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Monomeric and trimeric forms of photosystem I reaction center of *Mastigocladus laminosus*: Crystallization and preliminary characterization

(photosynthesis/cyanobacteria/diffraction/crystal structure)

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ABSTRACT **Photosystem I (PSI) reaction centers (RCs) of the thermophilic cyanobacterium *Mastigocladus laminosus* were purified and characterized. The PSI RC was obtained in two forms, monomeric and trimeric. The two forms contained the same number of pigments per P_{700} and displayed similar photochemical activities. The two forms had nearly identical polypeptide subunit compositions; the only observed difference was an additional subunit of about 12 kDa observed in the trimeric form. The purified preparations of both the monomeric and the trimeric forms were used for crystallization and preliminary crystallographic analysis. The trimeric PSI RC preparations produced several three-dimensional crystal forms, one of which, the "hexagonal needle" form (T_{HN}), had a hexagonal unit cell with dimensions of 300 × 300 × 160 Å, containing four PSI RC trimers. The monomeric preparations also produced single crystals of several forms under various crystallization conditions. One of these crystal forms, the "hexagonal plate" (M_{HP}), diffracted to a resolution of about 5.5 Å. It had a hexagonal unit cell with dimensions of 192 × 192 × 163 Å, containing six PSI RC monomers. Comparison of the PSI RCs in the crystals with those in the precrystallization preparations demonstrated that neither the monomeric nor the trimeric form of PSI RC was altered by the crystallization process. Both forms retained their original polypeptide subunit composition and their pigment content.**

Photosystem I (PSI) plays a major role in the oxygenic photosynthetic process. Its function in the reducing site of the electron transfer chain enables the reduction of ferredoxin and eventually the reduction of NADP.

The primary photochemical event of charge separation takes place in the core complex of PSI (CCI). The CCI complex (also designated the PSI reaction center, PSI RC) is composed of two homologous subunits of about 82 kDa, Ia and Ib, and at least nine other polypeptide subunits ranging from 1.5 to 25 kDa and designated as subunits II–X. Subunits Ia, Ib, II, . . . , X are encoded by the *psaA, psaB, psaC, . . . , psaK* genes (1, 2). Subunits Ia and Ib (the *psaA* and *psaB* gene products) contain the reaction center, P_{700}, all the pigments associated with the complex (chlorophyll *a* and β-carotene molecules), the primary electron acceptor A_0 (chlorophyll *a*), and the secondary acceptors A_1 (phylloquinone) and A_2 (the Fe-S center Fx) (2, 3). The two other electron acceptors, A_3 and A_4 (the Fe-S centers Fa and Fb), are associated with subunit VII, an 8-kDa polypeptide encoded by the *psaC* gene (3).

The recent isolation of all the genes coding for the different subunits of PSI RC enabled the determination of the protein sequences (1). Knowledge of the primary structure of the individual polypeptides enabled one to predict the major secondary structure elements, the function of different parts of the polypeptide chains, and the spatial arrangement of the subunits with respect to each other and the lipid bilayer (2).

However, only a detailed, high-resolution, three-dimensional structure of the PSI RC complex can provide details of the structure, function, and mechanism of action of this complex. The most direct way to get an accurate three-dimensional structure of macromolecules is x-ray crystallography. Crystallographic studies of the bacterial RC have led to the determination of the three-dimensional structure of the RCs of *Rhodopseudomonas viridis* (4, 5) and *Rhodobacter sphaeroides* (6–10). These high-resolution structural analyses enabled the determination of the spatial arrangement of the chromophores within the protein backbone and the arrangement of the entire complex within the photosynthetic membrane.

Subsequently, other photosynthetic complexes have been crystallized and studied. Among these are PSI RCs from cyanobacteria (11–14). Cyanobacterial PSI RCs have been shown to be analogous to those of higher plants with respect to pigment content, photochemical functions, and immunological crossreactivity between some of their subunits and the analogous subunits of higher plants (15). The genes coding for some of the polypeptide subunits of cyanobacterial PSI RCs have been cloned and sequenced. The sequences confirmed the close relationships between these subunits and the analogous subunits of PSI RC in higher plants (16–18).

