A requirement for EDTA in the separation of Photosystems 1 and 2 from the cyanobacterium *Chlorogloea fritschii*

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Fractions enriched in Photosystem 1 or Photosystem 2 activity have been isolated from the cyanobacterium *Chlorogloea fritschii* after extraction of the membranes with digitonin and Triton X-100. Separation of the extract into the two components was achieved by using a Sepharose 6B column, calibration of which gave K_d values of 0.3 for the Photosystem 1 fraction and 0.53 for Photosystem 2. These values corresponded to molecular weights of approx. 500 000 and 90 000 respectively. The Photosystem 1 particle was shown to aggregate on storage and EDTA was shown to be necessary to separate the Photosystem 1 and 2 fractions.

Considerable interest has been shown recently in the preparation and characterization of chlorophyll–protein complexes from cyanobacteria (blue–green algae) (Reinman & Thornber, 1979; Newman & Sherman, 1979). Although prokaryotic in structure, cyanobacteria contain two photosystems and normally perform O_2 -evolving photosynthesis in the same manner as higher-plant and algal chloroplasts. The accessory pigments of cyanobacteria are phyco-cyanins and phycoerythrins rather than chlorophyll *b* (Krogmann, 1973), but it has been suggested that Photosystem 2 and Photosystem 1 are essentially similar to those of chloroplasts (Krogmann, 1973; Thornber, 1975). However, Ogawa *et al.* (1969) showed that solubilization of membranes from *Anabaena variabilis* by Triton X-100 gave two fragments, the heavier of which contained Photosystem 1, the lighter being proposed to be derived from Photosystem 2. This was the reverse of the activities of fragments obtained in the same way from higher-plant chloroplasts by Vernon & Shaw (1971). Recently Photosystem 1 extracted from *Chlorogloea fritschii* has been shown to contain a small amount of the accessory pigment allophyco-cyanin (Pullin *et al.*, 1979).

Whatever differences exist between the cyanobacterial photosynthetic apparatus and that of higher-plant chloroplasts, they do not affect the fundamental similarity of the O_2 -evolving processes. The prokaryotic nature of cyanobacteria renders them powerful tools in the elucidation of these processes by the production of photosynthetic mutants (Sherman & Cunningham, 1977; Astier,

1975). It is therefore essential that membrane fragments containing Photosystems 1 and 2 isolated from cyanobacteria be well characterized. The present paper describes a relatively simple method for isolation of such preparations, and discusses the requirement for EDTA in their separation.

Methods

C. fritschii (*Chlorogloeopsis fritschii*; Culture Collection of Algae and Protozoa no. 1411/1, Department of Botany, University of Cambridge, Cambridge, U.K.) was grown and maintained as previously described (Evans *et al.*, 1976).

Isolation of Photosystem 1 and Photosystem 2 fragments

Membrane fragments were isolated by a procedure similar to that used by Bengis & Nelson (1975) to isolate Photosystem 1. Cells were washed with buffer (0.06 M-Tris/HCl/0.05 M-mannitol/0.03 M-EDTA, pH 7.8), resuspended in the same buffer and passed through a French Press at 150 Mpa (10 tonnes/in²). A temperature of 3°C was maintained throughout the preparation. Crude membrane fragments, cell debris and whole cells were isolated by centrifuging for 2 h at 130 000g. The resulting pellet was incubated in 0.06 M-Tris/HCl/0.03 M-EDTA/0.1 M-NaCl and 1.5% digitonin overnight. This mixture was then centrifuged for 15 min at 18 000g, the pellet discarded, and the supernatant was further centrifuged at 130 000g for 4 h. The green precipitate of this spin was incubated in 0.06 M-Tris/HCl/0.03 M-EDTA and 1.5% Triton X-100 overnight. This fraction was either centrifuged on a sucrose gradient

Abbreviation used: SDS, sodium dodecyl sulphate.

in the same manner as described by Ogawa *et al.* (1969), with similar results, or, more conveniently, centrifuged for 4 h at 130 000 *g* giving a pellet that was discarded, the supernatant then being centrifuged for 13 h at 130 000 *g*. This gave a blue-green pellet and yellow-green supernatant, the former expressing the characteristics of Photosystem 1, and the latter of Photosystem 2 (see below). An alternative method of separation of the Triton extract into Photosystem 1 and Photosystem 2 was by passage of this fraction down a Sepharose 6B column. The column was also used for further purification of Photosystems 1 and 2 fractions after centrifugation. Samples (0.2–1.0 ml) were applied to a Sepharose 6B-C1 column (90 cm $\times$ 1.5 cm) previously equilibrated with a pH 7.8 buffer containing 0.06 M-Tris/HCl, with or without 0.08 M-EDTA or 0.2% Triton X-100 as indicated. The column was calibrated for molecular size with bovine serum albumin, γ -globulin and ferritin as known standards.

