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Structural studies on improved crystals of the photosystem I reaction centre from *Phormidium laminosum*

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Improved crystallization methods led to the growth of large single crystals of the photosystem I reaction centre isolated from the thermophilic cyanobacterium *Phormidium laminosum*. Although the crystals exhibited a high degree of mosaicity, some preliminary X-ray diffraction studies were performed. The highest order reflections found correspond to interplanar spacings of approx. 0.8 nm. In conjunction with electron microscopy of thin three-dimensional crystals, a triclinic or monoclinic crystal system with unit cell dimensions $30 \times 18 \times 18$ nm was assigned. In addition, our data suggest the presence of four trimeric complexes per unit cell, which may be organised as two face-to-face pairs.

Membrane protein; Crystallization; Photosystem I; Reaction center

1. INTRODUCTION

Photosynthetic membrane protein complexes have been strongly involved in the development of the relatively new field of membrane protein crystallization [1–7]. Of special interest are the photosynthetic reaction centres, which convert light energy into chemical potential [1,2,6,7]. This report concerns the reaction centre of PS I which drives the second step of oxygenic photosynthesis in plants and cyanobacteria. The PS I reaction centre employs iron-sulphur centres as stable electron acceptors with redox potentials of about −500 mV. In contrast to the purple bacterial and PS II reaction centres, PS I houses light-harvesting capacity: 50–60 chlorophyll *a* molecules and several carotenoid molecules are bound by the two large polypeptide chains that bind the core of primary electron-transfer components [6]. The reaction centres of the green sulphur bacteria and the heliobacteria show similarities to the PS I reaction centre [8].

This paper describes the development of new methods which now allow the growth of large crystals of the PS I reaction centre from *Phormidium laminosum*. The complex which crystallizes is a trimer in solution [9,10], although the reaction centre probably exists as a monomer in the native membrane [9,11]. The first X-ray diffraction measurements are presented and the prospects for structure determination are assessed. Additional information on the packing of the complexes in the crystalline state is revealed by electron microscopy of thin crystals.

2. MATERIALS AND METHODS

Trimeric PS I reaction centre complexes were isolated as in [9]. In order to remove small amounts of detergent-solubilized ('free') chlorophyll, two runs on a Sepharose-CL 6B (Pharmacia) sizing column (63 × 2.5 cm), equilibrated with standard

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Abbreviations: Chl, chlorophyll; PS, photosystem

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buffer [10 mM Hepes, 10 mM $MgCl_2$, 5 mM $Na_2HPO_4 \cdot 12H_2O$, 0.05% (w/v) dodecyl maltoside (Calbiochem) and NaN_3 (0.2%, w/v); pH 7.5] were carried out. Samples equivalent to 10 mg chlorophyll at 2 mg/ml were applied. Fractions from the first run which contained free chlorophyll (identified by a blue-shift in the chlorophyll absorption peak) were discarded; remaining fractions were pooled and concentrated as described [6], before reloading. For the second run, fluorescence spectroscopy was used as a very sensitive test for free chlorophyll (see below). The purified material was concentrated, resuspended in standard buffer and stored in batches at −70°C.

For crystallization, purified protein at 1 mg Chl/ml was used. The concentration of $MgCl_2$ was adjusted to 90 mM using a 1 M stock solution. The concentration of PEG 6000 (Merck) was slowly increased to 3% (w/v) and finely adjusted so that the PS I reaction centre complexes precipitated slowly on ice, but redissolved upon warming to room temperature. Using a long-needle Hamilton syringe, 15–50 μl were loaded into glass capillaries sealed at one end only (7 × 0.10–0.15 cm) and placed in a refrigerator at 9°C for 3–6 weeks during which the volume was halved by dehydration. In a second approach, $MgCl_2$ was omitted and the PEG concentration was increased to about 4% (w/v). 25–50 μl of this solution were injected into capillaries and 10 μl of 0.1 M $MgCl_2$ were carefully loaded on top to form a sharp liquid/liquid interface at which a small amount of precipitate appeared. These capillaries were also left for several weeks as above. Crystal density was measured by isopycnic centrifugation on sucrose density step gradients.

