The 9 Å Projection Structure of Cytochrome $b_6f$ Complex Determined by Electron Crystallography

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Thin three-dimensional crystals of the cytochrome $b_6f$ complex from the unicellular algae Chlamydomonas reinhardtii have been grown by Bio-Beads-mediated detergent removal from a mixture of protein and lipid solubilized in Hecameg. Frozen-hydrated crystals, exhibiting $p2_{2}12_{1}$ plane group symmetry, were studied by electron crystallography and a projection map at 9 Å resolution was calculated. The crystals (unit cell dimensions of $a \approx 173.5$ Å, $b \approx 70.0$ Å and $\gamma = 90.0^\circ$) showed the presence of dimers, and within each monomer 14 domains of electron density were observed. The combination of the projection map obtained from ice-embedded crystals of cytochrome $b_6f$ with a previous map obtained from negatively stained samples brings new insight in the organization of the complex. For example, it distinguishes some peaks and/or domains that are only extramembrane or transmembrane, and reveals the possible localization of single-stranded transmembrane $\alpha$-helices (Pet subunits). Furthermore, the cross-correlation of our projection map from frozen hydrated samples with the atomic model of the transmembrane part of the cytochrome $bc_1$ complex has allowed us to localize the cytochrome $b_6$ at the dimer interface and to reveal structural differences between the two complexes.

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Introduction

The cytochrome $b_6f$ complex (plastoquinol:plastocyanin oxidoreductase) is a constituent of the photosynthetic membrane of plants, algae, and some cyanobacteria (Malkin, 1992; Hope, 1993). It is a large transmembrane protein composed of seven subunits in a 1:1 ratio: four large molecular mass subunits, three of which contain redox active prosthetic groups (cytochrome $f$, 31.8 kDa; cytochrome $b_6$, 25.4 kDa; subunit IV, 17.4 kDa; and the Rieske Iron-Sulfur protein, 18.6 kDa) and three additional small hydrophobic subunits (PetG, PetM and PetL of 3-4 kDa each; Haley & Bogorad, 1989; Pierre & Popot, 1993; Schmidt & Malkin, 1993; Pierre et al., 1995; Takahashi et al., 1996; ). While cytochrome $f$ contains only a single transmembrane $\alpha$-helix, four and three transmembrane segments are predicted to be present in cytochrome $b_6$ and subunit IV, respectively. Each of the three 3-4 kDa subunits (PetG, PetL and PetM) consist of one single-stranded $\alpha$-helix. The location of the N-terminal $\alpha$-helix of the Rieske protein with respect to the membrane remains uncertain, since it has been suggested to be either trans- (Karnauchov, 1997) or extramembrane (Breyton et al., 1994; de Vitry, 1994).

The cytochrome $b_6f$ complex is involved, together with plastoquinol and plastocyanin, in electron transfer between photosystem II and photosystem I, and the generation of a transmembrane electrochemical proton gradient that drives the synthesis of ATP by the CFo-CF1 ATP-synthase (for reviews, see Widger & Cramer, 1991; Hope, 1993; Cramer et al., 1994, 1996; Wollman, 1998). The electron transfer and proton translocation mechanism, which is described as the proton-motive Q cycle, involves two active sites $Q_0$ and
Q$_b$, located close to the luminal and stromal sides of the membrane, respectively. A better understanding of the reaction mechanisms of cytochrome $b_{6}f$ complex requires the knowledge of the atomic model of the complex. A density projection map at 8 Å resolution has been obtained by electron crystallographic studies of negatively stained thin three-dimensional (3D) crystals of the cytochrome $b_{6}f$ from Chlamydomonas reinhardtii (Mosser et al., 1997). This structural study showed that cytochrome $b_{6}f$ existed in the lipid bilayer as a dimer in agreement with earlier biochemical, biophysical as well as freeze-fracture electron microscopic studies (Huang et al., 1994; Breyton et al., 1997). This map provided information on the stain accessible regions of the protein, i.e. the large extramembrane parts, thereby allowing a tentative location of the Rieske protein and cytochrome $f$ subunits in the complex (Mosser et al., 1997).

