane as defined by the X and Y transitions instead of angles of individual transitions. From Equation 2, we can evaluate the resultant errors, \(\Delta \alpha\), on the tilt angle \(\alpha\) of this normal, as shown in Equation 5.

\[
\Delta \alpha = \arcsin \left[ \frac{1/3}{2} \sqrt{2 \Delta LD / A_{\text{normal}}} \right] + 1/2
\]

The error again depends on that of \(\Delta LD\), but in the worst case we calculate a maximum theoretical margin of error of \(\pm 3.5^\circ\).

**Circular Dichroism Spectroscopy—** CD spectra were measured on a Mark V Jobin & Yvon spectropolarimeter, using 0.7-ml samples in 2-mm path length quartz cuvettes (Hellma, Q5 0.200 series 282). Spectra were scanned between 350 and 600 nm in 0.2-nm steps, using a response time of 1 s and a sensitivity of 2–5 × 10^{-4}, first after reducing the samples with solid ascorbate and then following reduction by solid dithionite. Absolute spectra were obtained as the average of two scans minus that of two buffer blanks. Data are shown as the difference between absolute spectra recorded in the two redox conditions. Monomerization of Cyt bf f was achieved by supplementing a dimeric sample in 400 mM ammonium phosphate, 20 mM Mg, 0.1% liver egg phosphati-
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dychochine with solid HG in order to make it 100 mM HG and incubating for 1 h at 4 °C in the dark (cf. Ref. 39).

Examination of Crystal Structures—Examination of protein crystal structure and geometrical measurements were carried out using the CCP4 program package (44). Unless otherwise stated, distance and angle measurements on Cyt b$_6$f refer to the complex from chicken muscle mitochondria (Protein Data Bank code 1BCC; Ref. 2).

RESULTS

Determining the orientation of chromophores in a complex requires the use of an oriented sample. In the present experiments, immobilization was achieved by supplementing samples with polyacrylamide and inducing polymerization. It did not affect the spectrum of the hemes in the b$_6$f complex, but depending on the exact experimental conditions, chlorophyll a, on the other hand, could be bleached to different extents (see “Experimental Procedures”). Orientation was then induced by compressing the gel (as detailed in Ref. 45). We have performed LD studies both on isolated C. reinhardtii Cyt b$_6$f and on liposomes containing Cyt b$_6$f co-reconstituted along with Rb. sphaeroides RC. The first type of samples allows only the relative orientation of chromophores with respect to each other to be determined but offers a good resolution of complex spectral contributions. Reconstituted samples enable one to determine the absolute orientation of chromophores with respect to the membrane plane by including a protein of known structure as an internal reference while limiting the overlapping contribution of the large number of chromophores that would be present in native membranes.

Resolution of the Optical Spectra of Individual Hemes—The orientation of a tetrapyrole ring can be deduced from that of its X and Y electronic transitions. Establishing the orientation of the three hemes in the b$_6$f complex, therefore, requires determining the orientations, for each heme, either of the directions of its X and Y transitions (if they are distinguishable) or that of the normal to the ring plane in which they lie (if they are degenerate). In the fully reduced complex, the corresponding absorbance bands overlap. Since the three hemes, however, have different midpoint redox potentials, their spectra can be separated by analyzing spectra of the complex recorded at 100 K at various redox potentials. Given the E$_{m}$ values of the three hemes (19), only Cyt$_f$ contributes to spectrum a of Fig. 1A. Under the redox conditions of spectrum b, the totality of Cyt$_f$ is reduced together with part of heme b$_{H}$. Spectrum c corresponds to the sum of Cyt$_f$ and heme b$_{H}$. Spectrum d also contains a contribution from partially reduced heme b$_{L}$, whereas in spectrum e all of the three hemes are totally reduced. Decomposition of the ensemble of spectra allows one to resolve the contributions of each heme, as shown in Fig. 1B. The spectrum of Cyt$_f$ features a-band maxima at 547.2 and 551.4 nm and that of heme b$_{H}$ a-band maxima at 557.5 and 561 nm (calculated by subtracting spectrum a from spectrum c), whereas the spectrum of heme b$_{L}$ (obtained by subtracting spectrum d from spectrum e) presents a single a-band maximum at 560 nm. In this, as in most respects, the Cyt b$_6$f complex from C. reinhardtii is similar to the complex from spinach (46). Time-resolved measurements have shown that, at room temperature, the a-band maxima of b$_{H}$ and b$_{L}$ differ by only 0.6 nm (47), making them indistinguishable in static measurements (19). As our results show, they can, however, be resolved from each other at 100 K, a prerequisite to a detailed LD analysis.

