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Crystallization of the photosystem I reaction centre

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The reaction centre of the photosynthetic membrane complex photosystem I (PSI) from the thermophilic cyanobacterium *Phormidium laminosum* was found to crystallize under a range of conditions. The crystallization method, which can occur in the presence of larger detergent molecules than those used previously for the crystallization of membrane proteins, is presented in this report. Several crystal forms have been observed, and some of these show birefringence and linear dichroism. Optical measurements on crystals thicker than ~5 μm were severely restricted because of the very high chlorophyll density within the crystals, but linear dichroism measurements on thin single crystals were possible and the results are presented here. By comparing the data with earlier measurements on oriented PSI complexes, a working model for the orientation of the PSI complexes within the crystal could be proposed. The PSI reaction centre is one of the largest and most complex membrane protein units that have been crystallized to date.

Key words: photosynthesis/membrane protein/crystallization/linear dichroism/*Phormidium laminosum*

Introduction

The reaction centre of photosystem I (PSI) is an intrinsic membrane protein complex which is involved in photosynthetic electron transfer in plant chloroplasts and in cyanobacteria. Light energy absorbed by the complex is eventually trapped by a special chlorophyll species termed P700 which lies at the heart of the reaction centre of PSI. P700 uses the light excitation energy to push an electron on to a sequence of electron-accepting components which are also bound within the reaction centre. The electron eventually reduces the water-soluble iron–sulphur protein, ferredoxin, which, in turn can reduce NADP via an enzyme termed ferredoxin-NADP reductase. Finally, the reduced NADP can be used for the reduction of carbon dioxide in the Calvin cycle. The oxidized P700 is reduced by a water-soluble, copper-containing protein called plastocyanin, although in some cyanobacteria cytochrome *c* fulfills this role. For a general review of PSI, see Malkin (1982) and Rutherford and Heathcote (1985).

The PSI reaction-centre complex used in this study was obtained from the thermophilic cyanobacterium *Phormidium laminosum*. The reaction centre contains a minimum of three polypeptides, two of relatively low mol. wt (15 and 10 kd), and one of higher mol. wt (65 kd). The polypeptide composition of the *P. laminosum* PSI reaction centre resembles that from *Synechococcus* sp. (Takahashi *et al.*, 1982). The 65-kd polypeptide is the core protein of the reaction centre and is thought to bind P700, the early electron acceptors termed A0 and A1 and ~60 light-harvesting pigment molecules, a number which can vary between different PSI preparations. A third electron acceptor termed X, a (4Fe–4S) iron–sulphur centre, may also be bound by the 65-kd polypeptide (Golbeck and Cornelius, 1986; Hoj and Moller, 1986). There are strong reasons to suspect that the 65-kd band seen on SDS–PAGE is actually composed of two different polypeptides of similar mol. wt and with high amino acid sequence homology (see Fish *et al.*, 1985 for a discussion of this problem). It is likely that the two lower mol. wt polypeptides are involved in the binding of the (4Fe–4S) iron–sulphur acceptors called A and B (Takahashi *et al.*, 1982; R.C.Ford, P.Setif and K.Brettel, unpublished results).

Apart from binding electron transfer and light-harvesting components, the PSI reaction centre must interact with other proteins such as the water-soluble electron transfer proteins ferredoxin and plastocyanin as well as membrane-bound, light-harvesting proteins. Clearly, the PSI reaction centre must carry out a variety of functions, but perhaps the most intriguing pro-