Microcrystals suitable for electron microscopy were grown according to [12]. Protein equivalent to 18 μg Chl/ml was suspended in 50 mM Tris, 50 mM $MgCl_2$, 0.05% (w/v) dodecyl maltoside, 0.2% (w/v) NaN_3 (pH 8.2), and 2% (w/v) PEG 6000. Droplets (5 μl) were placed on parafilm and incubated at 25°C for 20–25 min. 30 s after transferring them to carbon-coated copper grids, excess liquid was blotted off and the material was stained with a 0.75% (w/v) solution of uranyl formate (pH 4.25). Large linear aggregates were obtained by mixing 1 μl of a 50% (w/v) ammonium sulphate solution (pH 8.2) with 1 μl of the protein solution at 1.1 mg Chl/ml in the buffer (without PEG). The droplets were left overnight at 9°C in a petri dish with a well containing a saturated $ZnSO_4$ solution. Mounting for electron microscopy was carried out as detailed above. Grids were observed in a Hitachi H-7000 transmission electron microscope operated at an accelerating voltage of 80 kV. Electron micrographs were recorded at calibrated magnification (50000 ×) on Kodak SO-163 electron image sheet film. Optical diffraction patterns were calibrated using thin 3D crystals of D-ribulose-1,5-bisphosphate carboxylase/oxygenase [13].

The crystals were mounted in thin-walled glass capillaries prior to X-ray exposure. They were irradiated with nickel-filtered CuK_α X-radiation from an Elliott GX-20 rotating-anode generator operating at 38 kV and 38 mA. The still photographs were recorded on Kodak DEF-5 X-ray film at 5°C using an Enraf-Nonius rotation camera, with a crystal-to-film distance of 100 or 150 mm and exposure times of 5–10 h.

Fluorescence spectra from 650 to 850 nm (20°C) were obtained with a Schoeffel RRS 1000 fluorometer using an excitation wavelength of 430 nm. Samples were diluted to 2–5 μM Chl in 3 ml standard buffer without detergent.

3. RESULTS AND DISCUSSION

Since free chlorophyll caused phase separation and inhibited crystallization, its removal from the purified reaction centre material was essential for reproducible growth of large crystals. The presence of low amounts of (strongly fluorescent) free chlorophyll could be detected by the appearance of a peak or shoulder at around 670 nm in the fluorescence emission spectrum (fig.1B–D), whereas the PS I reaction centre was characterized by a peak at 684 nm and a shoulder at 704 nm (fig.1A).

The improved crystal growth conditions resulted in crystals of prismatic habit [6] with lengths of 0.5–1 mm and thicknesses about 0.2 mm (fig.2a,c). The first crystallization method described above (dehydration method) generally produced the larger crystals, but the second (liquid/liquid interface diffusion) was preferred for testing fresh batches of material, since crystals often grew within a few days. The crystals diffracted X-rays weakly (fig.2b,d), and thus long exposure times were required. Because of the high intrinsic mosaicity of the crystals, estimation of the unit cell dimensions was possible by examining portions of the reciprocal lattice revealed by still

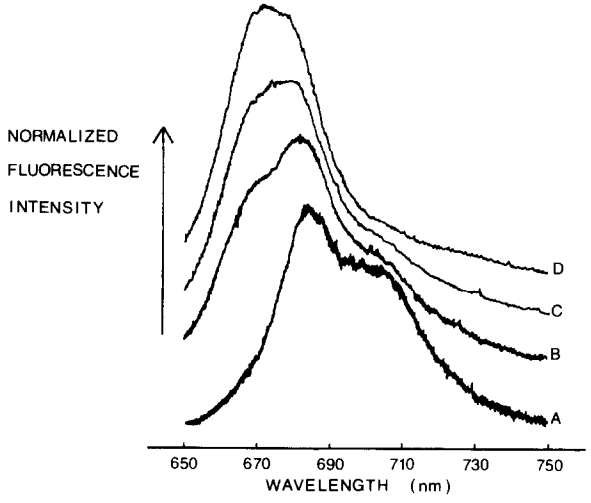

Fig.1. Fluorescence emission spectra of (A) the final purified PS I reaction centre material, and (B–D) discarded fractions from the column containing increasing amounts of free chlorophyll. Spectra were normalized at 682 nm. The fluorescence intensity of (D) at 670 nm was approx. 10-fold that of (A) at 684 nm.