Because of the 4-fold symmetry of metalloporphyrins, a transitions are expected to be degenerate in the heme plane (the X and Y transitions being isoenergetic). However, imbalance along these two axes may arise from asymmetric electrostatic fields created by the surrounding protein (36). As will be shown below, at 100 K the X and Y transitions of both b$_{H}$ and b$_{L}$ are separated by ~3.5 nm. In b$_{H}$, the absorbance of the two bands is in such a ratio (0.39:1) that the blue-shifted band appears as a distinct shoulder to the left of the a peak, whereas in b$_{L}$ its contribution is smaller (0.26:1) and is mainly reflected in the asymmetry of the peak (Fig. 1B) (see below in the “Discussion”).

Linear Dichroism of the Isolated Complex—Well resolved spectra recorded on the isolated complex were used to ascertain the amplitude and position of each A and LD band. Absorbance and LD spectra recorded on the dithionite-reduced complex (Fig. 2) show relatively well resolved X and Y transitions of heme f at 547.2 and 551.4 nm, whereas peaks at 560.5 nm in the absorbance spectrum and at 556.7 and 561 nm in the LD spectrum reflect the superposition of spectral contributions of b$_{H}$ and b$_{L}$. At 665.2 nm, the spectra show the pure contribution of the Q$_{Y}$ transition of Chla, and at 627.7 nm the smaller contribution of the Q$_{X}$ transition of chlorophyll overlapped with a vibrational component of the Q$_{Y}$ one (36). The normalized dichroism (LD/A ratio) did not change significantly across the 665.2 nm band, showing that it is indeed a pure single transition. It was not affected by the variable degree of bleaching occurring upon gel polymerization, indicating that, from the point of view of their orientation, Chla molecules were a homogeneous population.

In order to resolve the complex spectra in the Cyt a-band region, we first calculated LD/A ratios for the Cyt transitions using spectra recorded on an ascorbate-treated complex, where only Cyt$_f$ was reduced (Fig. 3A and Table I). Spectra obtained under more reducing conditions, where the b$_{H}$-heme appeared partially reduced (see “Experimental Procedures”), allowed us to determine the LD/A values for the X and Y transitions of heme b$_{H}$ (Table I; spectra not shown). After subtracting the f and b$_{H}$ contributions from the LD spectrum of a dithionite-
and 603.4 nm are due to the accessory BChl dized samples (not shown). The spectral contributions at 596 nm arise from the overlapped contribution of the RC bacteriopheophytin (BPheo) and the 547 nm transition of Cyt f. In these samples, where membranes tend to align with the membrane normal parallel to the direction of compression, the Q transition of P therefore should be tilted by an angle $\theta = 39-40^\circ$ with respect to the compression axis. Combining the information on $\theta$ with the experimental LD/A ratio for P yields the orientation parameter $\alpha$ (see Equation 1 under “Experimental Procedures”). Knowing $\alpha$, the absolute values of LD/A and therefore absolute orientations with respect to the membrane plane can then be determined for the other transitions in the sample.

An angle $\theta = 39-40^\circ$ between the Q transition of P and the normal to the membrane plane, however, yielded values for $\alpha$ that lead to mathematical inconsistencies when treating the LD data on the Cyt b$_6$f complex transitions. The smallest angle that yields a self-consistent set of orientations is 46°, the largest 54° (magic angle). Taking experimental errors (±2.5°, see “Experimental Procedures”) into account and considering the possibility of some variation in compression effects on the alignment of the particles, an angle of 46° is in fair agreement with previous LD studies. In order to check on the reliability of our approach, however, we calculated the orientation of the Q transition of P (peaking at 870 nm; data not shown) using the assumption that $\theta_{903.4} = 46^\circ$. This hypothesis yielded a tilt of 65° for the Q transition with respect to the membrane plane, in close agreement with the value of 68° determined by Tiede (45). Assuming $\theta_{903.4}$ to be greater than 46° would move the orientation of Q (and of all other transitions) closer to 54°, i.e. further away from the previous experimental determination.