Here, we describe the electron crystallographic analysis of the frozen-hydrated thin three-dimensional crystals of the cytochrome $b_{6}f$ complex. Images and electron diffraction data have been used to calculate a projection map of the complex at 9 Å resolution. The map, which confirms that cytochrome $b_{6}f$ complex exists as a dimer in reconstituted membranes, presents a significantly different electron densities distribution compared to map obtained from negatively stained samples. The combination of the two maps enable us to make a preliminary study of the protein densities in and out of the membrane. Finally, we compare our data with the published atomic structure of the cytochrome bc$_1$ complex.

**Results and Discussion**

**Projection map of ice-embedded sample**

Multilayered crystals of cytochrome $b_{6}f$ were obtained according to the method described by Mosser et al. (1997), using SM2-BioBeads as the detergent removing agent. However, crystallization procedure has been improved by: (1) increasing the protein concentration from 0.5 mg/ml to 1 mg/ml, which leads to a significant increase in the number of crystals; and (2) carrying out the freeze/thaw cycles at $-196^\circ$C and 4°C instead of $-196^\circ$C and 37°C, which leads to a reduction in the stacking of 2D layers. The best crystals, exhibiting an overall size of about 2 μm, were obtained after 24 to 48 hours incubation at 4°C following the freeze/thaw cycles.

Images of frozen-hydrated crystals were recorded as described in Materials and Methods. Figure 1(a) shows a typical computed transform with well-defined reflections up to 14 Å and reliable reflections to 9 Å resolution. A total of 15 micrographs were selected by optical diffraction and six were finally used for image analysis. The images clearly indicated an orthorhombic unit cell with $a = 173.5$ Å and $b = 70.0$ Å. Examination of the phase relationships indicated $p22,2_1$ plane group (Valpuesta et al., 1994) as previously found with negatively stained crystals (Mosser et al., 1997). The phase error of the Fourier components obtained after averaging are plotted in Figure 2. The overall phase residual up to 9 Å resolution was 23.7° (45° would be random), indicating that all the symmetry-related reflections were reliable up to this resolution (Table 1).

A typical electron diffraction pattern of frozen-hydrated crystals is shown in Figure 1(b). Strong and sharp diffraction spots can be seen up to 7 Å and lines of diffuse spots are observed in a resolution range of 5-4 Å. The presence of such lines can be explained by sample instability during data acquisition and/or the quality of the crystals. The presence of intense diffraction spots in the range of 10-7 Å resolution usually suggests the presence of α-helices that are perpendicularly oriented with respect to the membrane plane (Henderson & Unwin, 1975; Kühlbrandt et al., 1994). Four electron diffraction patterns were selected based on the lack of tilt and thickness of the specimen. Indeed, crystal thickness induces differences in intensities of Friedel-related reflections due to dynamical scattering by each layer, thus decreasing the accuracy of the final measurement of structure factor amplitudes (Grigorieff & Henderson, 1995). The average R-value between Friedel-related high intensity spots used in the analysis was 0.39 and, taking all spots into account, the merging R-factor was 0.62 between the four electron diffraction patterns. These values are comparable with those obtained with 2D crystals of microsomal glutathione transferase (Hebert et al., 1997).

A fully symmetrized projection map at 9.0 Å resolution (Figure 3) has been generated by merging phases from images and amplitudes from electron diffraction patterns as described in Materials and Methods. The unit cell in Figure 3, contains four monomers of cytochrome $b_{6}f$. The monomers form dimers, packed in rows parallel with the $b$ axis, with proteins facing alternatively up and down in the membrane as revealed by the $p22,2_1$ symmetry. Each dimer, arranged around a 2-fold axis of symmetry, has an elongated shape of 90 Å long and 62 Å wide.