Polyacrylamide-gel electrophoresis

This was performed by the method of Weber & Osborn (1969). Molecular-weight standards were γ -globulin, myosin, bovine serum albumin, alcohol dehydrogenase, hexokinase, alkaline phosphatase and myoglobin.

Absorption spectroscopy

Absorption changes were measured with either a Pye-Unicam SP.8100 or a Perkin-Elmer 550 spectrophotometer. Light-induced changes of photopigment P-700 were measured after covering the actinic light source (150 W tungsten halogen lamp connected by a fibre optic to the sample compartment) with a Corning 9592 filter, and the photomultiplier with a Schott RG 10 filter.

Photosystem 2 was assayed by reduction of dichlorophenol-indophenol essentially by the method of Vernon & Shaw (1969), except that the reaction medium was 0.06 M-Tris/0.03 M-EDTA, pH 7.8. These conditions were not expected to give maximal assay rates, but it was felt appropriate to leave the buffering conditions unaltered from the preparation medium for reasons discussed below.

P-700 and cytochrome *b*-559 estimations were obtained from difference spectra of oxidized compared with reduced samples with potassium ferricyanide as the oxidant and sodium dithionite as the reductant; absorption coefficients for P-700 of $64 \times 10^4 \text{ M}^{-1}$ (Ke, 1973) and for cytochrome *b*-559 of 20 mm^{-1} (Bendall *et al.*, 1971) were used.

Chlorophyll *a* was estimated by the method of Strain *et al.* (1971) after extraction into 80% acetone in water.

All chemicals were AnalaR wherever possible, otherwise of the highest grade commercially available. Digitonin and diuron [3-(3,4-dichlorophenyl)-

1,1-dimethylurea; DCMU] were obtained from Sigma Chemical Co.

Results and discussion

Fig. 1 shows typical absorption spectra of the Photosystem 1 and 2 preparations isolated after the final centrifugation. The red band of chlorophyll *a* absorbs at 678 nm in Photosystem 1 compared with 670 nm in Photosystem 2. This variation is also a characteristic of fractions characterized by other workers (Ogawa *et al.*, 1969; Reinman & Thornber, 1979; Newman & Sherman, 1978). In contrast with the results of Reinman & Thornber (1979), however, a higher absorption is seen at 490 nm in Photosystem 2 than in Photosystem 1, corresponding to an increased carotenoid/chlorophyll ratio in this fraction. The ratio A_{675}/A_{490} can therefore be used to characterize partially the fractions during elution from a Sepharose 6B column. The main characteristics of the two fractions are summarized in Table 1. The presence of a high P-700/chlorophyll *a* ratio and light-induced P-700 oxidation in the precipitate of the last centrifugation strongly suggested that this was a preparation of Photosystem 1. This was confirmed by the e.p.r. characteristics of

Fig. 1. Absorption spectra of Photosystem 1 and 2 fractions

—, Photosystem 1 (9.6 μg of chlorophyll/ml in 0.06 M-Tris, pH 7.8, containing 0.03 M-EDTA); ----, Photosystem 2 (8.0 μg of chlorophyll/ml in the same buffer as for Photosystem 1). Both preparations were derived from a Sepharose column as indicated in Fig. 2(a).

Table 1. *Characteristics of Photosystem 1 and 2 preparations*

Photosystem 1 was prepared from a Sepharose column as indicated in Fig. 2(a). Photosystem 2 was from the final centrifugation (see the Methods section). Abbreviation used: N.D., not detected.

	A_{675}/A_{490}	P-700/ chlorophyll	Cytochrome <i>b</i> -559/ chlorophyll <i>a</i>	Rate of dichlorophenol- indophenol reduction (nmol/mg of chlorophyll per h)
Photosystem 1 preparation	2.8–3.0	~1:40	<1:2400	N.D.
Photosystem 2 preparation	0.8–1.1	<1:400	~1:125	1380 (124*)

* + 16 μ M-diuron.