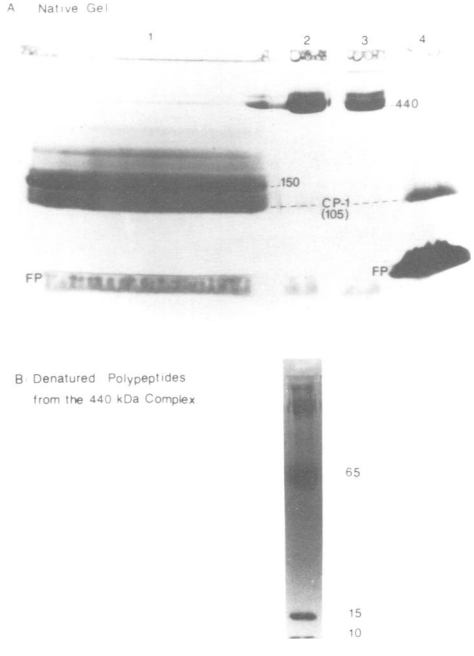

Fig. 1. (**a**) Migration of PSI complexes and free pigment (FP) on an unstained 5–10% polyacrylamide gel. **Tracks 2** and **3** show the migration of material (containing 100 μg Chl and 50 μg Chl respectively) from the lower band on the sucrose density gradient. **Track 1** shows the migration of PSI complexes harvested from the upper band on the sucrose gradient (1.05 mg Chl was loaded). **Track 4** shows the migration of spinach PSI material, 200 μg Chl was loaded. The numbers represent the estimated mol. wts of the various complexes in kd. (**b**) Denatured polypeptides from the 440-kd complex revealed by Coomassie stain after separation on a 10–15% polyacrylamide gel. The numbers represent the estimated mol. wt in kd.

