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Isolation and characterisation of a D1/D2/cytochrome *b*-559 complex from *Synechocystis* 6803

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A D1/D2/cytochrome *b*-559 complex has been isolated from the cyanobacterium *Synechocystis* 6803. The isolated complex shows reversible absorbance changes due to the photochemical accumulation of reduced pheophytin. Photoaccumulation of a chlorophyll radical, assumed to be the oxidised primary donor chlorophyll (P-680$^+$), can also be observed in the presence of the exogenous electron acceptor, silicomolybdate. Sodium dodecylsulphate urea polyacrylamide gel electrophoresis resolved three polypeptides of apparent molecular masses 50, 38 and 34 kDa. Spectrophotometric measurements demonstrated the presence of cytochrome *b*-559 in the preparation. The isolated complex seems to contain approximately 8 chlorophyll *a*, 1 pheophytin *a*, 1 β-carotene and 1 cytochrome *b*-559 haem as a molar ratio.

Introduction

The sequencing of the genes encoding the L and M subunits of purple bacterial reaction centres [1–4] coupled with X-ray crystallography of these proteins [5,6] indicated that the D1 and D2 polypeptides form similar subunits in the reaction centre of Photosystem II. This homology was first advocated by Michel and Deisenhofer [7] and by Trebst [8]. Their predictions were reinforced by the isolation of a D1/D2/cytochrome *b*-559 complex which showed properties indicative of the PS II reaction centre [10–13]. This complex was isolated from the chloroplast thylakoids of spinach [9,13] and peas [12,14] and was shown also to contain cytochrome *b*-559.

The isolation and characterisation of the D1/D2/cytochrome *b*-559 complex of higher plants is an important step forward in the elucidation of the molecular mechanisms of PS II and the associated water-splitting reactions. As yet, however, there have been no reports that such a complex can be isolated from cyanobacteria. Despite this, cyanobacteria offer a convenient system for mutagenesis experiments which can help to unravel the details of PS II functioning. An organism which has great potential for this purpose, particularly in the study of PS II, is *Synechocystis* sp PCC 6803. This organism is readily transformable and can grow photoheterotrophically on glucose. Indeed it has already proven to be an excellent system for site-specific mutagenesis experiments and has been used to show that the component, known as D, which gives rise to long-lived EPR Signal II at $g = 2.0046$ is a tyrosine radical at position 161 on the D2 polypeptide [15,16]. To continue to benefit from the application of molecular biological techniques using *Synechocystis* 6803 it is important to build up skills in the isolation and characterisation of functionally active PS II units from this organism. In this communication we report the isolation and characterisation of a photochemically active PS II reaction centre.

Materials and Methods

A glucose-tolerant strain of *Synechocystis* 6803 was grown at 30 °C in BG-11 medium [17] supplemented with 5 mM glucose. Cultures were perfused with air passing through 0.2 μm filters. The cells were harvested in the late-log phase by concentration through a Millipore Pellicon transmembrane tangential flow cell and by subsequent centrifugation at $8000 \times g$. Cells were then frozen in liquid N_2 and stored at −80 °C. For isolation of the membranes, the cells were suspended in

Abbreviations: PS II, Photosystem II; PS I, Photosystem I; Chl, chlorophyll; Mes, 4-morpholineethanesulphonic acid.

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50 mM Mes (pH 6.5), 10 mM $MgCl_2$, 1 mM benzamidine and 1 mM ε-amino caproic acid, and broken using a French Press. The cells were passed through the press twice at a pressure of 138 MPa. The suspension was centrifuged at $1000 \times g$ for 5 min and the pellet was discarded. The supernatant was diluted 2-fold with medium containing 50 mM Mes (pH 6.5), 1 mM benzamidine, 1 mM ε-amino caproic acid (buffer A) containing 20 mM sodium pyrophosphate and centrifuged at $100000 \times g$ for 60 min. The pellet was resuspended in buffer A containing 10 mM sodium pyrophosphate and centrifuged again as above. Through this procedure the majority of phycobilisomes were removed from the membranes. The resultant pellet was suspended in buffer A, frozen in liquid N_2 and stored at $-80\,^{\circ}C$ for further use.

