PREPARATION OF AN ACTIVE, OXYGEN-EVOLVING PHOTOSYSTEM 2 PARTICLE FROM A BLUE–GREEN ALGA

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1. Introduction

The supply of atmospheric oxygen is largely dependent on a unique biological process, the photo-oxidation of water by photosystem 2 of plants and algae. Attempts to understand the mechanism of this reaction have been hampered by the lack of a pure, oxygen-evolving PS2 particle which could be subjected to detailed physical and chemical studies; most attempts to prepare such a particle have failed because of the extreme lability of the O_2-evolving enzyme to many of the techniques, particularly detergent treatment, that are used for the extraction and purification of membrane-bound proteins. In recent years digitonin has been used in the preparation of increasingly pure PS2 particles from higher plant chloroplasts [1] and from the blue–green alga *Synechococcus cedrorum* [2]. Some of these preparations show high rates of electron transport from artificial electron donors, but all are completely inactive in O_2 evolution.

This report describes the purification of a highly-active, O_2-evolving PS2 preparation from membranes of the thermophilic blue–green alga, *Phormidium laminosum*.

Abbreviations: PS1 and PS2, photosystem 1 and photosystem 2; chl, chlorophyll; DMBQ, 2,6-dimethyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; LDAO, lauryldimethylamine oxide; P700, the reaction centre of photosystem 1

2. Materials and methods

Membrane fragments showing good rates of photosynthetic oxygen evolution (~200 μmol O_2 .mg chl a^{-1} .h^{-1} in the presence of 1 mM 2,6-dimethyl-*p*-benzoquinone and 2 mM potassium ferricyanide) were prepared from cells of *P. laminosum* (strain OH-1-p. Cl 1 [3]) by lysozyme treatment followed by osmotic shock to lyse the spheroplasts. The method used was based on that in [4] for *Phormidium luridum*. Lysozyme treatment was for 1 h at 37°C, in buffer A containing 0.5 M sorbitol, 10 mM $MgCl_2$, 10 mM Hepes/NaOH and 5 mM phosphate buffer (pH 7.5) plus 12.5 mM EDTA and 0.1% solid lysozyme (BDH). The spheroplasts were washed once in buffer A (without lysozyme or EDTA) then lysed by resuspension in hypoosmotic buffer B containing 10 mM $MgCl_2$, 10 mM Hepes/NaOH and 5 mM phosphate buffer (pH 7.5). Membrane fragments were collected by centrifuging at 27 000 × *g* for 15 min, washed once in buffer B then resuspended in buffer C containing 25% (v/v) glycerol, 10 mM $MgCl_2$, 10 mM Hepes/NaOH and 5 mM phosphate buffer (pH 7.5). The characteristics of these membrane fragments will be described in more detail in a subsequent publication.

For the preparation of an O_2-evolving PS2 particle, membrane fragments were resuspended at 1.0 mg chl *a*/ml in buffer C plus 0.35% (w/v) lauryldimethylamine oxide (LDAO, Onyx Chemical Co., Jersey City) giving a detergent:chl *a* ratio of 3.5:1 (w/w). The mixture was incubated for 40 min in the dark at 4°C,

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then centrifuged at 100 000 × *g* for 1 h. The clear supernatant, which was highly active in O_2 evolution, was passed through a Sepharose 6B column (3.3. × 50 cm) equilibrated with buffer C and containing no detergent. This treatment caused the PS2 particles to aggregate so that they could be concentrated by centrifuging (16 h at 100 000 × *g*). The pellet was resuspended in the minimum volume of buffer C.

Chl *a* concentrations were measured by the method in [5].

Rates of O_2 evolution were measured at 25°C using a Hansatech oxygen electrode (Hansatech Ltd, King's Lynn, Norfolk). The reaction mixture contained particles equivalent to 10 μg chl *a* in 1 ml buffer A plus 1 mM 2,6-dimethyl-*p*-benzoquinone and 2 mM potassium ferricyanide. Saturating illumination was provided by light from a 150 W projector lamp filtered through a Schott RG620 filter.