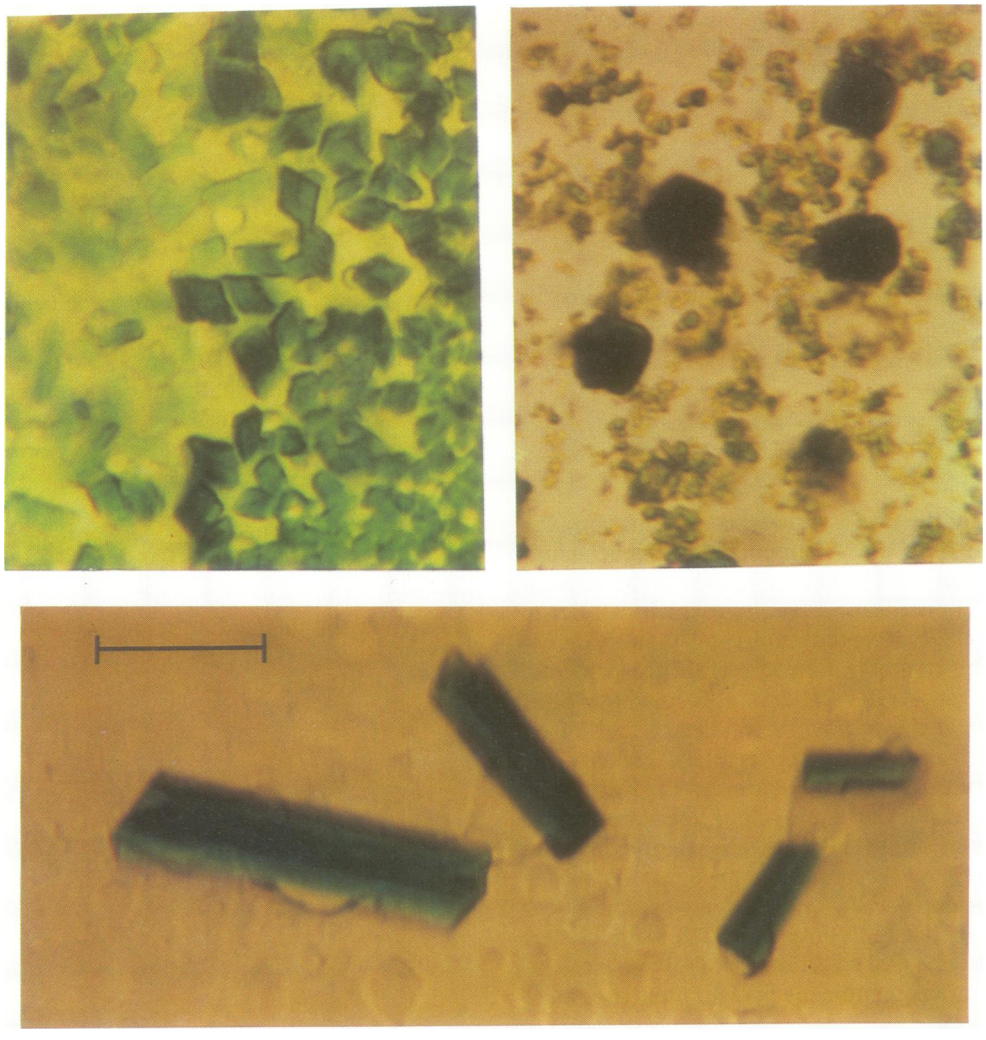

Fig. 2. Multiple forms of the PSI reaction centre crystals. **Top left**: Diamond- and rectangular-shaped crystals grown in the presence of Triton X-100 with a starting Chl concentration of 1 mg/ml and a PEG concentration of 9% (w/v). **Top right:** Square and irregular-shaped crystals grown in the presence of SDS (4 mg/ml Chl, 4.5% PEG at start). **Bottom:** Rectangular-shaped crystals grown with dodecyl maltoside (2 mg/ml Chl, 7% PEG). The bar represents 50 μm in all the photographs.

perty of this complex is its ability to accommodate so much non-protein material including the 50–60 rather large and rigid pigment molecules (chlorophyll *a* and carotenoid). In comparison, the photosynthetic reaction centre of the purple bacteria contains only five pigment molecules, three of which are directly involved in electron transfer (Deisenhofer *et al.*, 1985). The resolution of the three-dimensional structure of the PSI complex should provide a new insight into the mechanism of excitation energy transfer between the various light-harvesting pigments as well as highlighting a remarkable piece of protein engineering. The crystallization of this complex from *P. laminosum* is an initial step towards this goal.

The crystallization of other membrane proteins has been reported in recent years. In the pioneering work (Garavito and Rosenbusch, 1980; Michel, 1982; Garavito *et al.*, 1983), and in subsequent studies (Allen and Feher, 1984; Garavito *et al.*, 1984; Kühlbrandt, 1984; Welte *et al.*, 1985; Wacker *et al.*, 1986), small amphiphilic and detergent molecules were employed [heptane-1,2,3-triol, octyl glucoside and lauryldimethylamine oxide (LDAO)]. In current models of the crystallization process for membrane proteins (Garavito *et al.*, 1986), the small amphiphilic and detergent molecules form a water-soluble complex with the protein, but are small enough to allow the close packing required in the crystalline state. In the case of the PSI reaction centre, similarly sized detergent molecules have been found to be effective for crystallization (sodium dodecyl sulphate and dodecyl maltoside). Perhaps surprisingly, however, crystallization has also been observed in the presence of a rather large detergent (Triton X-100). The implications of this observation are discussed later.

Results and Discussion

Figure 1a shows the final stage in the purification of PSI reaction centre material from *P. laminosum*. Preparative SDS–PAGE resolved multiple green bands from the upper band of the sucrose density gradient (track 1), but only two bands from the material harvested from the lower band on the sucrose gradient (tracks 2 and 3). The darker of the two bands shown in tracks 2 and 3 represents the material taken for crystallization. Very little free pigment (FP) is liberated by the SDS–PAGE purification of the *P. laminosum* complexes. In comparison, track 4 was loaded with spinach (higher plant) PSI, and, under the same conditions, almost all of the pigments were liberated by the SDS, emphasizing the advantages of working with the very stable material from thermophilic cyanobacteria. The chlorophyll–protein complex in the upper green band in track 4 is termed CP-1 and corresponds with