The angles between heme or Chla planes and the membrane plane (Table I) therefore were calculated assuming $\theta_{903.4} = 46^\circ$.

**Orientation of Tetrapyrrrole Rings with Respect to the Plane of the Membrane**—Equation 2 (see "Experimental Procedures") can be used to deduce the orientation of the plane of the heme with respect to the membrane, provided it is assumed that the X and Y transitions are orthogonal and lie in the heme plane. This assumption yields orientations (averaged on the five independent b$_6$f-containing samples studied) between heme planes and the membrane plane of $52^\circ \pm 2$, $74^\circ \pm 2$, and $83^\circ$ for hemes f, b$_{13}$, and b$_{14}$, respectively (Table I).

The situation is more complex as regards the chlorin ring of Chla, because only the Q transition can be assigned a defined orientation, namely $49^\circ \pm 2^\circ$ with respect to the compression axis (Table I). The direction of the Q transition cannot be determined unambiguously from the present data, because it overlaps with a vibrational component of Q. Limits can be set, however, as to its value. Because the 627.7 nm band represents a mixture of the Q transition and the vibrational component of Q, the LD/A ratio at this wavelength also represents a combination of the contributions of both components. The respective weight of these contributions is unknown, but from optical theory the direction of the vibrational component and the electronic transition of Q$_f$ must be the same. The dichroism of the Q$_f$ vibrational component is therefore negative. The positive dichroism of the 627.5 nm band therefore indicates that the contribution of Q$_f$ is positive. The LD/A ratio of the 627.5 nm band therefore corresponds to the minimal value associated with the Q$_f$ transition. This implies that the angle between the Q$_f$ transition and the compression axis must be $\approx 60^\circ \pm 2^\circ$.

Deducing the orientation of the chlorin ring with respect to the membrane plane from the directions of the Q$_X$ and Q$_Y$ transitions further depends on assumptions regarding the an-
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![Resolution of the X and Y transitions of Cyt f, Cytb₁, and Cytb₂ at 10 K. A, decomposition of the absorbance and LD spectra of the ascorbate-reduced complex into two Cyt f transitions. B, decomposition of the absorbance and LD spectra of the dithionite-reduced complex into Cyt f, Cytb₁, and Cytb₂ transitions.](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Transition</th>
<th>Wavelength (nm)</th>
<th>LD/A (arbitrary units)</th>
<th>Orientation of Transition relative to compression axis (°)</th>
<th>Plane relative to membrane plane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heme f&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q₁</td>
<td>547.2</td>
<td>+0.297</td>
<td>61 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Q₂</td>
<td>551.4</td>
<td>−0.273</td>
<td>50 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Heme b&lt;sub&gt;H&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q₁</td>
<td>557.5</td>
<td>−1.0</td>
<td>37 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Q₂</td>
<td>561</td>
<td>+0.069</td>
<td>56 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Heme b&lt;sub&gt;L&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q₁</td>
<td>556.4</td>
<td>−1.09</td>
<td>35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Q₂</td>
<td>559.8</td>
<td>+0.049</td>
<td>56&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Qₓ</td>
<td>627.7</td>
<td>+0.272</td>
<td>60°–90°</td>
<td>41–55° or 73.5–90°</td>
</tr>
<tr>
<td>Qᵧ</td>
<td>665.2</td>
<td>−0.316</td>
<td>49 ± 2°</td>
<td></td>
</tr>
<tr>
<td>P&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Qₓ</td>
<td>603.4</td>
<td>−0.485</td>
<td>46&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Qᵧ</td>
<td>870</td>
<td>+0.503</td>
<td>65&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Experimental LD/A values depend on α, the orientation parameter, and on the characteristics of the LD spectrophotometer (see text). LD/A values therefore are given in arbitrary units.

