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Characterization by Electron Microscopy of Isolated Particles and Two-Dimensional Crystals of the CP47-D1-D2-Cytochrome *b*-559 Complex of Photosystem II[†]

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ABSTRACT: A photosystem II complex containing the reaction center proteins D1 and D2, a 47-kDa chlorophyll-binding protein (CP47), and cytochrome *b*-559 was isolated with high yield, purity, and homogeneity; small but well-ordered two-dimensional crystals were prepared from the particles. The crystals and the isolated particles were analyzed by electron microscopy using negatively stained specimens. The information of 20 different digitized crystals was combined by alignment programs based on correlation methods to obtain a final average. The calculated diffraction pattern, with spots up to a resolution of 2.5 nm, and the optical diffraction pattern of a single crystal indicate that the plane group is $p2_1$ (also called $p2gg$) and that the unit cell is rectangular with parameters of 23.5×16.0 nm, containing four stain-excluding monomers (two face-up and two face-down). In projection, the monomers have an asymmetrical shape with a length of 10 nm, a maximal width of 7.5 nm, and a height of 6 nm; their molecular mass is 175 ± 40 kDa.

Substantial progress has been made in understanding the structure and function of photosystem II (PS II)¹ both in terms of the catalytic activity of the manganese atom involved in water oxidation and in terms of identifying polypeptides which provide ligation sites for metals, pigments, and quinones. Several groups have reduced the oxygen-evolving reaction to a complex comprised of seven major polypeptides (Ikeuchi et al., 1985; Ghanotakis & Yocum, 1986; Franzen et al., 1986; Ghanotakis et al., 1987; Rogner et al., 1987; Yamada et al., 1987; Bowlby et al., 1988), including six intrinsic membrane proteins, which serve essential photochemical and structural functions, an extrinsic 33-kDa protein necessary for the stabilization of oxygen evolution, and one or more very small polypeptides (<5 kDa) of unknown function.

In the absence of refined structural information on PS II, the structures of reaction centers of purple bacteria (Deisenhofer et al., 1985; Allen et al., 1986) are cited as model systems for PS II, owing to similarities in amino acid sequences between the reaction center proteins of the two systems (Michel & Deisenhofer, 1988). Recent studies have shown that important components of the PS II electron-transfer process are tyrosine residues, called Y_Z (Tyr-161 of the 32-kDa PS II polypeptide denoted D1) (Debus et al., 1988b; Metz et al., 1989) and Y_D (Tyr-160 of the 32-kDa PS II polypeptide called D2) (Debus et al., 1988a; Vermaas et al., 1988). Identification of D1 and D2 tyrosine residues as intermediates in PS II primary electron transfer correlates with the report by Nanba and Satoh (1987) of the isolation of a pigment-protein complex

containing the D1 and D2 proteins and cytochrome *b*-559. A partial characterization of this preparation has revealed the presence of a spin-polarized triplet which is proposed to result from an interaction between the reaction center Chl *a* (P_{680}) and the intermediate acceptor pheophytin *a*. Other reports (Akabori et al., 1988; Seibert et al., 1988) also point to the D1-D2 heterodimer as the site of reaction center function in PS II.

An alternate technique for the isolation of the D1-D2-*b*-559 complex (Ghanotakis et al., 1989) avoids Triton X-100 and permits the sequential removal of Chl-binding proteins of apparent molecular weights (on SDS-polyacrylamide gel electrophoresis) 47K and 43K. An intermediate preparation derived from the procedure consists of a plastoquinone-depleted CP47-D1-D2-*b*-559 complex (Ghanotakis et al., 1989; Dekker et al., 1989). Spectroscopic investigations have shown that it is possible to photoaccumulate an EPR signal ascribed to reaction center tyrosine radicals (Petersen et al., 1990), suggesting that CP47-D1-D2-*b*-559 represents a more native PS II complex than D1-D2-*b*-559, where tyrosine radicals cannot be photoaccumulated. As a possible first step in the unraveling of the structure of PS II, this paper describes the preparation of two-dimensional crystals of CP47-D1-D2-*b*-559 and the results of analyses of these crystals by electron microscopy and image reconstruction techniques.