The laboratories of Witt (13, 14) and Ford (11, 12) reported three-dimensional crystals of PSI preparations purified from *Synechococcus* sp. and *Phormidium laminosum*, respectively. They obtained diffraction data (12, 13); Witt *et al.* (14) reported x-ray diffraction at a resolution of about 4 Å from their trimeric PSI RC crystals.

Here we describe the crystallization of the PSI RC of the thermophilic cyanobacterium *Mastigocladus laminosus*. In a previous report we presented preliminary results on the crystallization and characterization of the trimeric form of this complex (19). Now we demonstrate that the *M. laminosus* PSI RC can be isolated and crystallized also in its monomeric form. We have characterized both the monomeric and trimeric forms in their crystalline and solubilized states.

MATERIALS AND METHODS

Analytical Methods. Chlorophyll concentration was determined spectroscopically at 663 nm (20) and protein concentration was determined with the standard Bio-Rad protein assay kit. Gel electrophoresis of the fully denatured complex was carried out in slab gels containing a gradient of 12–20% acrylamide and 4 M urea (21). Gel electrophoresis of the nondenatured complex was used to analyze the fully assembled (i.e., pigmented) PSI RC complexes (22).

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Abbreviations: PSI, photosystem I; RC, reaction center.

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Isolation of PSI RC. *M. laminosus* cells were grown at 55°C, harvested, and broken by a French pressure cell as described (15, 19). The thylakoid membranes were isolated and the PSI RCs were purified from the thylakoids (15, 19). The purification included three main steps: extraction of the PSI RCs from the thylakoids by Triton X-100 or dodecyl β-D-maltoside, separation of the extracted material by ion-exchange chromatography, and further purification of the relevant fractions by sucrose gradient centrifugation (15, 19). In the last step of purification the monomers were separated from the trimers. When the sucrose gradient contained 0.1% dodecyl β-D-maltoside, the monomeric and the trimeric forms of PSI RC were recovered at about 12% and 18% sucrose, respectively.

The purity of the monomeric and trimeric forms was analyzed by SDS/PAGE, by immunoblotting, and by determination of the chlorophyll/P_{700} ratio. Preparations exhibiting the characteristics typical of PSI RC (2, 15) were used for the crystallization experiments.

Crystallization. We used the general approaches developed by Michel and Oesterhelt (23–25) and Garavito and Rosenbusch (26, 27) for the crystallization of membrane proteins. Crystallization experiments were carried out mainly by the "sitting drop" technique, on a vinyl plate with a 5 × 5 array of V-shaped wells (Costar catalogue no. 2897). A drop of about 15 μl, containing the protein complex, buffer solution, detergent, electrolyte, and a precipitating agent, was placed in each of the wells and allowed to equilibrate (by vapor diffusion) with a reservoir of about 1 ml containing a similar (but more concentrated) proteinless buffer solution (28). All the crystal forms of both the PSI RC monomer and the PSI RC trimer that were found suitable for crystallographic analysis were obtained in protein concentrations of 5–15 mg/ml with dodecyl β-D-maltoside as detergent (0.03–0.1%, wt/vol); with $MgSO_4$ as electrolyte (25–100 mM); with Mes as buffer (8–25 mM, pH 5.5–8.5); and with polyethylene glycol (PEG 6000; 1–5%, wt/wt) as the precipitating agent. The hexagonal crystals of the PSI RC monomer (the M_{HP} form) were obtained when the mother liquor contained protein at 10.5 mg/ml, 0.03% dodecyl β-D-maltoside, 25 mM $MgSO_4$, and 3.25% PEG 6000 at pH 6.5, while the reservoir solution contained twice the concentration of each of the nonprotein components. These crystals were used for the crystallographic analysis described below.