P700 was assayed chemically by recording reduced minus oxidised difference spectra on a Cary 118 spectrophotometer. Particles were diluted to 20 μg chl *a*/ml in 50 mM phosphate buffer (pH 7.0). The reductant was 2 mM ascorbate and the oxidant 1 mM ferricyanide. *A* $\Delta\epsilon_{700} = 64\ mM^{-1}\ .cm^{-1}$ was assumed and the isosbestic wavelength was taken as 727 nm [6].

Measurements of the half-time of activation of O_2 evolution and the acceptor 'pool' size were made using a modulated oxygen electrode as in [7]. PS2 particles and lettuce chloroplasts were dark-equilibrated for 15–20 min before measurements were taken.

3. Results and discussion

3.1. *Fractionation of* P. laminosum *membranes by LDAO*

By a single extraction with LDAO it was possible to fractionate *P. laminosum* membranes into a light (supernatant) fraction enriched 35–40-fold in the ratio O_2 evolution/P700 compared with the original membrane fragments, and a heavy (pellet) fraction containing the bulk of the P700 (table 1). The conditions for detergent treatment in section 2 were those found to give optimal fractionation; for example, higher ratios of LDAO:chl led to extensive solubilisation of PS1 as well as PS2. The presence of 25% glycerol at all stages of the preparation did not affect fractionation, but was found to increase the stability of O_2 evolution in the presence of detergents (A.C.S., unpublished results). O_2 evolution in the PS2-enriched preparations was light-dependent and 100% sensitive to 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), indicating that it was a true PS2 reaction.

When the PS2-enriched supernatant fraction was passed through Sepharose 6B the particles aggregated

Table 1
Characteristics of PS1 and PS2 fractions prepared by treatment of *P. laminosum* membranes with LDAO

	Original membrane fragments	Supernatant fraction	Pellet fraction	Concentrated PS2 particles
Chl *a* (mg/ml)	1.0	0.151	1.06	0.79
% of total chl *a*	100	13.5	86.5	9.1
Rate of O_2 evolution ($\mu mol\ O_2\ .mg\ chl\ a^{-1}\ .h^{-1}$)[a]	191	1217	10.3	1342
% recovery of O_2 evolution	100	86.0	4.7	63.9
P700 (nmol/mg chl *a*)	6.72	1.06	7.42	1.25
% recovery of P700	100	2.1	95.5	1.7
O_2 evolution/P700	1.0	41.0	0.049	37.6

[a] With 1 mM DMBQ and 2 mM $K_3Fe(CN)_6$ as electron acceptors

with the result that they were separated from the more slowly eluted soluble contaminants such as the bulk of the accessory phycobilin pigments and some free, detergent-solubilised chlorophyll. The PS2 particles could then be concentrated by high-speed centrifugation. The removal of free chlorophyll was reflected in slightly increased ratios of both O_2 evolution/chl and P700/chl in the concentrated particles, but the degree of enrichment in the ratio PS2/PS1 remained virtually unchanged (table 1).

3.2. *Characteristics of* P. laminosum *PS2 particles*

3.2.1. Size of the O_2-evolving particles

The concentrated preparation of PS2 particles could be re-solubilised without loss of activity by addition of a low concentration of LDAO (5 mg LDAO/mg chl *a*). The re-solubilised preparation was apparently heterogeneous with respect to size, since if it was passed through a second Sepharose 6B column (1.25 × 30 cm) equilibrated with buffer C plus 0.05% LDAO, O_2-evolution activity was eluted in a broad peak corresponding to a distribution of mol. wt from $\sim 2 \times 10^5$ to $>1 \times 10^6$ (calculated from molecular weight selectivity curves published by Pharmacia Fine Chemicals, Uppsala). However the fact that activity was present in particles of only a few hundred thousand molecular weight is significant since it suggests that, contrary to previous suggestions [8], the O_2-evolving enzyme does not need to be incorporated into a membrane vesicle (which would be expected to have mol. wt $>> 2 \times 10^5$) in order to remain active.

The second Sepharose column also separated the remaining P700, which was eluted in the void volume, from later O_2-evolving fractions in which P700 was undetectable.

3.2.2. Spectral characteristics

The room temperature absorption spectra in fig.1 show that both the initial detergent supernatant and the concentrated preparation of PS2 particles were enriched, compared with the original membrane fragments and the pellet after detergent treatment, in shorter wavelength forms of chlorophyll, and had negligible absorbance at wavelengths >700 nm. This is consistent with the results of studies showing that shorter wavelength forms of chlorophyll are associated predominantly with PS2 [9].