For isolation of PS II reaction centres, the membranes were diluted to a concentration of 0.3 mg Chl per ml with buffer A containing 6 mM β-lauryl maltoside. The solution was kept on ice for 60 min in the dark and then centrifuged at $100000 \times g$ for 60 min. The pellet was discarded, the supernatant was then diluted to 0.1 mg Chl per ml with buffer A containing 4.5% (w/v) Triton X-100 (specially purified for membrane research from Boehringer Mannheim), and further incubated on ice, in the dark for 45 min. This solution was applied to a column (9×120 mm) of Fractogel TSK DEAE-650(S) (Merck-BDH) maintained at 4 °C and equilibrated with buffer A containing 0.1% (w/v) Triton X-100 (running buffer). Application at 1 ml per min was followed by extensive washing at 0.5 ml per min with running buffer. The column was then washed with running buffer containing 50 mM NaCl. At this salt concentration, Photosystem I contained in the sample was eluted from the column. When the absorbance (at 450 nm) of the eluant returned to the same level as before the salt wash, the column was further washed with about 3 × column volume of running buffer containing 75 mM NaCl and the eluant collected. When continued washing with 75 mM NaCl in running buffer gave an absorbance equal to that of the eluant before the salt wash, a NaCl gradient in running buffer (75–400 mM) was applied at 2 mM NaCl per ml and a flow rate of 0.5 ml per min. Fractions eluted at about 120 mM NaCl were pooled after being tested for cytochrome *b*-559 content. The cytochrome-enriched fractions were diluted 4-fold in running buffer and reapplied onto a column (9×90 mm) as above. After washing with running buffer containing 75 mM NaCl the PS II reaction centre was eluted with a 75–300 mM NaCl gradient in running buffer, with other conditions as described above. The reaction centre sample was eluted at about 120 mM NaCl.

SDS-polyacrylamide gel electrophoresis was performed on slab gels using 6% acrylamide stacking gel and 10–17% gradient resolving gel containing 6 M urea and run at 12 °C. Samples were solubilised according to Laemmli [18] and were incubated at 100 °C for 3 min.

Immunoblotting using antibodies raised against *psb*A (D1), *psb*D (D2) [19] and *psb*C (CP43) gene products as well as with a monoclonal antibody raised against the CP47 polypeptide was carried out as described in Ref. 20. Pigment analyses and plastoquinone-9 (PQ-9) estimations were carried out using reverse-phase high-performance liquid chromatography (HPLC) on a column of Spherisorb ODS using a gradient of 56 : 19 : 25 (v/v/v) acetonitrile/methanol/water to 75 : 25 (v/v) acetonitrile/methanol in 20 min at 1.5 ml per min and at 30 °C. Quantification was carried out by spectrophotometric assays in an 8 μl flow cell at 255 nm and 450 nm for PQ-9 and β-carotene, respectively, and at 663 nm for chlorophyll *a* and pheophytin *a* and comparison with standard curves for each compound.

Chlorophyll *a*-to-pheophytin *a* ratios were further checked by pheophytinisation of chlorophyll *a* by acidification (1 nM HCl). Cytochrome *b*-559 content was estimated spectrophotometrically by the reduced (dithionite or ascorbate) minus oxidised (ferricyanide) spectrum at 559–570 nm according to Ref. 20.

Absorption and emission spectra were measured using a Perkin-Elmer UV-visible spectrophotometer and a Perkin-Elmer MPF 44A fluorimeter, respectively. Light-induced absorbance changes were recorded with a Perkin-Elmer dual-beam spectrophotometer with side illumination of a 1 cm pathlength sample cuvette from a quartz-iodine source equipped with light guides and transmission filters (Calflex heat filter and 2 mm Schott RG660 cut-off filter). The intensity of this red actinic light at the cuvette surface was 300 $\mu E \cdot m^{-2} \cdot s^{-1}$. To avoid artefacts due to scattering, the photomultiplier was shielded by a 4 mm Schott BG 38 broad pass filter. Light-induced measurements were carried out on samples kept at 4 °C.