<sup>b</sup> No attempt was made to assign heme optical bands to either the X or Y porphyrin axes. Data therefore refer to Q₁ and Q₂ rather than Qₓ and Qᵧ.

<sup>c</sup> The orientation of hemes f, b<sub>H</sub>, and b<sub>L</sub> as well as that of Chla, have been determined in five independent experiments, two on the liposome-integrated complex and three on the isolated complex. In the two first experiments, the Q₁ transition of the reaction center was taken as an internal standard whereas in the other three b<sub>L</sub> was taken as the reference. The orientations determined for f, b<sub>H</sub>, and Chla therefore can be given with experimental errors, whereas those for heme b<sub>L</sub> and the Q<sub>f</sub> transition of P cannot.

<sup>d</sup> The assignment of the optical bands to the Qₓ and Qᵧ transitions is known.

<sup>e</sup> The two ranges of angles correspond to alternative assumptions made on the relative orientations of the Qₓ and Qᵧ transitions in the Chla plane (see text). The first range results from assuming orthogonality of Qₓ and Qᵧ, the second from assuming an angle of only 23.5° between the two transitions (50).

<sup>f</sup> The orientation of the Qₓ transition of P is not deduced from the data but taken as an internal reference (see text).

The orientation of the chlorin ring might suggest a lobed signal similar to that reported for Cyt<sub>bc₁</sub> in the Soret region of the CD spectrum of Cyt<sub>bc₁</sub>. This has been attributed to interaction between hemes b₁₀ and b₁₁ (Refs. 52–54 and references therein). Since excitonic CD signals are sensitive both to the distance and relative orientation between the interacting chromophores, it seemed interesting to examine the CD spectrum of Cyt<sub>bc</sub> in the Soret region. As shown in Fig. 5 (h), the dithionite-reduced minus ascorbate-reduced CD spectrum features a bilobed signal similar to that reported for Cyt<sub>bc₁</sub>. There are, however, significant differences as follows: (i) expressed as the molar differential extinction coefficient, the b₁₁ signal appears much stronger; (ii) it is slightly more asymmetric; (iii) it is slightly red-shifted, so that it crosses the zero line at 433 nm, i.e., at the absorbance maximum in the difference spectrum of the b₁₁-hemes (19).

In the crystal structure of dimeric Cyt<sub>bc₁</sub>, the b₁₁-hemes of
FIG. 4. Absorbance and LD spectra of purified Cyt b<sub>6</sub>f complex from C. reinhardtii and reaction center from Rb. sphaeroides co-reconstituted into lipid vesicles. The spectra recorded on the dithionite-reduced sample show contributions of the Cyt b<sub>6</sub>f complex in the 547–561 nm region overlapping with that of the RC bacteriopheophytin (BPheo) at 545 nm. Overlapping spectral contributions of the RC bacteriochlorophylls L and M (BChl) and special pair (P) are visible at 595–604 nm.

the two monomers are as close in space one to another as b<sub>L</sub> and b<sub>H</sub>, are within one monomer (1). A similar situation is likely to prevail in the dimeric b<sub>6</sub>f complex. In order to find out whether b<sub>H</sub>/b<sub>L</sub> interactions contribute to the excitation signal, purified Cyt b<sub>6</sub>f was converted to monomers by incubation with a high concentration of detergent (a treatment that also removes the Rieske protein and Chla; see Ref. 39). This treatment had only a small effect on the amplitude of the short wavelength, positive lobe of the CD signal but strongly increased its asymmetry (Fig. 5).

DISCUSSION

Earlier LD measurements on Cyt b<sub>6</sub>f had been performed on the isolated spinach complex at room temperature (55). These conditions did not allow the resolution of individual electronic transitions. The present study, performed at 10 K and including photosynthetic RC as an internal standard, makes it possible to determine the orientation of all three hemes with respect to the plane of the membrane. The orientation of the Q<sub>Y</sub> transition of the red light absorbance band of Chla has also been established and restrictions placed as to those of the Q<sub>X</sub> transition and the chlorin ring. In order to obtain mathematically meaningful orientations for the heme transitions, the orientation of our reference, the double transition of the red light absorbance band of Chl<sub>a</sub> (46), was taken to be 46° off the normal to the membrane plane. Using this assumption, the Q<sub>X</sub> transition of Chla is oriented at 0°–30° with respect to the membrane plane, in agreement with previous work (56). This orientation cannot be more narrowly defined because, in the visible spectrum of Chla, the Q<sub>X</sub> transition overlaps with a vibrational component of the Q<sub>X</sub> transition (36) and only the minimal value of the linear dichroism of Q<sub>X</sub> can be ascertained.