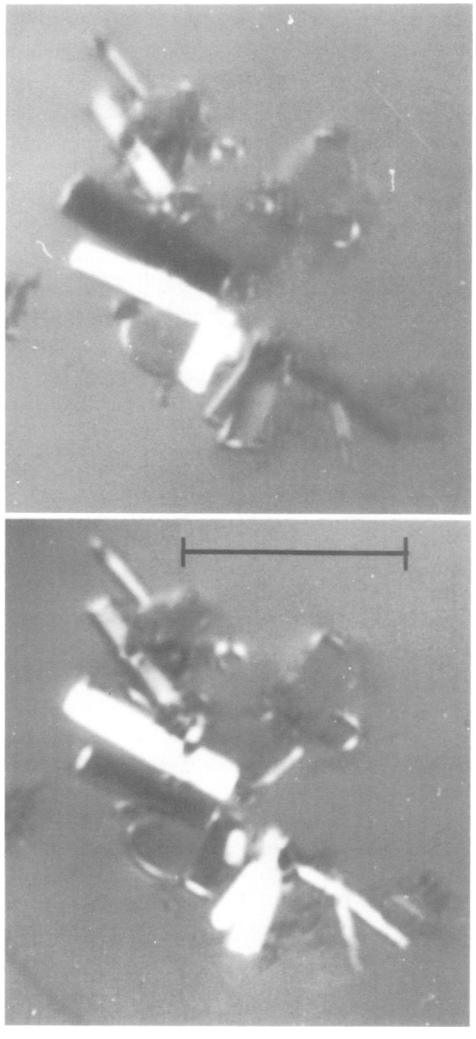

Fig. 3. Birefringence of rectangular-shaped crystals grown with Triton X-100. The second photograph represents the same field as the first, but rotated by 45°. The bar represents 10 μm.

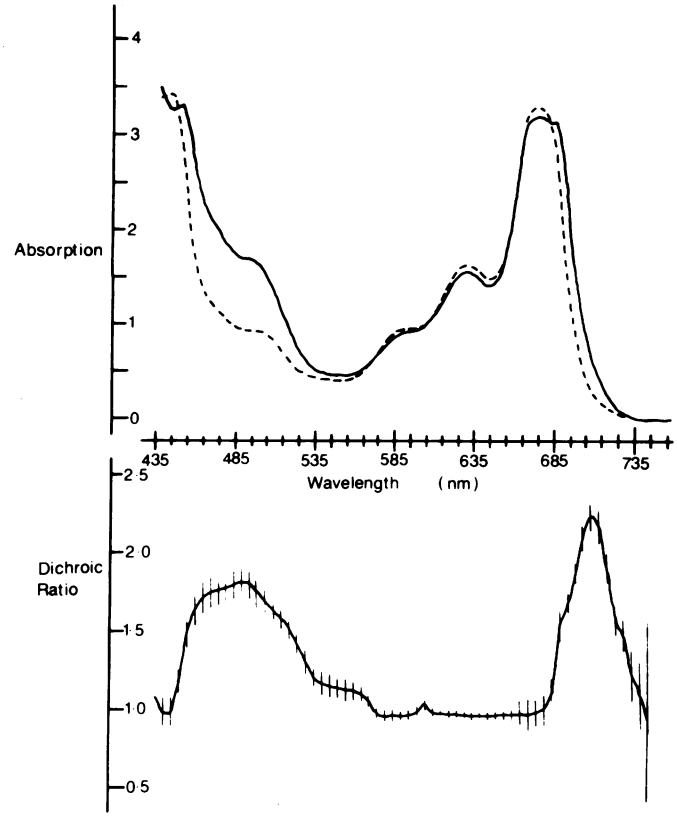

Fig. 4. Linear dichroism of a single rectangular-shaped crystal grown with dodecyl maltoside. The measuring beam was 3.1 μm diameter and passed through the crystal face of dimensions 18 × 7 μm. Spectra were recorded with a spectral bandwidth of 10 nm. Absorption parallel (dashed line, A_a) and perpendicular (solid line, A_b) to the long axis of the crystal is shown after refinement as described in the text. The dichroic ratio (A_b/A_a) is plotted below. The vertical bars indicate the SD of the least-squares fit for each wavelength interval.

the lowest of the green bands in track 1. A detailed characterization of the complexes in the bands in tracks 1 to 3 has been carried out (R.C.Ford, P.Setif and K.Brettel, unpublished results). The migration of the chlorophyll–protein complexes and their properties appear to correspond to those studied in a different cyanobacterium, *Synechococcus* sp. by Takahashi *et al.* (1982). Molecular weights of the various complexes have been estimated by using water-soluble marker proteins. The complex used for crystallization migrated closely with ferritin (440 kd), and it seems likely that it is an oligomer of the complex in the darkest band in track 1 which had an estimated mol. wt of 150 kd (unpublished data).