Results

In order to isolate the PS II reaction centre complex from the cyanobacterium *Synechocystis* 6803 we employed a procedure utilising a mixed non-ionic detergent system for solubilisation of the membranes and anion-exchange column chromatography for the separation and purification of the protein complexes. The purification of PS II was monitored at all stages by spectrophotometric determinations of the cytochrome *b*-559 content relative to chlorophyll *a*. The supernatant fraction obtained after the solubilisation of the thylakoid membranes with β-lauryl maltoside typically contained 60–70 chlorophyll *a* molecules per cytochrome *b*-559 haem. The polypeptide composition of this fraction is shown in Fig. 1 (lane a) which indicates that components of both PS II and PS I were present. Further

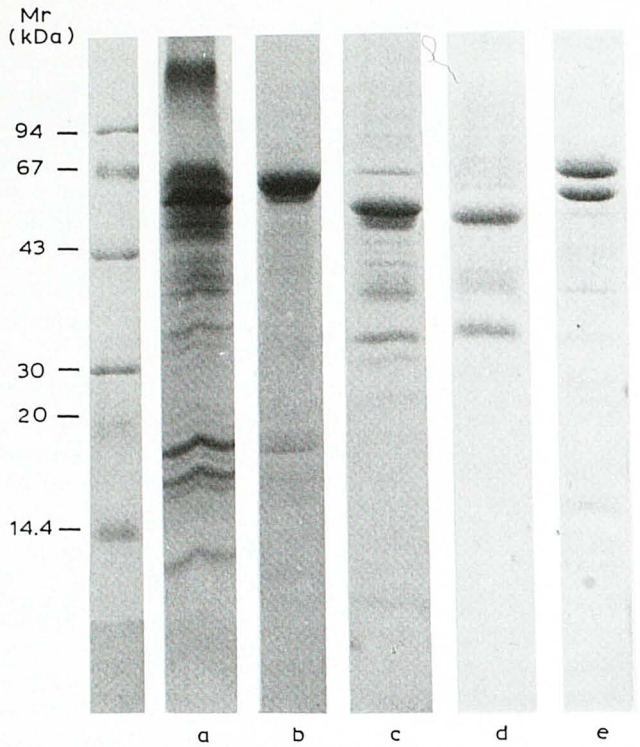

Fig. 1. SDS-PAGE of the total β-lauryl maltoside extract (a); PS I eluted at 50 mM NaCl (b); PS II complex from the first chromatographic elution (c); PS II complex resulting from two chromatographic steps (d); and material eluted at NaCl concentrations above 200 mM (e). Molecular mass markers are shown in the first lane.

solubilisation of this material with β-laurylmaltoside or β-octylglucoside and application to the column did not result in separation of the two photosystems. Addition of Triton X-100 to the maltoside extract, however, resulted in a chromatographic elution of PS I (as judged by the presence of P-700 and the polypeptides of CP1) at an NaCl concentration of 50 mM and retention of PS II on the column. The polypeptide composition of the material eluted at 50 mM NaCl is shown in Fig. 1 (lane b). The PS II enriched fraction which eluted at about 120 mM NaCl typically contained 12–14 chlorophyll *a* per cytochrome *b*-559 haem and its polypeptide composition is shown in Fig. 1 (lane c). Application of this fraction onto a second ion-exchange column resulted in further purification as seen in Fig. 1 (lane d). This latter fraction contained 7–8 chlorophyll *a* molecules per cytochrome *b*-559 haem and was resolved in essentially three polypeptides of apparent molecular masses 50, 38 and 34 kDa. The 38 kDa band appeared very diffuse spanning approx. 3 kDa. Based on chlorophyll levels, the estimated yield of this preparation was about 0.4%. Occasionally, another fraction was obtained from the first column at salt concentrations above 200 mM and its polypeptide composition is shown in Fig. 1 (lane e). The molecular masses of the two main bands are approx. 66 and 58 kDa and are probably the α- and β-subunits of the coupling factor component CF_1.