RESULTS AND DISCUSSION

The stability of cyanobacterial PSI RCs and their homology with the complexes of higher plants made them attractive candidates for structural studies, especially x-ray crystallography, that require a high degree of stability. Macromolecules purified from thermophiles are much more stable than the corresponding macromolecules purified from nonthermophiles (29). Since the PSI RC purified from the thermophilic cyanobacterium *M. laminosus* is fully active at 60–70°C (15), we decided to focus our structural study on this RC.

Purification and Biochemical Characterization. In the course of purifying the *M. laminosus* PSI RC with mild detergent (Fig. 1), we found the purified complex to exist in both a monomeric and a trimeric form. The monomeric form (M) sediments more slowly in the sucrose gradient than the trimeric form (T) (Fig. 1*a*). This difference in sedimentation results from the smaller size of the monomeric complex, which migrates further in nondenaturing gels (Fig. 1*b*). During electrophoresis some of the trimers dissociate into monomers.

The existence of a trimeric form of PSI RC in the thermophilic cyanobacterium *M. laminosus* correlates with similar results obtained from other thermophilic cyanobacteria: *P. laminosum* (30, 31), and *Synechococcus* sp. (32, 33). In these

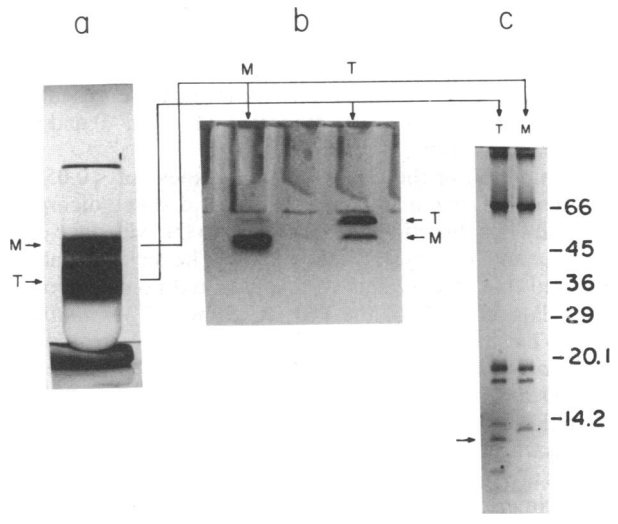

FIG. 1. Separation of the monomeric (M) and trimeric (T) forms of PSI RC of *M. laminosus*. The last step of the purification of the PSI RC complex was sedimentation in a sucrose density gradient (*a*). The two fractions obtained were analyzed in nondenaturing gels (*b*) and fully denaturing gels (*c*). The nondenaturing gel system used was the Deriphat gel (see ref. 22). The fully denaturing gels had a 12–20% acrylamide gradient and contained 4 M urea; molecular mass (kDa) markers are at right.

two thermophilic cyanobacteria, purification with mild detergent resulted in the trimeric form. To obtain smaller aggregates, treatment with a strong detergent such as SDS was required (34). This was recently demonstrated elegantly by Rögner *et al.* (35), who showed that in *Synechococcus* sp., the isolated PSI RC trimers could be disaggregated into both dimers and monomers. However, the smaller forms of the PSI RC obtained in this way (mainly the dimers) were less stable than the trimeric form (35).

Our finding that purification of the *M. laminosus* PSI RC with mild detergent resulted in both the monomeric and the trimeric form of the complex is more in accord with the results reported recently for the mesophilic cyanobacterium *Synechocystis* PCC 6803, in which treatment with mild detergent resulted in the purification of both trimers and monomers (36). The weight ratio of monomers to trimers was found to be about 1:2 for *M. laminosus* (Fig. 1*a*).

We conclude that under mild detergent conditions (up to 0.1% dodecyl β-D-maltoside), the monomeric and trimeric forms of the PSI RC of *M. laminosus* coexist. Examination of a solution of the PSI RC trimer (containing ≤0.1% dodecyl β-D-maltoside) a few days (at 4°C) after the separation and purification indicated partial disaggregation of the PSI RC trimers into monomers. However, attempts to obtain PSI RC trimers from purified monomers were unsuccessful even after a few weeks.