The polypeptide composition of the 440-kd complex after complete denaturation is shown in Figure 1b. Proteins revealed by Coomassie stain migrated to positions on the gel approximating to apparent mol. wts of 65, 15 and 10 kd. Two very slowly migrating bands (>180 kd) appear to be aggregation artefacts which are observed with the denaturation of all the *P. laminosum* PSI-containing chlorophyll–protein complexes including CP-1, and disappear when partially denaturing conditions are used. Similar artefacts have been discussed previously (Williams *et al.*, 1986).

Crystallization of the 440-kd complex can proceed very rapidly, and in one instance microcrystals were observed a few hours after the cooling of a saturated protein solution to 4°C from room temperature. Larger and better shaped crystals were grown by vapour diffusion over a period of 2–10 days. Crystals grown more rapidly or more slowly than this time period tended to be poorly shaped and very small. They rarely showed birefringence. Larger starting volumes tended to produce larger crystals, however crystallization did not occur or was too slow if the starting concentration of chlorophyll was <1 mg/ml. Large quantities of starting material are therefore required, a common problem of protein crystallization.

In the initial attempts to crystallize the complex, octyl glucoside and heptane-1,2,3-triol were tested, but there were found to only poorly solubilize the PSI complex, and at the very high concentrations required, dissociation of carotenoid pigments from the complex was observed, and a blue-shift in the absorption spectrum of the chlorophyll bound by the complex was detected. In comparison, high protein concentrations could be maintained in solution by Triton X-100, SDS or dodecyl maltoside at detergent concentrations <0.1% (w/v) and the absorption spectrum of the solubilized complex remained unaltered.

Figure 2 shows some examples of the crystals grown by the vapour diffusion method. A variety of regular shapes have been observed, but the diamond-shaped plates and rectangular prism forms tended to dominate. Rectangular-shaped crystals showed strong birefringence (Figure 3), but the diamond-shaped form

showed very weak or no birefringence through the diamond-shaped face, possibly suggesting a high symmetry axis along the viewing direction. The absorption spectra of various PSI crystals were recorded with a microspectrophotometer and compared with the spectrum of the starting solution. The spectra were very similar except that distortion of the crystal absorption spectrum occurred around the chlorophyll absorption peaks at ~435 and 680 nm (see Figure 4). The distortion was due to the very high density of chlorophyll within the crystal. By pressing the coverslip gently, it was possible to reorient the crystal so that its thickness could be estimated. It was found that a rectangular-shaped crystal of ~3 μm thickness had an optical density at 680 nm of 2.5. A chlorophyll concentration within the crystal of ~0.13 M could therefore be calculated, and with a protein to chlorophyll ratio of ~4:1 (unpublished data), a protein-plus-pigment concentration of 0.5–0.6 g/ml could be obtained. After resolubilization of crystals in a cuvette, light-induced P700 oxidation could be detected with no apparent loss of activity compared to the starting material. Linear dichroism could be detected by measuring the absorption spectra of single, rectangular-shaped crystals with polarized light, but in agreement with the birefringence measurements, no linear dichroism was observed for the diamond-shaped crystals. Similar differences in the absorption of polarized light were observed with rectangular crystals grown in the presence of Triton X-100, SDS and dodecyl maltoside. Linear dichroism was strongest in the wavelength region above 690 nm and between 450 and 540 nm, regions where chlorophyll *a* and carotenoid absorption (respectively) are strong.