Fig. 2. *Elution patterns of various fractions from a Sepharose-6B column*

Running buffer throughout was 0.06 M-Tris/0.03 M-EDTA, pH 7.8, (a) 'Triton extract' (see the text) of *C. fritschii* elution pattern (■); (b) ●, Photosystem 1 fraction; △, [P-700]; ○, Photosystem 2 fraction; (c) Photosystem 1 preparation after 8 weeks storage at -15°C ; (d) 'Triton extract' in the absence of EDTA in the preparation medium.

this fraction, which were similar to those reported by Evans *et al.* (1979) for a Photosystem 1 fraction. An unequivocal identification of a Photosystem 2 preparation lacking O_2 -evolving capacity is more difficult, but the supernatant fraction showed a high cytochrome *b*-559/chlorophyll *a* ratio and light-induced reduction of dichlorophenol-indophenol in the presence of diphenylcarbazine, two proposed characteristics of Photosystem 2 preparations (Vernon & Shaw, 1969; Newman & Sherman, 1979). The low rate of dichlorophenol-indophenol reduction is attributable to performance of the assay at pH 7.8 at relatively high ionic strength (Vernon & Shaw, 1969). Owing to the variation in Sepharose 6B column elution pattern on varying the buffer system it was decided not to alter the buffer system for this assay. The light-induced reduction of dichlorophenol-indophenol by the supernatant fraction in the presence of diphenylcarbazine was

inhibited substantially by diuron, confirming this activity to be catalysed by Photosystem 2.

Fig. 2 summarizes the characteristics of the Triton extract, the precipitate of the final centrifugation (Photosystem 1) and the supernatant of the final centrifugation (Photosystem 2) when applied to a Sepharose 6B column. Figure 2(a) shows the elution profile of the Triton extract, which is resolved into two distinct peaks, with different A_{675}/A_{490} ratios. The first peak to be eluted has a high ratio, and a high P-700/chlorophyll *a* ratio also. Fig. 2(b) shows that the precipitate of the final centrifugation is eluted at the same volume as this peak, which may therefore be identified with the Photosystem 1 fraction. Similarly the supernatant of the final centrifugation is identical with the second peak of the Triton extract. The column running buffer for these experiments contained no Triton, the only Triton being already present in these samples, and

the elution profile is typical of fresh unfrozen preparations. Calibration of the column gave K_d values for the two fractions of 0.38 for Photosystem 1 and 0.53 for Photosystem 2. These K_d values give apparent molecular weights for these two lipoprotein species of 500 000 and 90 000 respectively, confirming the original suggestion of Ogawa *et al.* (1969) that Photosystem 1 preparations of cyanobacteria are larger than Photosystem 2 preparations. Reinman & Thornber (1979) have separated SDS extracts of *Phormidium luridum* by gel electrophoresis to give three major pigment complexes, one of mol.wt. 255 000, one 118 000 (Photosystem 1) and one 58 000 (Photosystem 2). Only two fractions were isolated by us after Triton extraction, but both appear to be larger than those identified by Reinman & Thornber (1979). However, any comparisons are difficult to make as any molecular-weight determination of such a heterogeneous species may vary with the method employed. The characteristics of the Photosystem 1 and 2 fractions eluted from the Sepharose column, digested in SDS and run on polyacrylamide-gel electrophoresis are summarized in Table 2. The Photosystem 2 preparation polypeptide pattern is similar to the major bands reported by Newman & Sherman (1978) for a Photosystem 2 preparation from *Synechococcus cedrorum*. The largest polypeptide is a similar size to the Photosystem 2 component isolated by Reinman & Thornber (1979). The Photosystem 1 preparation shows a major band at mol.wt. 75 000 corresponding to the findings of Bengis & Nelson (1975) using Photosystem 1 preparations extracted in a similar way from spinach chloroplasts. The band at mol.wt. 110 000 may correspond to the species of this molecular weight

identified in many two-photosystem photosynthetic organisms (Thornber, 1975). In common with the results of Klein & Vernon (1977), it was found that the distribution of the protein between the mol.wt.-110 000 and -75 000 bands varied with the exact conditions of digestion. Using low SDS/chlorophyll *a* ratios for digestion, species as large as mol.wt. 300 000 were observed. Some similar effects were observed with the Photosystem 2 preparation, and the polyacrylamide-gel-electrophoresis characteristics of these preparations are therefore subject to further investigation.