MATERIALS AND METHODS

Subchloroplast PS II membranes and oxygen-evolving PS II reaction center complexes were prepared from spinach (Ghanotakis et al., 1987), and CP47-D1-D2-*b*-559 complexes were prepared as described by Dekker et al. (1989). For crystallization, the final fraction in buffer A (20 mM Bis-Tris, 20 mM NaCl, 10 mM $MgCl_2$, 1.5% taurine, and 0.03% dodecyl maltoside, pH 6.5) with 100 mM $MgSO_4$ was concen-

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¹ Abbreviations: Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Chl, chlorophyll; Cyt, cytochrome; Pheo, pheophytin; PS, photosystem; LHC, chlorophyll *a/b* binding protein.

FIGURE 1: Elution profiles of the purified CP47-D1-D2-*b*-559 fraction (dashed line) and of the solubilized, Tris-washed reaction center complex (solid line) on a Superose 12 HR 10/30 column, equilibrated in buffer A with 0.03% dodecyl maltoside. The flow rate was 0.5 mL/min.

trated slowly by dialysis against solid sucrose. Gel filtration was performed with a Superose 12 HR 10/30 column (Pharmacia) equilibrated with buffer A. An Aminco DW-2 spectrophotometer was used to quantify cytochrome *b*-559, using the dithionite-reduced minus ferricyanide-oxidized difference spectrum and assuming a differential extinction coefficient of $17\,500\text{ M}^{-1}\text{ cm}^{-1}$ (Cramer et al., 1986).

For electron microscopy, the PS II subcomplexes and/or crystals were diluted 20–50-fold with 20 mM Bis-Tris (pH 7.0), 20 mM NaCl, 10 mM MgCl_2 , 3.0% taurine, and 0.03% dodecyl maltoside. Diluted complexes were prepared with the negative-staining technique using a 1% unbuffered solution of uranyl acetate. A 5- μL drop of the sample was placed on carbon-coated copper grids for 1–2 min, blotted off with filter paper, and washed for a few seconds with distilled water just before adding the stain drop. After 2 min, excess stain was removed by blotting with filter paper, and the grids were air-dried. Micrographs were taken on Zeiss 10, Zeiss 902, or Philips EM 300 microscopes at a magnification of about 50000 $\times$.

Optical diffraction was carried out according to Markham (1968) using an optical bench equipped with a Spectraphysics Model 133 laser. Digitization was performed with a Datacopy Model 610F electronic digitizing camera. From 20 digitized electron micrographs (from the Philips EM 300 microscope), 20 crystalline areas were selected. Each of these areas was divided into overlapping fragments of 72×72 pixels (about $30 \times 30\text{ nm}$), each containing about 16 whole molecules. Image analysis was carried out with the IMAGIC software package (Van Heel & Keegstra, 1981) on a MicroVax-2 computer. The images of the 317 selected fragments could be combined by summation after they were brought into register by imposing the rotational and translational shifts calculated with alignment programs based on correlation methods.

RESULTS

Characterization of the Isolated Complexes. After purification, the CP47-D1-D2-*b*-559 complex was subjected to gel filtration in order to obtain an estimate of its structural integrity. A single peak was found (Figure 1, dashed line),

FIGURE 2: Room temperature absorbance spectrum of the purified CP47-D1-D2-*b*-559 complex at a concentration of $10\text{ }\mu\text{g}$ of Chl/mL. The vertical bar in the red absorbance peak of Chl indicates 675 nm. Inset: Dithionite-reduced minus ferricyanide-oxidized spectrum of the same fraction at $32\text{ }\mu\text{g}$ of Chl/mL, showing the difference spectrum of cytochrome *b*-559.

suggesting that the preparation consists of a homogeneous population of particles. Tris-washed, solubilized reaction center complexes show a second peak (Figure 1, solid line) which is due primarily to monomeric 43- and 28-kDa polypeptides and is not observed in the isolated CP47-D1-D2-*b*-559 complex [see Ghanotakis et al. (1987) for a similar experiment using oxygen-evolving fractions]. Comparison of these data with elution patterns of standard proteins produced an estimated molecular mass of 200–300 kDa for the CP47-D1-D2-*b*-559 material.

Figure 2 presents absorption spectra of the isolated complex. The red absorption maximum is at 675 nm; the inset shows the reduced-minus-oxidized difference spectrum of cytochrome *b*-559. When extinction coefficients of $17\,500\text{ M}^{-1}\text{ cm}^{-1}$ for the reduced-minus-oxidized absorption difference of the cytochrome at 559 nm and $74\,000\text{ M}^{-1}\text{ cm}^{-1}$ for the absorption of Chl at the red absorption maximum were used, a ratio of 15–17 Chl/*b*-559 for purified CP47-D1-D2-*b*-559 was found, which is consistent with the presence of 2 cytochromes and 30–34 pigments (Chl *a* + Pheo *a*) per reaction center (Dekker et al., 1989).