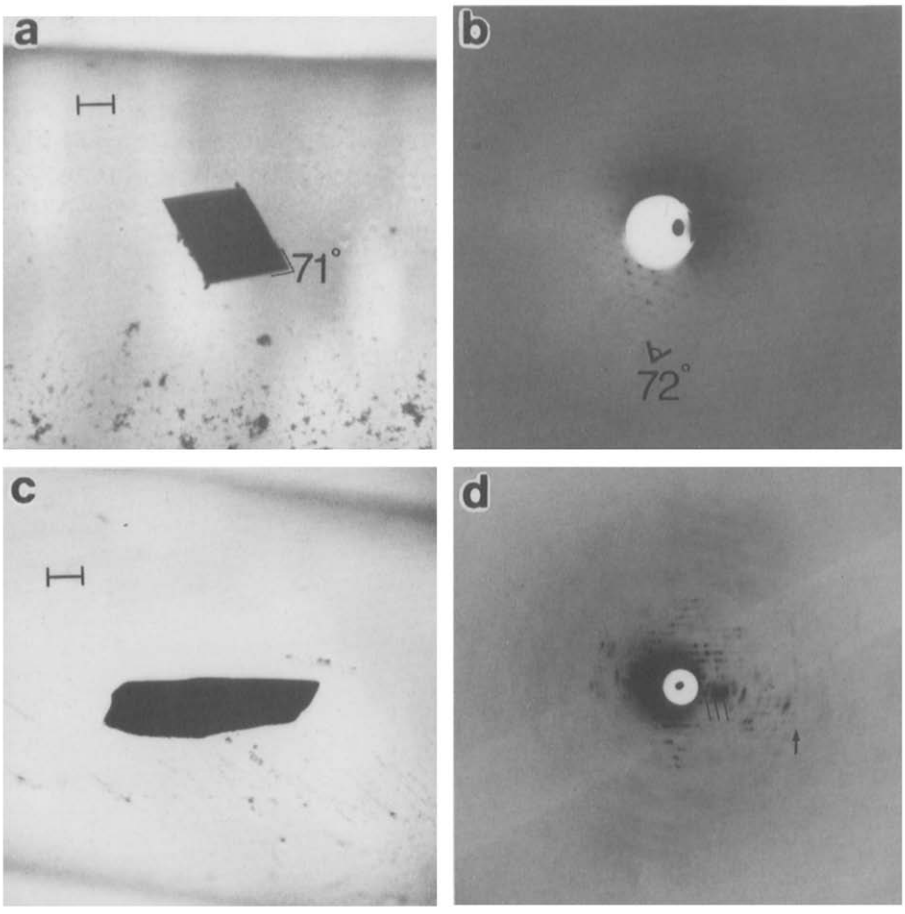

Fig.2. (a,c) Large crystals (in capillary) of the PS I reaction centre of *P. laminosum* looking (a) along and (c) normal to the long dimension of the crystal. The bar represents 0.1 mm. (b) X-ray diffraction from a crystal oriented with its longest axis approximately parallel to the beam (the reflections extend to a resolution of about 2.0 nm), and (d) normal to the beam (reflections extend to about 0.8 nm). Strong reflections (overexposed) possibly due to X-ray damage are marked with 3 parallel lines.

photographs (fig.2b,d) taken with the long dimension of the crystal oriented parallel and normal to the X-ray beam. Not surprisingly for a trimeric complex of molecular mass exceeding 700 kDa [7,9], the unit cell dimensions were large (30 × 18 × 18 nm). Since the concentrations of chlorophyll (0.1 g/ml) and protein (0.5–0.6 g/ml) within the crystals had been previously estimated by microspectrophotometry [6], a value of 4 trimeric complexes per unit cell could now be calculated, using a molecular mass of 780 kDa for the trimeric complex [6,7]. This corresponds to a V_m of 3.0 $Å^3$/Da, suggesting that the crystals contain only slightly more solvent than is typical for water-soluble proteins [14]. A crystal density value of 1.2 g/ml was measured using sucrose gradients, giving a higher estimate of 5 trimeric complexes per unit cell, but this may be less reliable because PEG and detergent may also be present in the crystal.