In order to identify the polypeptides seen in Fig. 1 (lane d) we carried out Western blotting using antisera raised against the *psb*A and *psb*D gene products [19]. As shown in Fig. 2 (lane c), the 34 kDa protein band cross-reacted with the antibodies raised against *psb*A, thus identifying it as the D1 protein, while the 38 kDa protein is identified as the D2 polypeptide due to its cross-reactivity with the *psb*D antiserum. The 50 kDa protein cross-reacted with both types of antisera. It therefore appears that this latter band is an aggregated form of both D1 and D2 polypeptides, possibly similar to the D1/D2 heterodimer band identified in reaction centre preparations of higher plants [9,12,13]. For comparison, in Fig. 2 (lane a) the polypeptide composition of the higher plant reaction centre is also shown. Western blotting using antisera against the *psb*C gene product (CP43) and a monoclonal antibody raised against CP47 showed reactivity with the cyanobacterial total extract as shown in Fig. 3. No reaction could be detected with the above antibodies when the PS II preparation was tested. It is worth noting that in the majority of cases the SDS-PAGE system used did not resolve the cytochrome *b*-559 polypeptides in the cyanobacterial preparation. Only rarely were we able to

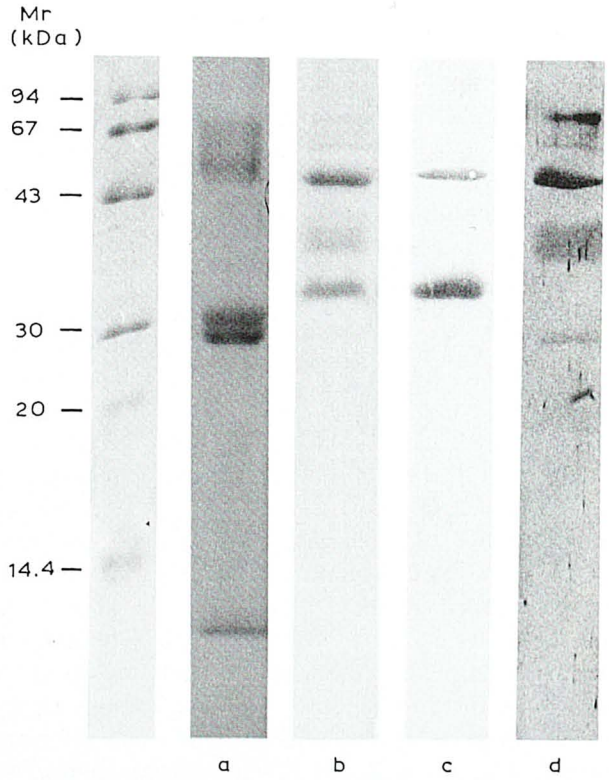

Fig. 2. SDS-PAGE of higher plant PS II reaction centre complex (isolated from peas according to Ref. 14) (a); and cyanobacterial PS II reaction centre complex (b). Similar gels as shown in (b) were subjected to immunoblotting using antibodies against the *psb*A gene product (D1) (c) and the *psb*D gene product (D2) (d). For both (a) and (b) 2 μg Chl *a* were loaded on the gel. Molecular mass markers are shown in the first lane.

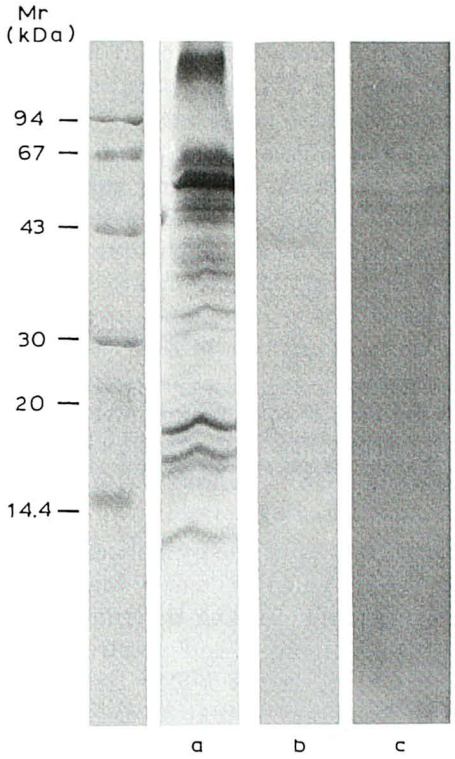

Fig. 3. SDS-PAGE of cyanobacterial total extract (a). Similar gels were subjected to immunoblotting using antibodies against the *psb*C gene product (CP43) (b) and the CP47 polypeptide (c).