In order to assess more accurately the linear dichroism measurements, the crystal was rotated around an axis parallel to the polarized measuring beam and spectra were recorded every 15° until a circle was completed. The transmission can be expressed as follows (Martin *et al.*, 1979):

$$T(\theta) = (T_a - T_b)\cos^2(\theta - \theta_0) + T_b$$

Where T_a and T_b represent the transmission maximum and minimum, θ defines the orientation of the crystal relative to an arbitrary reference system and θ_0 is the angle between the reference system and the direction of T_a. The values for T_a, T_b and θ_0 were refined by alternate least-squares fitting of T_a and T_b followed by θ_0, and the fit was repeated independently for each wavelength interval (every 5 nm). As shown in Figure 4, the 460–520 nm region and the 690–730 nm region clearly show dichroism, and the angle θ_0 indicated that (within experimental error) for both regions, the maximum absorption occurred when the long axis of the crystal was oriented approximately perpendicular to the direction of polarization of the measuring light. From the calculated transmission maxima and minima the dichroic ratio was plotted (Figure 4).

It was clear from all the measurements of PSI crystals that some regions of the absorption spectrum showed little or no linear dichroism. This may be explained by the presence of a population of randomly oriented chlorophyll molecules or alternatively by a population with a high degree of symmetry. The hypothesis is based on data for the 565–655 nm region (where the absorption is <2.0), and is supported by previous work on oriented PSI complexes (Tapie *et al.*, 1984). As discussed earlier, the maximum absorbance that could be measured by the microspectrophotometer in the experiment shown in Figure 4 was ~3.3, and this explains the flattening of the absorption spectrum seen around the chlorophyll absorption peaks at 680 and 435 nm. Values for the dichroic ratio in these regions of the spectrum are therefore close to 1.0, and must be discounted. In order to obtain information on pigment orientation for these strongly

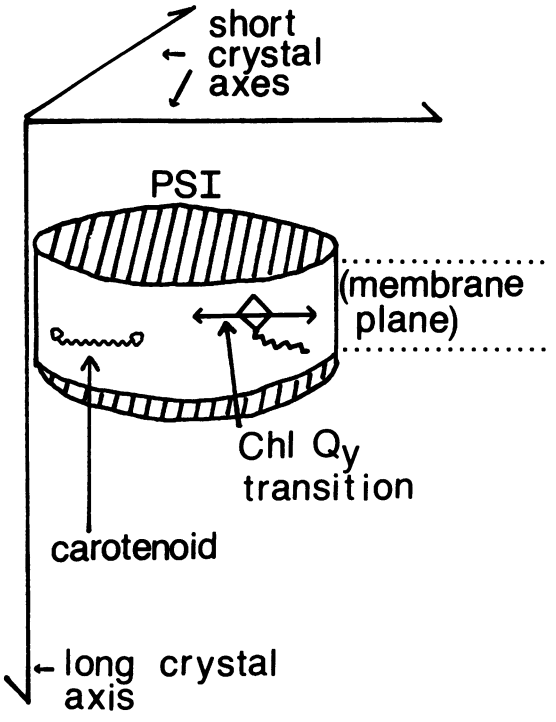

Fig. 5. Working model of the PSI reaction centre and its orientation in the rectangular-shaped crystals. The proposed average orientations of the carotenoids and the Q_y transitions of the long wavelength-absorbing chlorophylls are also shown.

absorbing regions, very thin, plate-like crystals are required, but to date such crystals have not been observed for the PSI reaction centre.