The problem of inconsistent polyacrylamide-gel-electrophoresis patterns of Photosystem 1 and 2 fractions experienced by many workers (e.g. Klein & Vernon, 1977) may be influenced by the aggregation of extracts during storage. Fig. 2(c) illustrates the column elution pattern for a Triton extract stored at -15°C for 8 weeks. The apparent molecular weight of the Photosystem 1 fraction was greatly increased, and the fraction was eluted in the void volume of the column. It seems likely that this effect was due to aggregation of this fraction, which could be reversed by increasing Triton concentration. Incubation of the fraction with 4% Triton for 16 h together with addition of 0.15% Triton to the column running buffer restored the profile to that observed in Fig. 2(a). Throughout the preparation of the Photosystem 1 and 2 fractions, 0.03 M-EDTA was present in the buffer system, in common with the preparations described by other workers (Newman & Sherman, 1978; Reinman & Thornber, 1979). Omission of EDTA from the preparation medium resulted in a lack of resolution of Photosystems 1 and 2. The final centrifugation resulted in only a green precipitate and colourless supernatant. The elution profile of the Triton extract is shown in Fig. 2(d), being a broad unresolved peak. This suggests that, in this organism, Triton alone is not responsible for extracting and resolving the photosynthetic apparatus into two distinct fractions (Vernon & Shaw, 1971). Subsequent addition of EDTA to the unresolved Triton extract had no effect. It may therefore be that association between the Photosystem 1 and 2 particles during the extraction is caused by cations, an effect reversed by EDTA.

Thus two pigment complexes only can be isolated from *C. fritschii* by extraction with digitonin and Triton followed by a comparatively simple column separation. One fraction has Photosystem 1 activity, the other has Photosystem 2 activity. This agrees with the findings of Ogawa *et al.* (1969) and Newman & Sherman (1978). The relationship between these fractions and those described by Reinman & Thornber (1979) has yet to be elucidated.

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Table 2. Polypeptide composition of Photosystem 1 and 2 preparations determined by polyacrylamide-gel electrophoresis

Photosystems 1 and 2 preparations were from a Sepharose column as indicated in Fig. 2(a). Samples containing 5–10 μg of chlorophyll/ml were incubated for 16 h at 37°C with SDS/chlorophyll (3000:1, w/w), 0.04% mercaptoethanol and 0.16% EDTA; 0.05 ml was loaded on each gel. Gels were scanned with a densitometer, the relative intensities of Coomassie Blue-stained bands being determined by the area under the curve.

Photosystem 2 preparation		Photosystem 1 preparation	
Molecular weight	Relative band intensity	Molecular weight	Relative band intensity
55 000	1	110 000	1
34 000	1	75 000	10
19 000	1	45 000	2
10 000	3	27 000	4

References

- Astier, C. (1976) Contribution a l'etude genetique d'une Algue Bleue, *Aphanocapsa 6714*, These du Docteur 3e cycle, l'Universite de Paris XI, Centre d'Orsay
- Bendall, D. R., Davenport, H. E. & Hill, R. (1971) *Methods Enzymol.* **23A**, 327–334
- Bengis, C. & Nelson, N. (1975) *J. Biol. Chem.* **250**, 2783–2788
- Evans, E. H., Foulds, I. & Carr, N. G. (1976) *J. Gen. Microbiol.* **92**, 147–155
- Evans, E. H., Rush, J. D., Johnson, C. E. & Evans, M. C. W. (1979) *Biochem. J.* **182**, 861–865
- Ke, B. (1973) *Biochim. Biophys. Acta* **301**, 1–33
- Klein, S. M. & Vernon, L. P. (1977) *Biochim. Biophys. Acta* **459**, 364–375
- Kroghmann, D. W. (1973) in *The Biology of the Blue-Green Algae* (Carr, N. G. & Whitton, B. A., eds.), pp. 80–98, Oxford University Press, Oxford
- Newman, P. J. & Sherman, L. A. (1979) *Biochim. Biophys. Acta* **503**, 343–361
- Ogawa, T., Vernon, L. P. & Mollenhauer, H. H. (1969) *Biochim. Biophys. Acta* **172**, 216–229
- Pullin, C. A., Brown, R. G. & Evans, E. H. (1979) *FEBS Lett.* **101**, 110–112
- Reinman, S. & Thornber, J. P. (1979) *Biochim. Biophys. Acta* **547**, 188–197
- Sherman, L. A. & Cunningham, J. (1977) *Plant Sci. Lett.* **8**, 319–326
- Strain, H. H., Cope, B. T. & Svec, W. A. (1971) *Methods Enzymol.* **23A**, 474
- Thornber, J. P. (1975) *Annu. Rev. Plant Physiol.* **26**, 127–158
- Vernon, L. P. & Shaw, E. I. (1969) *Plant Physiol.* **44**, 1645–1649
- Vernon, L. P. & Shaw, E. R. (1971) *Methods Enzymol.* **23A**, 277–289
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **224**, 4406–4412