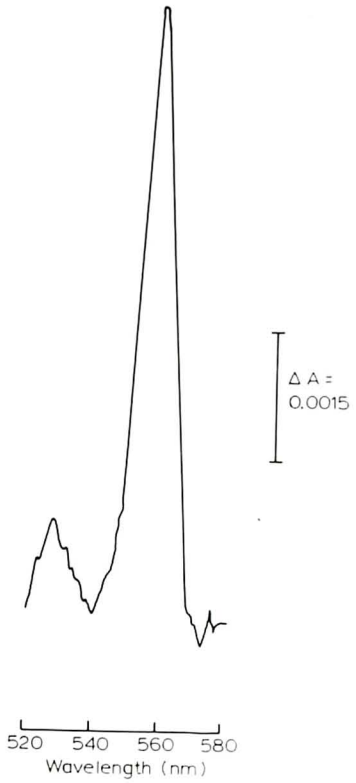

Fig. 4. Oxidised (potassium ferricyanide)-minus-reduced (sodium dithionite) spectrum of cytochrome *b*-559 in the cyanobacterial PS II preparation. The measurement was taken using samples at 3.5 μg chlorophyll *a* per ml.

observe two polypeptides with apparent molecular weights of 12 and 6 kDa, in our gel system, presumably representing the α- and β-subunits of cytochrome *b*-559.

In all preparations, however, we were able to spectrophotometrically determine cytochrome *b*-559. A typical oxidised-minus-reduced spectrum obtained with the PS II preparation is shown in Fig. 4. The room temperature absorption spectrum of this PS II complex is shown in Fig. 5. It shows both similarities and differences to that of the higher plant PS II reaction centre. The red absorption peak at 673 nm is identical to that previously reported for spinach and pea [9,12]. In the blue region, however, the peaks at 417 nm and 434 nm have about equal absorbances unlike the higher plant systems where the short-wavelength peak predominates.

Fig. 6 shows the emission spectra measured at room temperature (A) and at 77 K (B). The peaks at wavelengths of 683 nm and 685 nm are essentially identical to those obtained for the corresponding higher plant preparations [12].

When sodium dithionite and methyl viologen were added to the suspension of cyanobacterial PS II complex and actinic light applied, there were reversible absorbance changes which gave the spectrum shown in Fig. 7. This spectrum is indicative of the photo-accumulation of reduced pheophytin and therefore identifies the cyanobacterial preparation as a photoactive system similar to the higher plant complex [9,12]. Addition of silicomolybdate to higher plant PS II reaction centres has been shown to result in the formation of a chlorophyll cation, thought to be P-680$^+$, following illumination [12]. Similar absorption changes were observed in the cyanobacterial preparation with silicomolybdate present (Fig. 8). Table I summarises the data obtained

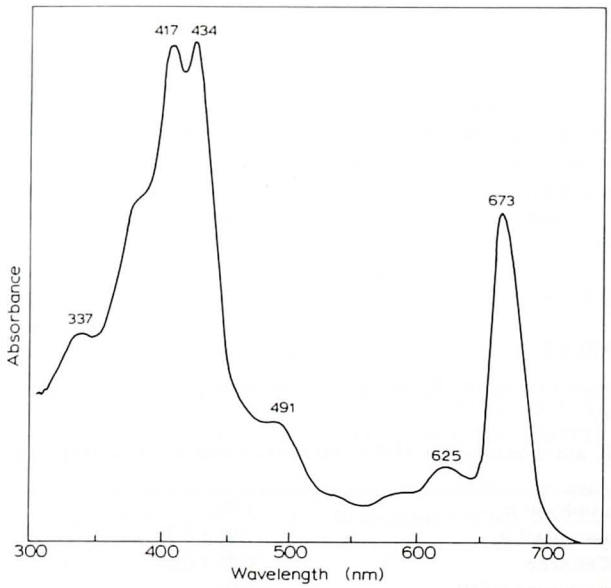

Fig. 5. Room temperature absorption spectrum of the cyanobacterial PS II reaction centre complex.