Electron Microscopy and Crystallization. Figure 3 shows electron micrographs of the purified CP47-D1-D2-*b*-559 complex, where ordered two-dimensional crystals and single particles are seen. The most abundant projection of the single particles (Figure 3a) is a circular or slightly elliptical shape with a diameter of 9–11 nm ($\pm 1\text{ nm}$, SD, $n = 70$). In a few cases, rodlike projections with dimensions of roughly $10 \times 6.5\text{ nm}$ are observed, which are sometimes aggregated in stacks (not shown). A similar aggregation behavior has been observed for several other membrane proteins, including photosystems I and II (Rogner et al., 1987; Boekema et al., 1987; Fromme et al., 1987). For these systems, there is evidence that the shortest dimension (about 6 nm) represents the distance across the membrane (Ford & Holzenburg, 1988; Dekker et al., 1988). Small stacks of crystals in the side-view position with a thickness of 6.5 nm were also occasionally observed (Figure 3b), and we therefore conclude that the nearly circular shape in Figure 3a represents the top view, i.e., the view perpendicular to the plane of the membrane. The $11 \times 9\text{ nm}$ top view in dodecyl maltoside is significantly smaller than the $15 \times 10\text{ nm}$ observed for cyanobacterial PS II material retaining the

FIGURE 3: Electron micrographs of negatively stained CP47-D1-D2-*b*-559 preparations: (a) a two-dimensional crystal surrounded by single particles in top-view position; (b) two-dimensional crystals in side-view and top-view projections; (c and d) examples of two-dimensional crystals used for image analysis; in (c), parts of an extra protein layer can be seen (the most stain-excluding regions). The bar represents 100 nm.

43-kDa polypeptide in the same detergent (Rogner et al., 1987) and for oxygen-evolving core preparations from spinach isolated according to Bowlby et al. (1988; data not shown). This suggests that removal of the 43-kDa protein results in a significantly smaller, less elongated, particle. We failed to observe the triangular-shaped particles reported by Irrgang et al. (1988). However, their spinach PSII material contains substantial amounts of Chl *b*, suggesting possible contamination by a light-harvesting Chl *a/b* binding protein (LHC II) which may have been mistaken for reaction centers. This possibility is supported by the report of Kuhlbrandt et al. (1983) of triangular particles associated with LHC II.

An estimate of the molecular mass of purified CP47-D1-D2-*b*-559 was made on the basis of its size and shape. This estimate is more reliable than that based on gel filtration because in the latter case a dependence on the shape of the particles and of standard proteins could influence gel filtration behavior. The mass of a cylinder with diameters 9 and 11 nm and a height of 6 nm is 230 ± 60 kDa, assuming a specific volume of 2 ± 0.5 nm³/kDa. Approximately 20–25% of this mass is due to the detergent shell, so the estimated mass of the protein complex itself is 180 ± 45 kDa. This suggests that the CP47-D1-D2-*b*-559 complex is monomeric, since the

molecular mass of the protein constituents is (when based on the amino acid sequences) about 150 kDa and that of the associated pigments about 30 kDa.

The most important feature visible in the micrographs of Figure 3, however, is the appearance of ordered, honeycomb-like arrays with dimensions of up to a few hundreds of nanometers. These small, two-dimensional crystals appear to be ordered in a hexagonal-like pattern with stain accumulation in the cavities. At first sight, this organization resembles the two-dimensional crystals of LHC II which occur in a *p3*-type symmetry (Kuhlbrandt et al., 1983). For the CP47-D1-D2-*b*-559 crystals, however, the distance between the spots of stain appears to be slightly larger in one direction, suggesting that they are ordered with a 2-fold, *p2*-type symmetry. Occasionally, fragments of a second layer on top of the first layer were observed (the highly stain-excluding regions in Figure 3c).

Experiments were performed in order to determine the circumstances which favored formation of the crystals, which form during dialysis of the preparation (suspended in buffer A containing 100 mM MgSO₄) against solid sucrose. High-salt conditions are essential, since at lower salt concentrations aggregations involving the hydrophilic regions of the complexes

FIGURE 4: Optical diffraction pattern of a single crystal with dimensions of approximately 100×100 nm, showing spots up to the fifth order and 2-fold symmetry along the reciprocal axes.

were observed in the electron micrographs; such interactions lead to the formation of random aggregates. Dilution of the crystals in buffers with detergent concentrations lower than about 0.015%, with taurine concentrations less than 0.5%, or at pH values lower than 6.5 also resulted in the appearance of large, irregular sheets or strings that obscured the crystals. A short, mild sonication resulted in a complete disappearance of crystals. Otherwise, however, the crystals were stable and could be frozen and stored at -60°C . Experiments are now under way to refine the conditions necessary for crystal formation.