Figure 4(a) shows an isolated dimer of cytochrome $b_{6}f$ and within each monomer 14 peaks of electron densities are resolved; 12 of these peaks are packed in a bundle, whereas peaks 13 and 14 are isolated. Within the bundle, two groups of densities are defined. The first one, consisting of peaks 6, 7 and 8, form a central square like motif that is involved in the dimer interface. The second one, consisting of peaks 9, 10, 11 and 12 stands on one side of the square motif and is only very lightly connected to it. Most of the peaks present a mean diameter around 11 Å, consistent with the presence of α-helices perpendicularly oriented (or slightly tilted) to the membrane as already suggested by the diffraction pattern. From sequence analysis, the cytochrome $b_{6}f$ from C. reinhardtii is expected to
Figure 1. Frozen-hydrated crystals of cytochrome $b_{6}$ complex from *C. reinhardtii*. (a) Fourier transform of an untilted image of a $b_{6}$ complex crystal. The size of the boxes indicates the IQ values, with IQ = 1 being the largest. The IQ is defined by Henderson et al. (1986) such that the signal-to-noise ratio of the selection amplitude for IQ grades 1-7 is given by $7/\text{grade}$. Concentric lines indicates the zero values of the phase-contrast transfer function for a defocus of 6600 Å. $a^{*}$ and $b^{*}$ refer to the reciprocal lattice vectors. The zeros of the CTF are at 1/12.7 Å$^{-1}$, 1/8.9 Å$^{-1}$, 1/7.1 Å$^{-1}$, 1/6.18 Å$^{-1}$ resolution, respectively. (b) Electron diffraction pattern of a $p22,2_{1}$ frozen-hydrated crystal of cytochrome $b_{6}$ complex recorded at a temperature of $-184^\circ$C and an accelerating voltage of 120 kV. The strongest spots are observed around 1/7-1/10 Å$^{-1}$. The diffuse lines marked by the arrow heads correspond to 1/4.4 Å$^{-1}$. 
contain 11-12 transmembrane helices (Pierre et al., 1995). The higher number of peaks densities observed (in comparison with these putative transmembrane helices) may have several explanations: (1) the contribution of extramembrane domains; (2) the tilt of some helices; (3) an unequal stoichiometry of the small subunits; or (4) an additional small subunit still not reported.

Comparison with the projection map of negatively stained sample

To interpret the densities that are observed in the present projection map of ice-embedded specimen, we have compared it with our previously reported projection map of the negatively stained specimen at a similar resolution (Figure 4). It is worth noting that structural information obtained from a projection map of negatively stained samples is expected to primarily concern the extramembrane domains of the complex (Mosser et al., 1997). In contrast, the cryo-projection map seems to reveal essentially the transmembrane part of the complex. Figure 4(c) shows the best superimposition of the two maps and reveals the differences and common features. Indeed, it points out some peaks and/or domains that are only extramembranous or transmembrane illustrating the complementarity of the two different preservation techniques. For instance, domain IV from the negative stain map finds no counterpart in the cryo-projection map, and thus likely represents extramembrane loops. In contrast, peaks 2 and 13, which are well defined in the cryo-projection map, are totally absent from the negative stain map, suggesting that these parts reside inside the membrane. The small subunits, which are single-stranded α-helices and with only small extramembrane loops, could account for those densities. It should also be noted that part of domain III from the negative stain map, overlays peaks 1 and 5 in the cryo-projection map, while the rest does not overlap any density.

In a similar way, most of the surface covered by the extramembrane domain of the Rieske protein (domain I in the negative stain map) exhibits little densities in the cryo-projection map (see Figure 4(c)). Only peak 14 lies unambiguously underneath domain I, and even if it appears isolated from the rest of the bundle this peak may reasonably be attributed to the Rieske protein.

Table 1. Crystallographic data

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Electron Cryo-crystallography of Cytochrome b₆f
Figure 3. Projection map of cytochrome $b_6f$ complex from *C. reinhardtii* at 9 Å resolution. The projection density map with a $p22_12_1$ symmetry imposed was calculated to 9 Å using the amplitudes from four electron diffraction patterns and phases from six images. One unit cell with $a = 173.5$ Å (horizontal), $b = 70.0$ Å (vertical) is outlined, with the symbols representing the crystallographic symmetry elements. Each corner of the unit cell is located on the 2-fold axis of symmetry of a dimer. The zero-level was derived from the mean density of the map.