Calculating the angle the chlorin ring makes with the membrane plane further depends on assumptions regarding the angle between the Q<sub>X</sub> and Q<sub>Y</sub> transitions. Assuming the two transition moments to be orthogonal would imply that the plane of Chla makes an angle of 41°–55° with that of the membrane. However, quantum mechanical calculations suggest the two transitions to make an angle of only 23° one with another (50). In this hypothesis, it would follow from the LD data that the chlorin ring is tilted by 73.5°–90° with respect to the membrane plane. Both hypotheses, actually, are open to criticism, the first one because it relies only on analogy with porphyrins, and the second because the quantum mechanical calculations assume an isotropic environment.

Arrangement of the Two Hemes in Cyt b<sub>6</sub>—This is the first study of the organization of hemes in Cyt b<sub>6</sub>f to resort to optical spectroscopy. From previous EPR measurements on the spinach complex, the plane of heme b<sub>H</sub> was proposed to lie roughly perpendicular to the membrane (26), a conclusion in agreement with the results of the present study. In the EPR studies, however, a single signal was observed at g = 3.6, most probably arising from the overlap of signals from the b<sub>H</sub> and b<sub>L</sub> hemes. The present LD measurements yield a complete set of assignments for the six electronic transitions comprising the a bands of the three b<sub>6</sub>f-hemes. As presented under “Results,” this decomposition assigns quite different weights to the X and Y transitions. Such a situation may seem surprising, since it implies that the probabilities of the two transitions are quite different. Nevertheless, there are many precedents for it, such as Cyt f (46), the c<sub>556</sub> High Potential heme of the photosynthetic RC from Blastochloris viridis (57), and the b<sub>L</sub>-heme of the bc<sub>1</sub> complex of Rb. sphaeroides (58).

The angles between the planes of porphyrin rings and the membrane plane found in this study (74° ± 2° and 83° ± 3° for hemes b<sub>H</sub> and b<sub>L</sub>, respectively) are identical, within theoretical experimental errors (±3.5°, see “Experimental Procedures”), to those (73°–76° for b<sub>H</sub>, 82°–83° for b<sub>L</sub>) determined crystallographically in the bc<sub>1</sub> complex from vertebrate mitochondria (2). The distance between the iron atoms of the b<sub>1</sub>- and b<sub>H</sub>-hemes had been expected to depend on the length of sequence separating the heme carrying histidines, 13 residues in Cyt b<sub>H</sub> versus 14 residues in Cyt b<sub>L</sub> (see “Introduction”). In an ideal a-helix, moving a residue by one sequence position translates its a-carbon by ~1.5 Å longitudinally and ~3.2 Å transversally. Our LD data suggest either that such a rearrangement does not take place or that it has no incidence on the tilt of the two b-hemes with respect to the membrane plane. The possibility that the sequence change is accommodated without any relative displacement of the hemes is supported by the observation that insertion of an extra residue in helix D leaves a bacterial bc<sub>1</sub> complex largely unaffected (18).

In order to probe further the arrangement of b<sub>H</sub> and b<sub>L</sub> in the b<sub>6</sub>f complex, we have resorted to CD measurements. Previous
work has shown that bc₁ complexes exhibit an excitonic signal, centered on 432 nm in the dithionite-reduced minus ascorbate-reduced difference spectrum, which has been attributed to electronic interactions between hemes b₁H and b₁L (see Refs. 53–54 and references therein). The strength of the signal is a function both of the distance between the hemes and of the relative orientation of their planes (54). At variance with LD, CD spectra therefore have the potential to detect relative rearrangements that would leave the tilt of each heme with respect to the membrane plane unchanged. We find that purified Cyt bc₁f in its native, dimeric state (—) and following monomerization by incubation with an excess of detergent (see “Experimental Procedures”; — —). The difference CD spectrum for beef heart Cyt bc₁ · · · · from Ref. 52) is shown for comparison.