When the two purified forms of *M. laminosus* PSI RC were analyzed in a fully denaturing gel (Fig. 1*c*), they were found to have almost identical polypeptide compositions. Under electrophoretic conditions that provided a better resolution in the region of 8–25 kDa, an additional polypeptide subunit, with an apparent molecular mass of about 12 kDa, was observed in the trimeric but not in the monomeric form. This additional subunit (see arrow in Fig. 1*c*), which has not been reported previously, may function as the linker that assembles the monomers into trimers.

Crystallization. After purifying and separating the two homogeneous fractions containing the monomeric and trimeric forms, we set up identical crystallization conditions for both of them in parallel. Microcrystals of both forms were detected within 1–2 days; fully grown crystals were obtained

in 1–2 weeks. For both the monomeric and trimeric forms several different crystal forms were obtained; they varied in morphology, size, and space group, depending on the exact crystallization conditions. Fig. 2 shows three representative crystal forms of the PSI RC monomeric form (*a–c*) and three of the PSI RC trimeric form (*d–f*).

Examination of the crystals (for thickness of <0.05 mm) with a polarizing microscope showed distinct dichroism, especially in a direction perpendicular to the wide face of the M_C, T_{SP}, and T_{TP} crystals (see Fig. 2). The dichroic patterns indicated that the crystals were single and homogeneous.

The T_{HN} crystals of the PSI RC trimer of *M. laminosus* seem to be similar to the crystals of the PSI RC isolated from *Synechococcus* sp. (13, 14) in the overall shape, size, and space group. The T_{SP} crystals of our PSI RC trimer seem to be similar to one of the crystal forms of the PSI RC of *P. laminosum* (11, 12). It should be noted that these similar crystals are from thermophilic cyanobacteria and they are all of the trimeric form of the PSI RC. In contrast to the trimeric PSI RC crystals, no monomeric PSI RC crystals have so far been reported.

A preliminary biochemical characterization was carried out to examine whether crystallization affected the pigment content of the monomeric and trimeric forms of PSI RC. Nondenaturing gel electrophoresis indicated that the pigments are bound to the PSI RC complex in the crystal in the same way as they are bound to the solubilized complex (Fig. 3, lanes C vs. lanes S). Hence, we conclude that for both forms the content of pigments and the composition of polypeptide subunits (data not shown) have not been altered by

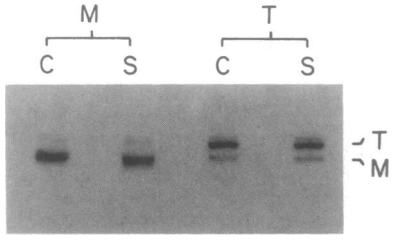

FIG. 3. Nondenaturing electrophoresis of monomeric (M) and trimeric (T) forms of the PSI RC in solution (lanes S) and in the crystal (lanes C).

crystallization. Moreover, the nondenaturing gels indicate that the form of aggregation (monomer or trimer) has not been altered during crystallization.

Crystallographic Analysis. It is not known whether the monomeric or the trimeric form of the PSI RC of *M. laminosus* is the active form *in vivo*, nor is the functional advantage or significance of the trimerization clear. Furthermore, the two forms remain essentially unchanged during crystallization. Consequently we decided to perform a crystallographic analysis on both forms.

Two of the crystal forms described above showed reasonable diffraction patterns. These are the "hexagonal plate" crystals of the monomeric form (M_{HP}; Fig. 2*b*) and the "hexagonal needle" crystals of the trimeric form (T_{HN}; Fig. 2*d*). Preliminary analysis of the x-ray diffraction patterns indicated that both the M_{HP} crystals and the T_{HN} crystals belong to a hexagonal crystal system, with a unit cell of a =