Figure 4. Projection maps of isolated dimer of cytochrome $b_6f$ complex from ice-embedded and negatively stained specimen. (a) Projection map of an isolated dimer of cytochrome $b_6f$ complex at 9 Å resolution obtained from ice-embedded specimens. A total of 14 peaks of electron densities (labeled as 1 to 14) can be resolved. (b) Projection map of an isolated dimer of cytochrome $b_6f$ complex at 8 Å resolution from the negatively stained specimen (Mosser et al., 1997). Four domains have been outlined and the two main extramembrane domains (noted I and II) have been attributed to the Rieske protein and the cytochrome $f$, respectively. (c) Superimposition of the projection maps from ice-embedded and negatively stained specimen. The cryo-projection map has been drawn as a grey surface underlayed by the external contour level of (a). Two contour levels from the negative projection map (b) were used to outline the external contours of the dimer and of the central groove on one hand, and of the four domains within each monomer on the other hand.
Although the sharpness of this peak would be characteristic of an z-helix perpendicularly oriented to the membrane, the absence of data in the z direction does not allow us to definitively determine its location in or out the membrane. However, the relative easy dissociation of the Rieske protein from cytochrome $b_{6}f$ complex during purification, delipidation, or incubation with chaotropic agents, has been taken as strong evidence for a non-transmembrane anchoring of this subunit (Breyton et al., 1994; de Vitry, 1994). If the N-terminal z-helix is revealed to be transmembrane, then these biochemical data could be reinterpreted with an isolated transmembrane anchoring z-helix of the Rieske protein, free of intra-membrane contact with other subunits and with a rather large extramembrane domain. Indeed, this could lead to a loose binding of this subunit and explain its easy dissociation from the complex. The apparent absence of density around peak 14 in the cryo-projection map could reflect either the leveling-off of the extramembrane density in the cryo-projection map or eventually the slight movement of the Rieske protein subunit as observed in cytochrome $b_{1}$ crystals (Zhang et al., 1998).

With respect to the cytochrome $f$, part of its extramembrane surface defined by domain II in the negative stain map (Mosser et al., 1997), overlaps four peaks (3, 4, 11 and 12) in the cryo-projection map. One of those peaks may be its membrane anchoring z-helix, whereas the others could be extramembrane z-helices of cytochrome $f$ or z-helices of other subunits.

In the absence of detailed data relating to the interaction within the different subunits of the complex, it seems difficult to further interpret the densities observed in our projection maps of the cytochrome $b_{6}f$ complex.

**Cross-correlations with the projection map of transmembrane part of the $bc_{1}$ complex**

The cytochrome $b_{6}f$ complex share functional similarities as well as sequence homologies (Cramer et al., 1987; Hauska et al., 1988; Malkin, 1992; Furbacher et al., 1996) with cytochrome $bc_{1}$ complex. For instance, all four major subunits of the cytochrome $b_{6}f$ complex have an equivalent in the cytochrome $bc_{1}$ complex: the cytochrome $b_{6}$ and subunit IV of the $b_{6}f$ complex have sequence homologies and correspond to the first four (A, B, C, D) and the three following transmembrane helices of the cytochrome $b$ (E, F, G), respectively; the Rieske protein has sequence homologies with the Rieske protein of the cytochrome $bc_{1}$ complex (Hauska et al., 1988), and the cytochrome $f$ of the $b_{6}f$ complex is equivalent to cytochrome $c_{1}$. In this context, the comparison of our cryo-projection map with the recently solved structure of the cytochrome $bc_{1}$ complex (Xia et al., 1997; Zhang et al., 1998) could bring some additional hints to the interpretation of the cryo-projection map.

Cross correlations have been performed, in real space, between our cryo-projection map (Figure 4(a)) and the 9 Å truncated projection map (Figure 5(a)) of the X-ray atomic model of the transmembrane region of the chicken cytochrome $bc_{1}$ dimeric complex (kindly provided by E. Berry). Two possible orientations, corresponding to the stromal and luminal views, were considered for the cytochrome $bc_{1}$ projections. Cross-correlations were calculated in both orientations and the best fits obtained are presented in Figure 5(b) (stromal view) and (c) (luminal view). The cross-correlation coefficients are of 43.6% and 56.7%, respectively. Cross-correlations are mostly sensitive to the overlap of centers of gravity. In the cytochrome $bc_{1}$ complex, the strong square motif composed by helices A, B, C, D of cytochrome $b$ will thus drive the correlation.