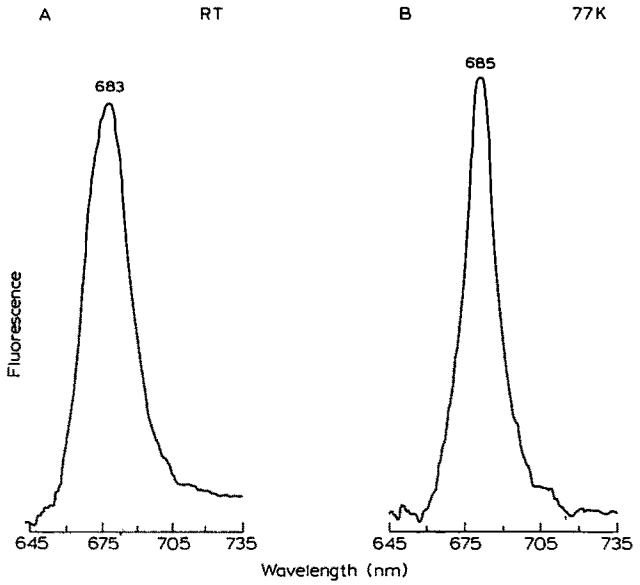

Fig. 6. Room temperature (A) and 77 K (B) fluorescence emission spectra of the PS II complex isolated from *Synechocystis 6803.*

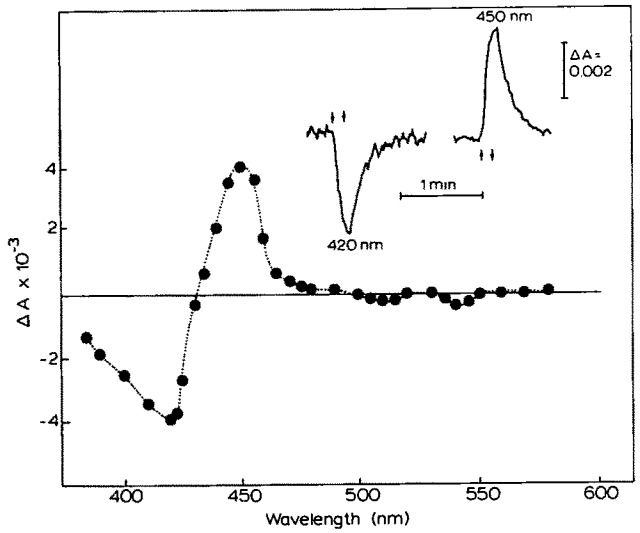

Fig. 7. Light-dark difference spectrum obtained in the presence of excess sodium dithionite plus 1 μM methyl viologen. The sample was suspended in 50 mM Tris-HCl (pH 8.0) at 3.8 μg per ml Chl and measurements were performed at 4° C. Inset: Light-induced absorption changes at two different wavelengths as indicated.

TABLE I

Composition of the PS II reaction centre complex from Synechocystis 6803

S.E. are shown for the HPLC analyses of four different preparations.

Pheophytin *a*	1.00
Chlorophyll *a*	7.97 ± 0.35
β-Carotene	0.75 ± 0.03
Cytochrome *b*-559	0.94
Plastoquinone-9	< 0.05

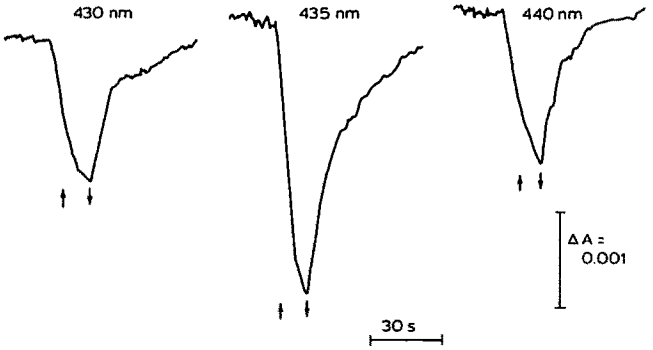

Fig. 8. Light-induced absorption changes at three different wavelengths as indicated, in the presence of 200 μg per ml silicomolybdate. The sample was suspended in 50 mM Tris-HCl (pH 8.0) at 4 μg per ml Chl and measurements were made at 4° C.