The interpretation of linear dichroism measurements on crystals is not simple, particularly when more than one chromophore is present and the form of the unit cell is unknown as in this case (Hofrichter and Eaton, 1976). Estimation of the number of chlorophylls per reaction centre for the 440-kd complex has been carried out (R.C.Ford, P.Setif and K.Brettel, unpublished results). About 55 chlorophyll *a* molecules and several carotenoids appear to be present per reaction centre, and there are probably three reaction centres per 440-kd complex. Thus, it seems likely that the dichroism observed in the crystals represents an average over many pigment molecules. Fortunately the data can be compared with earlier studies on PSI reaction centres which were oriented in stretched gels (Haworth *et al.*, 1982; Tapie *et al.*, 1984), and in the studies of Junge *et al.* (1977) using photoselection. In the photoselection studies, Junge and co-workers monitored the primary electron donor, P700, with polarized light, and observed that preferential excitation of P700 could be detected when an excitation pulse was polarized parallel to the measuring beam. The preferential excitation only occurred when the excitation wavelength fell between 450 and 520 nm or was >690 nm. Linear dichroism in the spectra of oriented PSI complexes was detected between 430 and 510 nm and between 675 and 740 nm in the studies of Haworth and colleagues and Tapie and co-workers, but very little dichroism was detected between 530 and 660 nm and <430 nm. In these studies, dichroic ratios up to ~1.4 were obtained, smaller than those detected in the PSI reaction centre crystals, but larger dichroic ratios might be expected for a crystalline system with a higher degree of order than the oriented complexes. Apart from this difference the linear dichroism measurements on single crystals

appear to be qualitatively identical to the earlier measurements on PSI complexes oriented in gels. By relating the dichroism measurements to studies on intact membranes, it was possible to propose that the long axes of carotenoid molecules and the Q_y transition of the long wavelength-absorbing chlorophylls were oriented parallel to the membrane plane (Haworth *et al.*, 1982; Tapie *et al.*, 1984).

A working model for the organization of PSI complexes within the rectangular crystals can be proposed using the simplest interpretation of the data, based on the earlier work on non-crystalline systems. The model (Figure 5) also borrows from the X-ray determined structures of the reaction centres of *Rhodopseudomonas viridis* (Deisenhofer *et al.*, 1985) and *Rhodobacter sphaeroides* (Chang *et al.*, 1986) where the hydrophobic region of the complex (located in the membrane in the absence of detergent) is sandwiched between two hydrophilic regions which are located in the aqueous phase. In our working model, hydrophilic interactions are predicted to be stronger along the long axis of the crystal whilst along the short axes, hydrophobic or detergent–detergent interactions are thought to exist. In the model, the long axes of the carotenoid molecules tend to be oriented perpendicular to the long axis of the crystal. A similar orientation for the Q_y transition of the long wavelength-absorbing chlorophyll molecules is predicted.

The working model is probably a crude oversimplification of the true three-dimensional orientation of the PSI complexes within the crystal since it is based on the stretched gel system which orients the complexes in only one direction. In addition, the present data cannot be used to distinguish between an upwards or downwards orientation of the complex within the crystal. It is nevertheless surprising that relatively strong linear dichroism can be detected within the crystals despite the high number of chromophores within the complex. Clearly a strong ordering of some of the light-harvesting molecules must exist in the reaction centre of PSI, and this feature probably has functional significance (Junge *et al.*, 1977). The model should greatly improve upon X-ray diffraction analysis of the crystals.

To date, very few membrane proteins have been crystallized and therefore it may be of interest to compare the conditions used for the crystallization of PSI reaction centres with those previously employed. Dodecyl maltoside and SDS, with saturated chains 12 carbon atoms in length are comparable to LDAO which has been used for the crystallization of other membrane proteins (Allen and Feher, 1984; Welte *et al.*, 1985). However, Triton X-100 is a large molecule in comparison with the other detergents, with a short, bulky aromatic tail with 14 carbon atoms and an extended region of polyethoxyethylene groups equivalent to ~20 carbon and 11 oxygen atoms. It is difficult to understand how Triton X-100 can be accommodated within the current models for the crystallization of membrane proteins. It seems likely that the unit cell of the PSI reaction centre crystals will be large since it must accommodate at least one 440-kd complex, and thus in comparison, Triton X-100 could be considered to be relatively small. It may also be feasible that residual LDAO, or SDS from earlier stages in the preparation could remain tightly bound to the complex and that these, rather than Triton X-100 are bound in the crystal. In general, it was found that the smaller detergents dodecyl maltoside and SDS produced larger crystals. Thus, although it is interesting that crystallization can occur in the presence of Triton X-100, the observation does not necessarily negate the current models of membrane protein crystallization. The extraction and analysis of the detergent content of large crystals should be carried out in order to clarify this problem.