In both correlations (Figure 5(b) and (c)), peaks 6, 7, and 8 of the cytochrome $b_{6}f$ cryo-projection map overlay with the square motif formed by helices A, B, C, D of cytochrome $b$. This fact, together with the sequence homologies of helices A, B, C, D of cytochrome $b$ shared with the cytochrome $b_{6}$ strongly suggests that peaks 6, 7 and 8 would correspond to the four helices of cytochrome $b_{6}$. Such an assignment of the cytochrome $b_{6}$ at the dimer interface is compatible with cross-linking experiments (Chain & Malkin, 1991; Vater et al., 1996). The exact superimpositions of the other subunits is not straightforward in either cross-correlations (models I and II, Figure 5(b) and (c), respectively), suggesting structural differences between the cytochrome $b_{6}f$ and $bc_{1}$ complexes. However, the luminal view of cytochrome $bc_{1}$ (model II) seems more favorable as it exhibits the best cross-correlation factor with our cryo-projection map of cytochrome $b_{6}f$. Moreover, in this model the two complexes could be almost superimposed with the same 2-fold symmetry axis. The dimeric packing of cytochrome $b_{6}f$ is roughly similar to that of cytochrome $bc_{1}$, involving an interface between the AD helices. It is worth noting that in the cytochrome $bc_{1}$ complex, helices C and D are nearly perpendicular to the membrane plane forming two close densities, whereas helices A and B are highly tilted, forming a croissant-like motif in projection (see Figure 5(a)). Model II exhibits the closest spatial organization, where tilted helices A and B may correspond to elongated peak 6 and helices C and D to peaks 8 and 7, respectively. Besides, in the cytochrome $bc_{1}$ the extramembrane part of the Rieske protein of one monomer is located on the second monomer and has its membrane insertion named FeS in Figure 5(a). A similar situation is found in model II where the Rieske protein FeS membrane insertion would be in close contact with the peak 14, and its extramembrane domain would cross over to the second monomer (Figure 5(c)). Functional constraints and in par-
Figure 5. Comparison of calculated 2D projection map of the transmembrane domain of the cytochrome bc₁ complex and the cryo-map of the cytochrome bc₆f complex. (a) Projection map of the transmembrane region of the chicken cytochrome bc₁ complex at 9 Å resolution calculated from the X-ray atomic model kindly provided by E. Berry. The attribution of each helix is indicated as follows: A to G, C₁, FeS, Su7 and Su10 for cytochrome b, cytochrome c₁, Rieske protein, subunits 7 and 10, respectively. The myxothiazol and antimycin A binding pockets are indicated as Q_o and Q_i, respectively. (b), (c) Best fits of the cross-correlation between our projection map in ice of the cytochrome bc₆f complex (black continuous lines) and the transmembrane region of the cytochrome bc₁ complex (light grey). (b) Fit obtained for a stromal view, i.e. the extramembrane domains of the cytochrome c₁ and the Rieske protein are located beneath the membrane structure. The cross-correlation coefficient was 46.3%. (c) Fit obtained for a luminal view, i.e. the extramembrane domains of the cytochrome c₁ and the Rieske protein are located above the membrane structure. The cross-correlation coefficient was 56.7%. In both fits, helix H of cytochrome b as well as the small subunits 7 and 10 were conserved on purpose, although they have no counterpart in the cytochrome bc₆f complex. It should be noted that their removal did not significantly affect the final overlap of the two maps. The cross-correlation coefficients dropped to 29.3% in the first fit while it remained at 55.5% in the second fit. This difference is mainly due to the absence of helix H which has a strong contribution in the first superimposition.

Conclusion

This study presents a projection map of the cytochrome bc₆f complex at 9 Å resolution obtained from merging images and electron diffraction data of ice-embedded specimens. The combination of this map with the previous study in negative stain enabled us to characterize the mass distribution of the cytochrome bc₆f complex both in and out of the membrane. In particular, some peaks are only present in the cryo-projection map and may reveal the possible localization of some small subunits. The comparison with the cytochrome bc₁ structure allowed us to localize the cytochrome bc₆ at the
dimer interface and indicated structural similarities and differences between the two complexes. Moreover, this comparison gave rise to two structural models, with one satisfying better the structural and functional constraints considered. Nevertheless, the determination of a three-dimensional model of the cytochrome $b_{6f}$ complex remains necessary as it appears difficult to transpose all structural data from the cytochrome $b_{c_1}$ complex to the cytochrome $b_{6f}$ complex.