The development of several intense reflections along a single radial line was noticed after about 2 h of irradiation (see fig.2d), and these reflections increased in relative intensity with time until they were the only feature in the diffraction pattern, suggesting that they were in some way a product of X-ray damage. Presumably, the order in the crystals had collapsed to sets of planes with disordered populations. The reflections always occurred at positions equivalent to spacings of 2.9, 3.6 and 4.8 nm. In the paper of Witt and coworkers [7], identical spacings were calculated

from rings observed in the powder diffraction of a batch of microcrystals of the *Synechococcus* sp. PS I reaction centre. This raises the question of whether the rings at these spacings in the powder diffraction pattern might also have arisen from X-ray-induced damage.

Prospects for structure determination of the PS I reaction centre can now be assessed. Problems with the *P. laminosum* crystals include high mosaicity and X-ray sensitivity. Since X-ray damage is apparent after only a few hours of irradiation, and it is generally the higher order reflections which disappear first, it might be expected that the current resolution limit could be

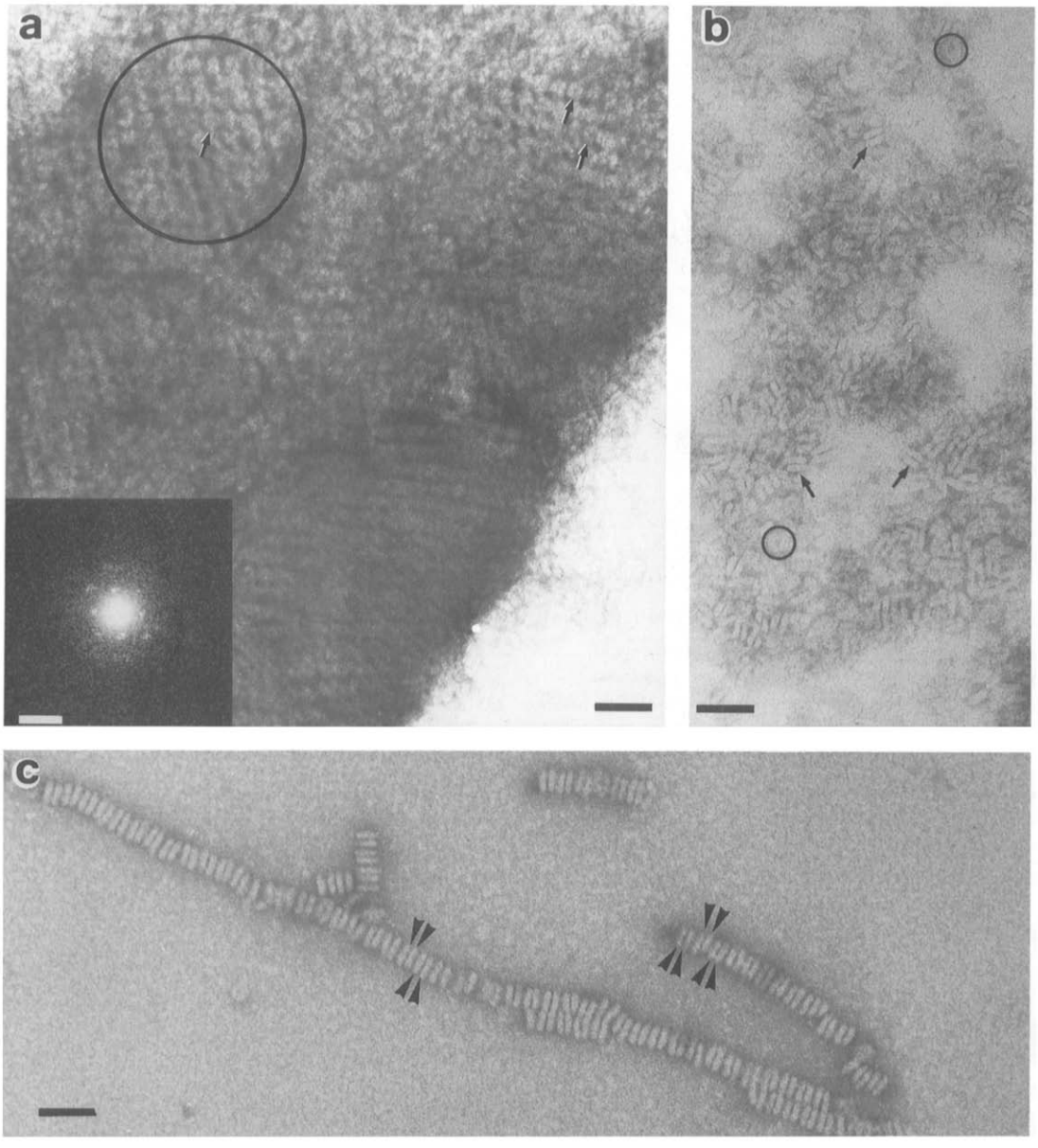

Fig.3. Transmission electron micrographs of specimens negatively stained with uranyl formate. (a) Section of a thin 3D crystal of the PS I reaction centre. The circled area was used for optical diffraction (inset). (b) Area surrounding the crystal showing paired trimeric complexes side-on (arrows) and face-on (circles). (c) Ammonium sulphate-induced stacks of trimeric complexes. Bars represent 50 nm for each micrograph and 0.1 nm^{-1} for the optical diffraction pattern. For further information see text.

significantly improved by the stabilization of the crystals, for example by use of very low temperatures. If these problems can be overcome, the prospects for structure determination seem good. Moreover, the methods applied here for the growth of large crystals may now allow the screening of PS I reaction centres from a wide range of organisms and thus open up an alternative source for higher quality crystals.