Figure 4 shows an optical diffraction pattern of an area (about 100×100 nm) of the crystals containing about 50 stain spots, equivalent to about 100 ordered monomeric particles. Spots up to the fifth order can be observed, which is, in view of the small area, sufficient to indicate that the particles are well-ordered. The diffraction pattern shows two characteristic features: mirror-symmetry along the perpendicular axes and strong, even reflections along both crystal axes. The odd reflections are absent.

Image Analysis. Since the crystals are well-ordered, but relatively small, it was necessary to combine the information from several small crystals to obtain a suitable signal-to-noise ratio. Signal enhancement is easily performed by the so-called correlation averaging method (Frank, 1982; Saxton & Baumeister, 1982; Boekema & Van Bruggen, 1983). We selected 317 fragments from the best crystalline arrays of 20 digitized electron micrographs. These fragments (72×72 pixels or about 30×30 nm, each containing 8 stain spots and 16 monomeric units) were brought into translational and rotational equivalent positions by alignment procedures. Sums of fragments of the same crystal revealed that the crystals do not show a handedness; i.e., the way the crystals are attached to the carbon support film is irrelevant for the analysis. Therefore, the fragments of different crystals were summed after being brought into equivalent positions by the alignment procedure. The 300 fragments with the highest correlation factor between fragment and reference image were summed (Figure 5A).

From the signal-to-noise-enhanced image of the 300 summed fragments, a diffraction pattern was calculated by Fourier transformation (Figure 5B) to reveal a rectangular lattice with unit cell parameters of 23.5×16.0 nm, with an angle between both crystal axes of 90° . Two 10th order spots can be seen along the reciprocal long axes. Furthermore, the 7,5 and 7,-5 spots are visible, indicating that the sum of the aligned fragments contains information with a maximal resolution of about 2.5 nm. The pattern shows the same characteristics as those of the optical diffraction pattern of a single crystal (Figure 4). The reflections across the two perpendicular crystal axes

FIGURE 5: (A) Computer-averaged two-dimensional projection of the PS II crystals, representing the sum of 300 fragments of crystals by correlation averaging. No symmetry is imposed on the sum, which has been treated with a high-pass filter to suppress low-frequency noise of more than 10 nm^{-1} . (B) Computer-generated Fourier transform of (A). The diffraction pattern shows spots to about 2.5 nm in reciprocal space. Systematic absences of odd reflections along the perpendicular axes and the fairly good mirror symmetry across the two axes indicate that the two-sided plane group is $p22_12_1$. The bar represents 0.266 nm^{-1} .

show fairly good mirror-symmetry (Figure 5B). Furthermore, the calculated diffraction pattern reveals that the amplitudes of all even reflections along both crystal axes are strong and that the odd reflections are absent or very weak. The latter observation is strong evidence for the presence of screw axes in the plane of the crystal indicating a two-dimensional plane group $p2gg$ (Hahn, 1983), which is described as $p22_12_1$ in the notation of Holser (1958).

After taking into account the considerations just described on the symmetry of the crystal, the alignment procedure of the 317 fragments was repeated again, but with an improved reference made from the sum of the best 300 fragments with imposed screw axes and with 2-fold symmetry. In addition, the center of the sum was refined with a precision of better than 0.05 nm and 0.5° to the center of the unit cell, which is located on one of the 2-fold axes perpendicular to the crystal. The result of the final alignment, again a sum of the best-shifted 300 fragments, is presented in Figure 6.