from chemical analyses of the cyanobacterial D1/D2/cytochrome *b*-559 complex using high-performance liquid chromatography for the determination of pigments and plastoquinone-9 and spectroscopy for the determination of cytochrome *b*-559. The analysis indicated that the complex contains, as a molar ratio, approx. 8 chlorophyll *a*, 1 β-carotene, 1 cytochrome *b*-559 haem and 1 pheophytin *a*. No plastoquinone-9 was detected.

Discussion

A chlorophyll *a* binding complex composed of the D1 and D2 polypeptides has been isolated from *Synechocystis* 6803. This complex did not contain significant levels of the apoproteins of CP47 and CP43 (i.e., products of the *psb*B and *psb*C genes, respectively) but does bind cytochrome *b*-559. No such preparation has been previously isolated from cyanobacteria. Immunoblotting has identified not only the D1 and D2 monomers, but also a higher molecular aggregate form of both of these polypeptides. A similar aggregate of D1 and D2 has been found in SDS-PAGE of the isolated higher plant PS II reaction centre [9,12,13]. A striking difference between the higher plant PS II reaction centre and that of the cyanobacterial system, however, is that in the latter the D1 and D2 polypeptides have higher molecular weights as judged by gel electrophoresis. In actual fact, according to gene sequences there should be very little difference between the eukaryotic and prokaryotic D1 and D2 molecular weights [22]. Also of interest is that in the case of *Synechocystis,* the D1/D2 aggregate gives a much sharper band on SDS-polyacrylamide gels compared with the higher plant PS II reaction center preparation [12,13]. Presumably, these differences result from slightly different surface properties of the proteins which dictate their interactions with the solubilizing detergent. Like the higher plant system, the D1/D2/cytochrome *b*-559 complex shows light-induced signals, indicating that it harbours the chromo-

phores which give rise to primary charge separation. On the other hand there is a distinct difference between absorption spectra of the higher plant and cyanobacterial D1/D2/cytochrome *b*-559 complexes. Chemical analyses indicate that this difference is due to more chlorophyll being present in the PS II reaction centre complex of *Synechocystis*. There is no evidence that the additional chlorophyll is due to a high level of 'free' pigment in the preparation or that there is significant contamination by other chlorophyll binding proteins such as CP43 or CP47. In our hands we routinely obtain four chlorophyll *a* molecules per cytochrome *b*-559 for the isolated higher plant D1/D2/cytochrome *b*-559 complex [12,23] while a value of eight was obtained for the *Synechocystis* preparation. Based on pheophytin, we have estimated two chlorophyll molecules per pheophytin for the PS II reaction centre isolated from pea chloroplasts [23]. In contrast, we estimate by HPLC that in the *Synechocystis* D1/D2/cytochrome *b*-559 complex there are eight chlorophylls per pheophytin. The reason for these significant differences is at present unknown. The pigment contents estimated for the higher plant systems seem to compare well with those of the related reaction centre of purple bacteria, such as *Rhodobacter sphaeroides* or *Rhodopseudomonas viridis*, which have a bacteriochlorophyll-to-bacteriophenophytin ratio of 2. It is possible, however, that in *Synechocystis* there is only one pheophytin molecule which is on the active branch and which gives rise to the photoreduced form shown in Fig. 7 and that the other expected pheophytin is replaced by a monomeric chlorophyll. If this were the case, then based on homology with the bacterial or higher plant systems, a chlorophyll-to-pheophytin ratio of 5 would be anticipated. Such a higher ratio would be more in line with our findings. On the other hand, if the cyanobacterial reaction centre contained two pheophytin molecules we would have to account for 16 chlorophyll *a* molecules per complex. This high value seems inconceivable based on the expected similarities between reaction centres of purple bacteria and Photosystem II. The argument for only one pheophytin per cyanobacterial reaction centre is also supported by the observation that the carotenoid-to-pheophytin ratio is 1 rather than 0.5 as in the higher plant and bacterial reaction centres. There is always the possibility that during the solubilisation procedures some of the pheophytin molecules are displaced.