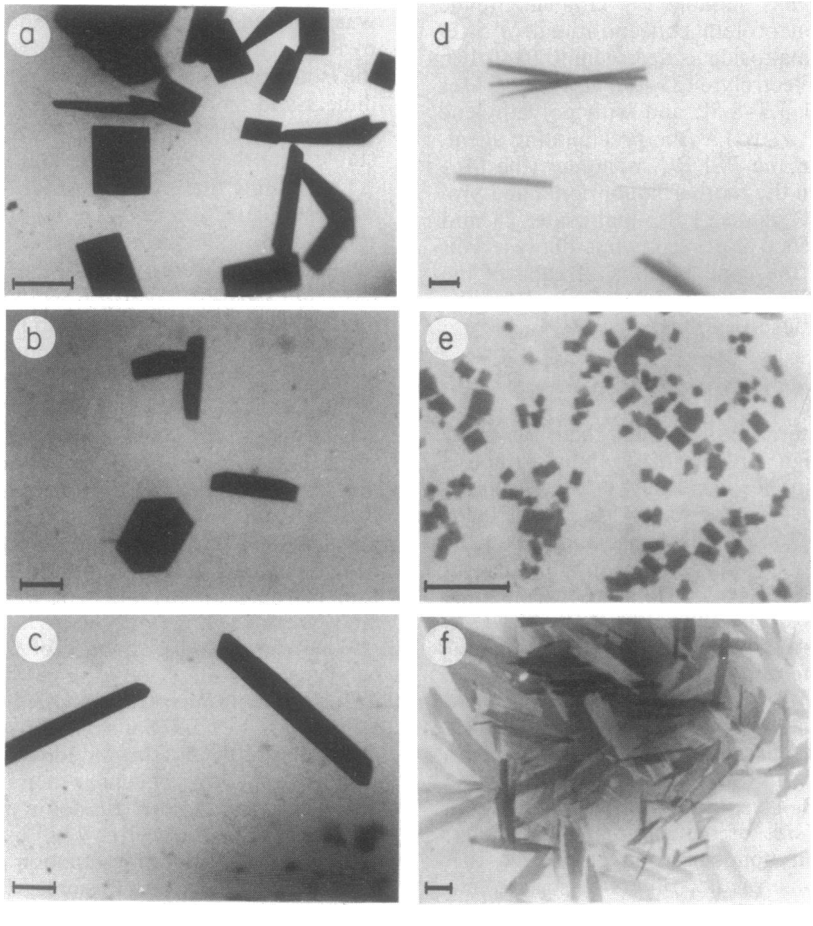

FIG. 2. Representative crystal forms of the monomeric form (*a–c*) and the trimeric form (*d–f*) of the PSI RC of *M. laminosus*. All crystal forms presented were obtained by the sitting-drop method, using dodecyl β-D-maltoside as detergent and PEG 6000 as precipitant (see details in the text). (*a*) The "coffin" form (M_C). (*b*) The "hexagonal plate" form (M_{HP}). (*c*) The "rod-like" form (M_{RL}). (*d*) The "hexagonal needle" form (T_{HN}). (*e*) The "square plate" form (T_{SP}). (*f*) The "thin plate" form (T_{TP}). (Bars = 0.2 mm.)

$b = 192 \pm 3$ Å and $c = 163 \pm 4$ Å for M_{HP}, and a unit cell of $a = b = 300 \pm 10$ Å and $c = 160 \pm 8$ Å for T_{HN}. The unique hexagonal c axis is perpendicular to the face of the hexagonal plates of the M_{HP} crystals and is parallel to the needle axis of the T_{HN} crystals.

The hexagonal needle crystals of the PSI RC trimer (T_{HN}) have thus far proven to be the best trimer crystals for crystallographic analysis. From their diffraction pattern (extending often to 7-Å resolution) we concluded that the most probable space group is $P6_322$. This space group is identical, and the unit cell dimensions are very similar (300 × 300 × 160 Å vs. 285 × 285 × 167 Å), to those reported for the crystals of the trimeric PSI RC complex of *Synechococcus* (14). We therefore conclude that the T_{HN} crystals of the PSI RC trimer of *M. laminosus* are similar to the hexagonal crystals of the PSI RC trimer of *Synechococcus* sp.