DISCUSSION

Small but relatively well-ordered crystals have been obtained from PS II material containing the 47-kDa Chl-binding protein CP47, the reaction center proteins D1 and D2, and cytochrome

FIGURE 6: Projected structure of crystalline PS II subcomplex particles, shown with imposed 2-fold symmetry, and treated with a high-pass filter to remove low-frequency noise of more than 10 nm^{-1} . Only positive and equidistant contour lines have been drawn. The unit cell of $23.5 \times 16.0 \text{ nm}$, the 2-fold rotation axes (●) normal to the plane of the crystal, and the 2-fold screw axes (---) in the plane of the crystal are indicated.

b-559. The plane group of the crystals is $p22_12_1$ (also called " $p2gg$ "), and the unit cell is rectangular, with dimensions of $23.5 \times 16.0 \text{ nm}$. The unit cell contains four stain-excluding monomers (two face-up and two face-down), which are related to each other by 2-fold axes perpendicular to the plane of the crystal and screw axes in the plane of the crystal (Figure 6). The packing of the monomers results in large stain-filled cavities with diameters of about 6–7 nm. Similar cavities were observed in two-dimensional crystals of the light-harvesting complex LHC II (Kuhlbrandt et al., 1983; Li, 1985), but that crystal has a different symmetry ($p3$ type) and its motif is different as well.

Gel filtration experiments (Figure 1) demonstrate the monodisperse nature of the CP47–D1–D2–*b*-559 preparation, and no contaminating proteins are observed (Dekker et al., 1989). The shape of the monomeric unit in the crystals shows similarities with that of the isolated particles. As seen in Figure 6, the lowest contour level indicates the lowest positive density in the reconstructed monomers and shows the maximal dimensions of the monomer in projection. The maximal length is 10 nm; the maximal width occurs asymmetrically at one side of the particle and is about 7.5 nm. Both dimensions are 1–1.5 nm smaller than those seen in top views of the isolated mo-

nomers (9–11 nm). This difference is not unexpected since the isolated particles are surrounded by a shell of detergent molecules; for the formation of the two-dimensional crystals, at least a part of this shell has to be removed in order to facilitate the interactions between the hydrophobic regions of the proteins. The mass of the monomeric unit is $175 \pm 40 \text{ kDa}$ (assuming a distance of 6.0 nm across the plane of the crystal and a specific volume of $2 \pm 0.5 \text{ nm}^3/\text{kDa}$), which corresponds well with the estimated mass of the isolated particle corrected for the detergent contribution ($180 \pm 40 \text{ kDa}$; see Results).

The patterns of spots in the optical diffraction pattern of a single crystal (Figure 4) and in the calculated Fourier transform of the final average (Figure 5B) have similar characteristics, indicating a $p22_12_1$ plane group in the crystals. This plane group implies that there are four monomeric particles in the unit cell, two of which are oriented right-side-up and two right-side-down. This explains why no handedness was found for the individual crystals; the right-side-down orientation or a 180° turn of a crystal will not influence the shape of the asymmetric unit. If the crystal contained only unidirectionally oriented monomers, then different views of crystals will appear, giving rise to one type of shape in the final average and not, as in our case, to mirror images within the same average.

Several other membrane proteins have been crystallized into two-dimensional sheets with the same $p22_12_1$ crystal symmetry as the PS II particles. Cytochrome oxidase was the first crystallized membrane protein shown to have such a symmetry (Vanderkooi et al., 1972; Fuller et al., 1979). Cytochrome *c* reductase (Leonard et al., 1981) and NADH dehydrogenase (Leonard et al., 1987) extend up to 15 nm across the membrane, and projections of molecules oriented up and down in the crystal differ primarily because of considerable variations in height in the plane of the crystal, causing large differences in stain distribution around the building blocks of the crystals. In PS II crystals, the up and down orientations of the monomers appear similar in projection (Figure 6), indicating that they are packed at the same height within the plane of the crystal and therefore make good contact with the carbon support film.

It appears that the $p22_12_1$ plane group is very common; for most of the proteins which crystallize with this type of symmetry, it is believed that crystallization starts with dimerization of equally oriented monomers. The next step is then determined by interactions between right-side-up- and right-side-down-oriented dimers. It is possible that a similar process takes place in the formation of PS II crystals. Of the complexes studied thus far, the final resolution in NADH dehydrogenase and cytochrome *c* reductase has been hampered somewhat by the large distance across the membrane. The structure of bacteriorhodopsin in the orthorhombic crystal form ($=p22_12_1$), however, has been resolved to 0.7 nm (Michel et al., 1980), and with the $p3$ crystal form, the two-dimensional projection was recently determined to 0.28 nm (Baldwin et al., 1988). The two-dimensional projection of LHC II crystallized with $p3$ symmetry has been reported to 0.7-nm resolution (Kuhlbrandt, 1988). The PS II particles are characterized by a short distance across the membrane, similar to bacteriorhodopsin and LHC II, so if larger two-dimensional crystals can be obtained, a similar resolution may be obtained with PS II crystals when using cryoelectron microscopy.

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