The spectrum for the concentrated PS2 particles was very similar to that of PS2 particles (inactive in O_2 evolution) from the blue-green alga *Synechococcus cedrorum* [2]. The decreased A_{620} and the slight shift in the chlorophyll absorbance peak compared with the initial PS2-enriched detergent supernatant reflect the removal of phycobilins and free chlorophyll on the Sepharose 6B column.

3.2.3. Photosynthetic unit and acceptor pool size

These parameters were investigated by using a modulated oxygen electrode of the type designed in [7]. The area bounded by the 'oxygen gush' curve in saturating light (fig.2) has been shown to give a measure of the size of the PS2 acceptor 'pool', and can be calibrated by comparison with the activation area, which is assumed to represent 1 equiv./centre [10]. For the PS2 particles a pool size of ~9 equiv./centre was obtained by this method. Compared with reported values for spinach chloroplasts of 10–20 equiv./centre [10] this indicates that an appreciable proportion of the original plastoquinone acceptor pool remained associated with PS2 in the *P. laminosum* PS2 particles.

In continuous light, following total dark equilibration, the rise time of the activation curve for O_2 evolution gives a measure of how frequently the light beam hits each PS2 trap, and therefore is related to the photosynthetic unit size [11]. For *P. laminosum* PS2 particles the half-time for activation was ~900 ms, compared with ~280 ms, under the same conditions, for lettuce chloroplasts. Taking the photosynthetic unit size for higher plant chloroplasts as ~300 chl/PS2, this suggests a photosynthetic unit size of ~100 for PS2 in the *P. laminosum* particles. This value is likely to be an overestimate, since chl *b* in the lettuce chloroplasts would absorb the actinic orange light less strongly than chl *a*, thus increasing the apparent activation time compared with *P. laminosum*, which contains only chl *a*. In addition, the activation time for the *P. laminosum* preparation might be affected by the presence of some residual phycocyanin which, if active in light harvesting, would tend to increase the apparent photosynthetic unit size. Thus although the PS2 particles from *P. laminosum* still retained appreciable amounts of light-harvesting chlorophyll,