Since the monomeric M_{HP} crystals have considerably smaller unit cell dimensions, they offer advantages in both data collection and structure determination. Some of the thick and well-formed M_{HP} crystals of the PSI RC monomer, which later could be recognized and selected, showed a well-defined and reproducible diffraction pattern that extended to about 5.5-Å resolution (Fig. 4). The reflections produced sharp and round spots, and the crystals lasted for at least 20 hr in the x-ray beam before a noticeable deterioration of the diffraction pattern occurred. Several such diffraction patterns were recorded (only partial data were measured) on x-ray film and on a Xentronics area detector, from which it was concluded that the most probable space group of these crystals is $P6_3$. The packing of the PSI RC monomers in this hexagonal unit cell is shown in Fig. 5. Each unit cell contains six PSI RC monomers (as proved below by V_M calculations). Since in such a unit cell there are both threefold rotation axes (▲) and 2_1 screw axes ( ), there is only one PSI RC monomer per asymmetric unit.

In this unit cell, three monomers are arranged in the *ab* plane around the threefold axis and are related to the other three monomers of the unit cell by a symmetry operation of a 2_1 screw axis with a half-unit-cell translation along the c axis. As a result of these symmetry operations a "hexamer" of packed monomers is formed around the 6_3 screw axis, which is located at the corners of the unit cell. The hexamer is composed of two staggered trimers (formed from monomers) that are packed as a result of the crystallographic symmetry in a "head-to-tail" mode relative to each other. The head and tail, we believe, could correspond to the two sides of the PSI complex that stick out of the photosynthetic membrane. This kind of layered monomer packing may provide information about the similarities and polarities of the two sides of the PSI complex, as well as the possible packing of the PSI particles within the photosynthetic membranes. It should be noted, however, that the proposed packing of the trimeric form of the PSI RC particles of *M. laminosus* and *Synechococcus* sp. (14) is significantly different from that of the monomeric form. In both cases the trimers are packed in a head-to-head mode relative to each other.

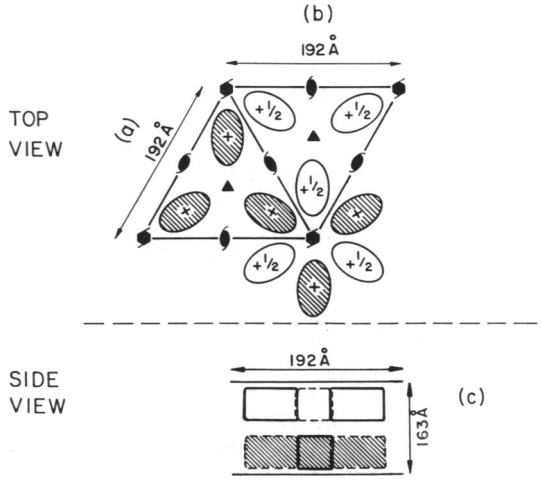

FIG. 5. Crystal packing of the PSI RC monomer in unit cell of the $P6_3$ space group. Each monomer is represented by an ellipsoid; shaded (+) is above the *ab* plane; clear (+½) is displaced by ½ of the unit-cell c axis from the shaded plane along the c axis; , 2_1 screw axis; ▲, threefold axis; , 6_3 screw axis.

To estimate the number of monomers per asymmetric unit, we calculated V_M, the crystal volume per unit molecular mass (protein plus detergent) (37, 38). The total volume of the $P6_3$ unit cell is 5.2×10^6 Å^3. Assuming a total molecular mass of about 250 kDa for the PSI RC monomer, and taking into account the amount of detergent attached to the protein [about 0.6 g of detergent per gram of protein (39)], one obtains