FIG. 5. Difference circular dichroism spectrum of the b-hemes in Cyt bc₁f. Difference CD spectra (dithionite-reduced minus ascorbate-reduced) were recorded on solutions (8.7 μM) of purified Cyt bc₁f in its native, dimeric state (—) and following monomerization by incubation with an excess of detergent (see “Experimental Procedures”; — —). The difference CD spectrum for beef heart Cyt bc₁ · · · · from Ref. 52) is shown for comparison.

It does not seem likely that the stronger intensity of the CD signal results from improper assumptions made in calculating the differential molar extinction coefficient. In the present analysis, b₆ concentrations were based on an extinction coefficient ε₅₆₄ = 20,000 M⁻¹ cm⁻¹ (37). Revising it to 24,000 M⁻¹ cm⁻¹, as recently suggested (60), would raise Δε to 360 M⁻¹ cm⁻¹, further increasing the difference between the two complexes. The stronger signal cannot result either from differences in dipole strengths between the various b-type hemes, given that those are probably somewhat smaller in the b₆f than in the bc₁ complex (60). Interpreting the more intense CD signal of Cyt bc₁f in terms of relative rearrangement of hemes b₁H and b₁L also raises difficulties. According to the theoretical treatment of heme/heme interactions given by Palmer and Degli Esposti (54), moving the two b-hemes apart by ~2 Å (the likely increase in distance between histidine α-carbons were the intervening α-helix elongated by one residue) is predicted to decrease the intensity of the excitonic signal by ~15%. In the crystallographic structure of chicken muscle Cyt bc₁ (2), the planes of hemes b₁H and b₁L make one with another an angle of ~48°, close to that (45°) considered to permit optimal excitonic interaction (54). Except if heme planes happened to be rotated by 90° with respect to each other, in which case no intensity change would result, any departure from the position observed in the bc₁ complex would therefore be expected to decrease the CD signal.

Given the proximity between b₁L-hemes in the bc₁ dimer (in chicken bc₁, 20.8 Å separate the two b₁L-heme iron, as compared with 20.5 Å between the b₁H and b₁L irons within a monomer (2)), it cannot be excluded a priori that part or all of the CD signal in the bc₁ complex may be due to b₁L-hemes or the bc₁ complex (bc₁ in the present work). Combined with the C2 geometry of the two dimers (1, 4), imposes that the angle between the planes of the two b₁L-hemes make one with another in the bc₁ complex but, given the unfavorable angle, this interaction is not expected to be strong interactions (54). A different geometry of the dimeric Cyt bc₁f could conceivably result in stronger b₁L/b₁L interactions. This difference cannot reside in the relative orientation of the two b₁L-hemes, however, since the conservation of the angle the plane of the b-hemes makes with the membrane (present work), combined with the C2 geometry of the two dimers (1, 4), imposes that the angle between the planes of the two b₁L-hemes be conserved. A stronger interaction could be generated if the distance between the two b₁L-hemes were shorter in Cyt bc₁f than in the bc₁ complex but, given the unfavorable angle, the contribution of this effect to the overall CD signal would be expected to be small. In order to experimentally test this hypothesis, we converted native, dimeric Cyt bc₁f to monomers by incubating it with detergent (39). Following this treatment, the intensity of the negative lobe of the CD signal decreased only slightly, whereas that of the smaller negative lobe was more strongly affected. The increased asymmetry of the spectrum implies the contribution of non-excitonic components in the detergent-treated complex, indicative of a perturbation of heme conformation and/or environment within a monomer. Neither theoretical considerations nor experimental observations, therefore, conclusively support the idea that increased b₁L/b₁L interactions may account for the strength of the b₁f CD signal.