Although we have no firm explanation at present for the high ratio of chlorophyll-to-pheophytin or cytochrome *b*-559 in the *Synechocystis* preparation, we can say with certainty that a complex composed of the D1 and D2 polypeptides, but free of CP47 and CP43, can be isolated from this cyanobacterium, showing photochemical activity indicative of the PS II reaction centre. Clearly, more work is required to refine this cyanobacterial preparation and to establish with certainty the stoichiometry of its prosthetic groups.

Acknowledgements

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References

1 Youvan, D.C., Bylina, E.J., Alberti, M., Begusch, H. and Hearst, J.E. (1984) Cell 37, 949–957.
2 Williams, J.C., Steiner, L.A., Feher, G. and Simon, M.I. (1984) Proc. Natl. Acad. Sci. USA 81, 7303–7307.
3 Williams, J.C., Steiner, L.A., Ogden, R.C., Simon, M.I. and Feher, G. (1983) Proc. Natl. Acad. Sci. USA 80, 6505–6509.
4 Michel, H., Weyer, K.A., Gruenberg, H., Dunger, I., Oesterhelt, D. and Lottspeich, F. (1986) EMBO J. 5, 1149–1158.
5 Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1985) Nature 318, 618–624.
6 Allen, J.P., Feher, G., Yeates, T.O., Komiya, H. and Rees, D.C. (1987) Proc. Natl. Acad. Sci. USA 84, 6162–6166.
7 Michel, H. and Deisenhofer, J. (1986) in: Encyclopaedia of Plant Physiology, Photosynthesis III (Staehelin, A.L. and Arntzen, C.J., eds.), Vol. 19, pp. 371–381, Springer-Verlag, Berlin.
8 Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.
9 Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
10 Okamura, M.Y., Satoh, K., Isaacson, R.A. and Feher, G. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. I, pp. 379–381, Martinus Nijhoff, Dordrecht.
11 Takahashi, Y., Hansson, Ö., Mathis, P. and Satoh, K. (1987) Biochim. Biophys. Acta 893, 49–59.
12 Barber, J., Chapman, D.J. and Telfer, A. (1987) FEBS Lett. 220, 67–73.
13 Marder, J.B., Chapman, D.J., Telfer, A., Nixon, P.J. and Barber, J. (1987) Plant Mol. Biol. 9, 325–333.
14 Chapman, D.J., Gounaris, K. and Barber, J. (1988) Biochim. Biophys. Acta 933, 423–431.
15 Debus, R.J., Barry, B.A., Babcock, G.T. and McIntosh, L. (1988) Proc. Natl. Acad. Sci. USA 85, 427–430.
16 Vermaas, W.F.J., Rutherford, A.W. and Hansson, Ö. (1988) Proc. Natl. Acad. Sci. USA, in press.
17 Allen, M. (1968) J. Phycol. 4, 1–4.
18 Laemmli, U.K. (1970) Nature 227, 680–685.
19 Nixon, P.J., Dyer, T.A., Barber, J. and Hunter, C.N. (1986) FEBS Lett. 209, 83–86.
20 Gounaris, K., Chapman, D.J. and Barber, J. (1988) FEBS Lett. 234, 374–378.
21 Wassermann, A.R. (1980) Methods Enzymol. 69, 181–202.
22 Golden, S.S., Brusslan, J. and Haselkorn, R. (1986) EMBO J. 5, 2789–2798.
23 Barber, J., Gounaris, K. and Chapman, D.J. (1987) in Cytochrome Systems: Molecular Biology and Bioenergetics (Papa, S., Chance, B. and Ernster, L., eds.), pp. 657–666, Plenum Press, New York.