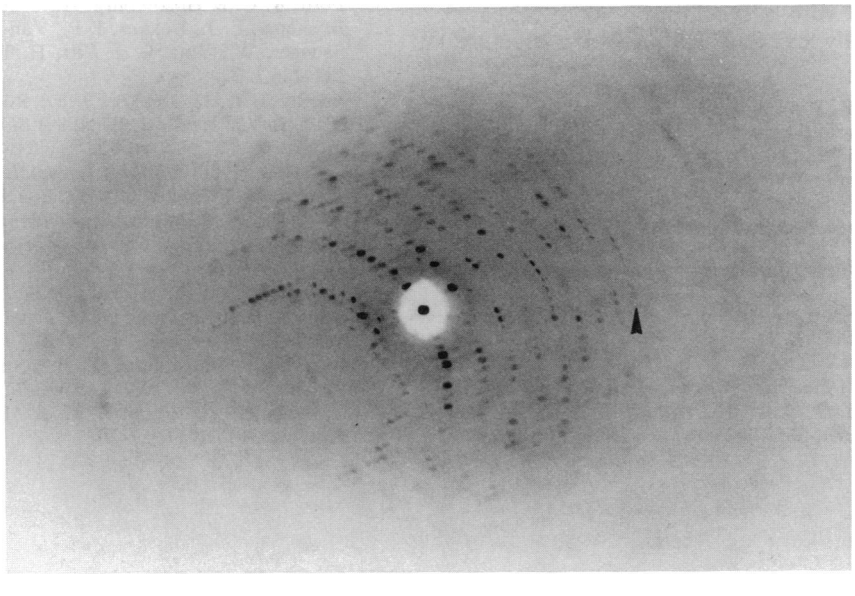

FIG. 4. Representative x-ray diffraction pattern obtained from crystals of the monomeric form (M_{HP}, Fig. 2*b*) of the PSI RC. The diffraction photograph was obtained using a rotating anode source at 40 kV and 240 mA and a 0.5-mm collimator. Exposure time was 8 hr. The diffraction pattern shows a resolution limit of about 5.5 Å (see arrowhead).

for one PSI RC monomer per asymmetric unit a V_M value of 2.17 Å^3/dalton. This value is within the range reported for other integral membrane proteins [for example, *Rb. sphaeroides* RC, 2.1 Å^3/dalton (38), and matrix porin, 2.9 Å^3/dalton (39)]. It is also in good agreement with the V_M values obtained for water-soluble proteins [most commonly observed value, 2.15 (37)]. In an alternative procedure one can ignore the detergent and solvent and calculate V_S, the specific volume of protein alone in the crystal, which gives for our $P6_3$ unit cell a value of 3.5 Å^3/dalton. This value is also within the range of reported values for membrane proteins [for example, 3.4 for the *P*2 form of *Rb. sphaeroides* RC (38) and 4.2 for the $P2_12_12_1$ form of *Rb. sphaeroides* RC (6)]. These values support our conclusion that the $P6_3$ unit cell of the monomeric form of *M. laminosus* PSI RC contains six monomers and has one monomer per asymmetric unit.

The volume available for each PSI RC monomer in the $P6_3$ unit cell is 8.7×10^5 Å^3, in fair agreement with the dimensions deduced from electron microscopy of PSI RC monomers of both *Synechococcus* sp. (33, 35) and spinach (40). We believe that the 5.5-Å diffraction data currently collected on the monomeric $P6_3$ crystals will enable us to determine not only the shape and dimensions of the PSI RC complex but also the spatial arrangement of the protein subunits and the various pigments within the complex.

SUMMARY AND CONCLUSIONS

We have characterized several forms of *M. laminosus* PSI RC crystals. Previously we showed that the crystalline complexes contain all the original components (both protein and pigments), that these components have not been altered by crystallization, and that the photochemical activity is retained in the crystals (19). Here we have demonstrated that the PSI RC can be isolated and crystallized in both monomeric and trimeric forms. Biochemical and biophysical analyses have shown that the two forms are essentially identical with respect to their photochemical activity. Of the several crystal forms, the two hexagonal forms (monomer and trimer) seem to be the most suitable for a detailed crystallographic analysis. The hexagonal form of the monomer (a $P6_3$ unit cell of 192 × 192 × 163 Å) is especially promising since its asymmetric unit contains only a single PSI monomeric complex and it is rather stable in the x-ray beam. Its crystals diffract to at least 5.5 Å with a rotating anode source; a higher resolution is expected with a synchrotron source. These studies should eventually lead to a high-resolution structure of the native RC.

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