We have also used electron microscopy to investigate the crystallization behaviour of the PS I reaction centre complexes and to try to confirm the X-ray diffraction measurements. The application of a 2D crystallization procedure [12] produced many microcrystals about 1 μm across but unfortunately no 2D arrays. Occasionally, microcrystals were observed that were thin enough for some structural details to be apparent (fig.3a). Typically, the crystal was surrounded by densely packed, but disordered trimeric complexes (fig.3b). Small crystalline arrays could be discerned within the microcrystal (circled area fig.3a) with repeat distances of 18.4 × 18.0 nm at an angle of 72°, as determined by optical diffraction. This is consistent with the X-ray diffraction measurements (fig.2b). In fig.3a, the units in the ordered arrays seem to consist of a rounded lighter area (presumably protein) with a stain trap in the middle. Occasionally stain-filled channels emerging from the centre can be observed (arrows). This image corresponds to the 'top' view of the isolated trimeric complex described earlier [9].

In fig.3b the disordered complexes surrounding the crystal appear to be present in pairs when viewed side-on (arrows). This pairing is particularly prevalent in the presence of ammonium sulphate (fig.3c). Note that there is an alternate spacing between complexes, suggestive of face-to-face pairing (arrowheads) and that a stack of four trimeric complexes corresponds to about 30 nm. Similar pairing has also been observed for the PS I reaction centre of *Synechococcus* sp. (Witt, H.T., personal communication).

By combining the measurements from X-ray diffraction and electron microscopy, some tentative conclusions can be reached. The angle of 72° suggests a monoclinic or triclinic crystal system. The other angles are close, but may be not exactly equal, to 90°. Because of the mosaicity problems, it might be necessary to collect a full data set of X-ray intensities before the space group can be conclusively determined. It is speculated that the four trimeric complexes are stacked on top of each other in the unit cell so that the three-fold axis of each trimer runs roughly parallel with the long (30 nm) unit cell dimension. This agrees with the linear dichroism measurements of the crystals [6,9]. By taking into account the tendency of the trimeric complexes to form face-to-face pairs under the crystallization conditions, a model could be proposed where two pairs, stacked on top of each other and related by a (perhaps non-crystallographic) screw axis, are envisaged to form the unit cell content. Such a model must be tested by further crystallographic investigation.

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