Materials and methods

The culture of *P. laminosum* (strain OH-1-p.C1.1) was a kind gift from Professor M.C.W.Evans at University College London. The procedure of Stewart and Bendall (1979) was employed for the growth of cells, preparation of spheroplasts and photosynthetic membranes, and for the removal of photosystem II. PSI-enriched membrane fragments were solubilized with SDS (1% w/v) at room temperature for 5 min at a chlorophyll (Chl) concentration of 1 mg/ml. After precipitation with polyethylene glycol (PEG) 6000 (20% w/v) and centrifugation (10 000 *g*, 3 min) the pelleted material was resuspended in a small volume of Buffer 1 (20 mM morpholino ethanesulphonate, 5 mM $MgCl_2$, 15 mM NaCl, 0.2% w/v Triton X-100, pH 6.3) and loaded onto linear sucrose density gradients prepared from 0.2 M and 1 M sucrose solutions in Buffer 1. Centrifugation at 45 000 r.p.m. in a Kontron TST60 rotor for 16 h produced two dark-green bands at ~0.7 and 0.5 M sucrose. SDS–PAGE of the harvested complexes was carried out according to Laemmli (1970), except that 0.1% SDS was used in the running buffer. Preparative gradient gels (5–10% polyacrylamide w/v) were poured with 5-mm-thick spacers. A 4.5% stacking gel was used. Gels were loaded with sample equivalent to ~0.2 mg Chl/cm^2 of gel area and then run for ~5 h in a cold room. Identification of the various chlorophyll–protein complexes is described in the results (Figure 1). Protein was extracted from the gel by homogenizing gel slices in Buffer 1 with a teflon-in-glass homogenizer. Centrifugation at 10 000 *g* for 5 min allowed the removal of gel fragments and then the supernatant was concentrated by dialysis against solid sucrose. A final concentration of the sample was achieved by PEG-precipitation as described above. The protein was resuspended in a small volume of Buffer 2 (50 mM Trishydroxymethylaminomethane(Tris)–HCl, 20 mM $MgCl_2$, 0.2% sodium azide, pH 8.2) and the detergent required for crystallization was added (if Triton X-100, 0.1%; if SDS, 0.05%; if dodecyl maltoside, 0.125%). The PEG-precipitation was again carried out and this resuspension–precipitation washing procedure was repeated four times.

Crystals were grown by rapid vapour diffusion from a saturated protein solution in PEG 6000. The starting concentration of PEG varied from 4 to 10% (w/v) depending on the protein concentration and detergent used. Specific examples are given in the Results and Discussion. Small volumes (100–400 μl) of sample were added to 0.5-ml Eppendorf tubes with the top removed and then placed inside a 2-ml Eppendorf tube containing 0.1 g of $MgCl_2$. The tube was sealed and then left in the dark at room temperature for up to 10 days. The rate of vapour diffusion could be reduced by covering the 0.5-ml Eppendorf tube with parafilm in which a pinhole was made. Crystals were mounted on a quartz microscope slide and were kept hydrated by a solution of 25% (w/v) PEG in Buffer 2. Spectra were recorded with a single-beam Zeiss microspectrophotometer with an Ultrafluar UF32 objective lens and stored in a Hewlett-Packard 9845B microcomputer. Fitting of linear dichroism measurements was performed by a non-linear regression program written for this purpose by one of us (D.P.).

SDS–PAGE of denatured polypeptides was carried out as above except that the gels were run at room temperature. Complexes were completely denatured by incubation for 10 min at 70°C in a buffer containing 90 mM Tris (HCl) pH 6.8, 6% (v/v) 2-mercaptoethanol, 5% (w/v) SDS, 12% (v/v) glycerol. Molecular weights were estimated with the following marker proteins: ferritin (440 kd), catalase (230 kd), lactate dehydrogenase (140, 35 kd), bovine serum albumin (67 kd), trypsin inhibitor (20 kd), cytochrome *c* (12.5 kd).

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