In summary, LD data indicate that the angles the planes of hemes b₁H and b₁L make with that of the membrane are very similar in Cyts bc₁L and bc₁f and CD data do not suggest changes in their distance or relative orientation. These data therefore suggest that the relative position of the hemes have changed.
very little in the course of evolution, an important point for homology modeling. How the extra residue in the sequence of helix D is accommodated cannot be predicted with certainty since either a slight change of pitch in the helix and/or the formation of an “o-aneuryism” (π-bulge) could permit the Cα positions of the two histidine residues to remain unchanged (61, 62). There are precedents for π-bulges in transmembrane α-helices (63, 64). In bacterial reaction centers, for instance, the single transmembrane helix of subunit H features one more residue in B. viridis than in Rb. sphaeroides. Comparison of the two structures (Protein Data Bank codes 1PRC and 1AIJ, Refs. 65 and 66) shows that the Cα coordinates of the other residues remain essentially unchanged, the extra residue being accommodated by a localized π-bulge (64). We note that a π-bulge localized close to Thr-188, the extra residue in C. reinhardtii Cyt b6f, would create a locally more hydrophilic environment, providing extra opportunities for hydrogen bond formation in the immediate vicinity of heme b6. Comparative Raman resonance spectroscopy of the hemes in bacterial Cyt bc1 and spinach Cyt b6f on the other hand suggests that the structure of heme b6 is very similar in the two complexes, whereas that of b53 is slightly more distorted in the b6f complex (67).

**Orientation of Heme f**—Our LD data indicate that the plane of heme f makes an angle of 52 ± 2° with the membrane plane, very different from the angle of 74–79° observed in the crystalographic structure of the bc1 complex (2, 3). Such a discrepancy is not unexpected, given the different evolutionary origins and folds of Cyt f and c1.

More surprisingly, a 52 ± 2° angle is also at odds with the results of EPR measurements on isolated and membrane-integrated spinach b6f complex, from which it was concluded that the plane of heme f makes a 25–30° angle with that of the membrane (24–28). This difference is much greater than the experimental variation observed in Cyt f orientation with respect to the other chromophores in our five independent experiments and to theoretical errors (±3.5°). The small difference between the calculated orientation of heme f in the solubilized (49–51°) and the reconstituted complex (54°) could result simply from the difficulty of measuring the contribution of f at 547.2 nm over the background of the large 545-nm BPheo peak and is within the range of theoretical errors. It does not seem likely either that the difference between the EPR and LD experiments be due to erroneous assumptions regarding the orthogonality of X and Y transitions; whatever assumption is made regarding the angle ϕ between these transitions (Equation 3), the angle γ between the plane of heme f and that of the membrane cannot be made smaller than 45°. Neither could the discrepancy be accounted for by an inappropriate assumption regarding the orientation of our reference transition (see “Results”).

On the other hand, we note that in LD experiments Cyt f has been studied in its reduced state, whereas it was in the oxidized state in EPR experiments. X-ray data indicate that the Rieske protein of the bc1 complex undergoes large scale displacements upon binding of inhibitors to the Qp site (2). Recent EPR measurements (23) and cryoelectronmicroscopy data show that similar events take place in the b6f complex. In bc1 crystals, the movement of the Rieske protein is not accompanied by a significant displacement of Cyt c1. However, recent EPR data indicate that the conformational change of the Rieske protein in both the bc1 and b6f complexes can also be induced by modulating its redox state (68). There are, to our knowledge, no data yet regarding the effect of redox potential on heme orientation in either the bc1 or the b6f complex. Interestingly, LD data collected on beef heart mitochondrial bc1 complex are in agreement with crystallographic data as regards the relative arrangement of hemes b5 and b1, but again at odds with them as regards the orientation of heme c1 (6). Whether the transition between redox states is accompanied by a reorientation of f and c1 hemes is under further examination.

**Acknowledgments**—We thank D. Picot for help with the examination of crystallographic structures; M. Desmaird for access to a Jobin & Yvon spectrophotograph and advice about CD spectroscopy; W. Nitschke for discussions on the manuscript; G. Palmer and H. Scheer for comments on the CD data; E. A. Berry for communication of the Cyt bc coordinates prior to publication; and J. Breton, P. Joliot, and Y. Pierre for useful discussions.

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