**Materials and Methods**

**Purification and crystallization**

The cytochrome $b_{6f}$ complex was purified in the presence of 6-O-(N-heptylcarbamoyl)-methyl-β-D-glycopyranoside (Hecameg) (Pierre et al., 1995). Crystallization of the cytochrome $b_{6f}$ complex was performed as described (Mosser et al., 1997) with several modifications. Briefly, the crystallization was started by addition of SM2-BioBeads (Rigaud et al., 1997) to a micellar solution containing the cytochrome $b_{6f}$ complex and a mixture of egg phosphatidylcholine and di-C18:1-phosphatidylglycerol (1:1 to 1.41 w/w). The final protein concentration was adjusted to 1 mg/ml and the lipid/protein ratio to 0.3 (w/w). After complete detergent removal at 4 °C, the reconstituted material was kept for 24 hours at 4 °C and then treated by three freeze (−196 °C)/thaw (4 °C) cycles to induce crystal growth.

**Electron microscopy**

The crystal suspensions were applied to carbon-coated EM grids that had been pre-treated with 7.5 mM Hecameg, to enhance spreading of the water. Excess suspension was blotted off and specimens were vitrified by plunging into liquid ethane (Dubochet et al., 1988) on a Leica freezing station EMCP. The vitreous specimens were transferred to a Gatan 626 cold stage and examined on a Philips CM120 cryo-microscope. Micrographs were recorded at 120 kV, 60,000 × magnification, with an electron dose of about 10 e/Å². Electron diffraction patterns were recorded with an exposure time of 5 s using an effective camera length of 2300 mm, and a beam diameter of approximately 2.5 μm on the grid.

**Image processing**

Micrographs were selected by optical diffraction and digitized at 10 μm pixel size with a Leaflscan 45 CCD-array microdensitometer. Initial areas of 3000 or 6000 pixels square were padded up to 3300 and 6600 pixels square before correction for crystal lattice distortions and contrast transfer function (Henderson et al., 1986; Henderson et al., 1990). Determination of the spacegroup was performed using the ALLSPACE program (Valpuesta et al., 1994). The processing of individual images as well as the merging of several images were performed using the MRC image analysis package (Crowther et al., 1996).

Electron diffraction micrographs were digitized with a Joyce-Loebl Mk4 densitometer at 15 μm pixel size. These patterns were corrected for the background subtraction. The intensities of reflections were measured, averaged and converted to amplitudes as described (Baldwin & Henderson, 1984; Ceska & Henderson, 1990) using the MRC image analysis package.

The projection map was generated with the CCP4 Program Suite (Collaborative Computational Project, 1994) using averaged phases values from several images and averaged amplitudes from electron diffraction patterns.

**Calculation of projection maps based on atomic models**

The coordinates of the atomic model for the mitochondrial cytochrome $b_{c_1}$ complex from chicken were kindly communicated by Dr. E. Berry. The following segments, of the pre-released version of entry 1bcc, were kept to calculate the projection map of the transmembrane regions: cytochrome $b$: Leu30-Ala53, Leu79-Tyr105, Lys111-Leu134, Thr175-Phe200, Ser226-Phe246, Leu289-Leu308, Leu521-Gln342, Pro347-Leu370; cytochrome $c_1$: Met204-Arg224; Rieske protein: Ser366-Val389; subunit 7: Ala43-Thr63; and subunit 10: Thr17-Ile42. The two hemes of cytochrome $b$ were also conserved. Projection electron density maps were calculated and displayed at 9 Å resolution using sequentially the pdbset, sfall, FFT and pluto programs from the CCP4 Program Suite (Collaborative Computational Project, 1994).

**Alignments and cross correlation of projection maps**

Two-dimensional projection maps of the transmembrane region of the cytochrome $b_{6f}$ complex were quantitatively aligned by translational and rotational cross-correlation against our projection map obtained from frozen-hydrated specimens, using the image processing system SPIDER (Franck et al., 1981).

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