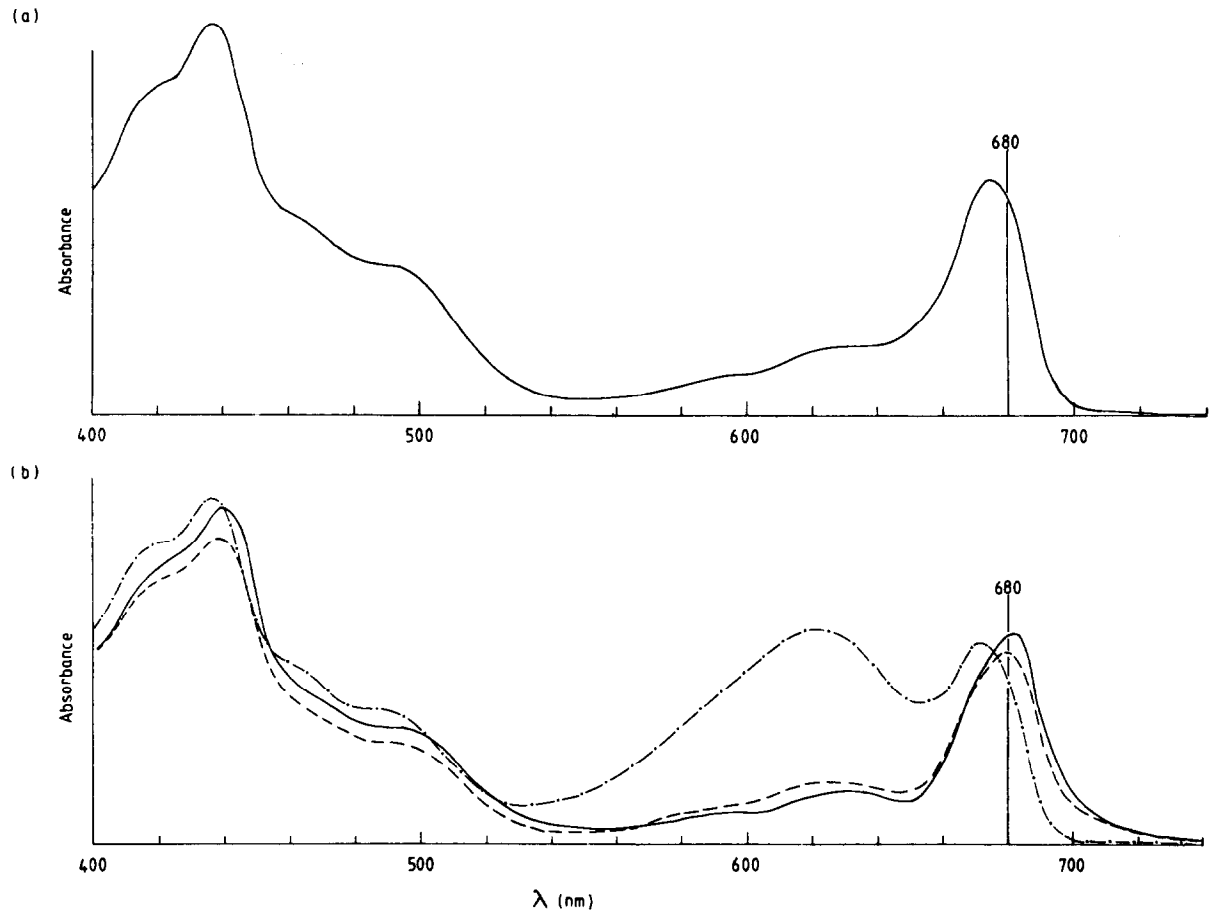

Fig.1. Absorption spectra of (a) *P. laminosum* concentrated PS2 particles; (b) membrane fragments (————), supernatant (—·—·—·—) and pellet (———) fractions after LDAO treatment.

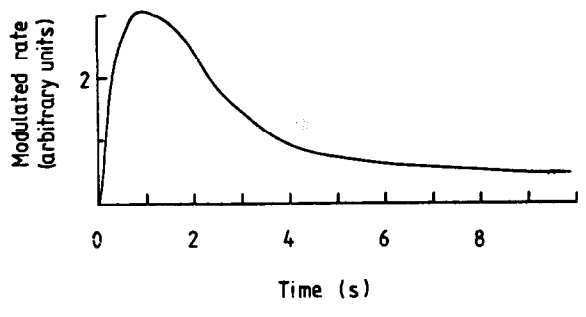

Fig.2. Oxygen gush from concentrated PS2 particles. The modulated beam was passed through a 2.6 mm heat filter (Corning 5600) and a 3 mm orange filter (Schott ORG 550). The frequency was 20 Hz and the temperature 25°C.

it is clear that they had a considerably smaller photosynthetic unit size than higher plant chloroplasts.

4. Conclusion

The PS2 preparation from *P. laminosum* is the most highly enriched preparation with respect to oxygen evolution that has been obtained from any oxygenic organism. It provides promising material for future chemical studies on the photosynthetic water dehydrogenase. The fact that it is almost totally devoid of P700 and is active in O_2 evolution in optically clear solution suggests that it will also prove useful in physical studies on the mechanism of photo-oxidation of water by photosystem 2.

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References

[1] Satoh, K. and Butler, W. L. (1978) Plant Physiol. 61, 373–379.
[2] Newman, P. J. and Sherman, L. A. (1978) Biochim. Biophys. Acta 503, 343–361.
[3] Castenholz, R. W. (1970) Schweiz. Z. Hydrol. 32, 538–551.
[4] Binder, A., Tel-Or, E. and Avron, M. (1976) Eur. J. Biochem. 67, 187–196.
[5] Arnon, D. I., McSwain, B. D., Tsujimoto, H. Y. and Wada, K. (1974) Biochim. Biophys. Acta 357, 231–245.
[6] Wood, P. M. and Bendall, D. S. (1975) Biochim. Biophys. Acta 387, 115–128.
[7] Joliot, P. (1972) Methods Enzymol. 24, 123–134.
[8] Stillwell, W. and Ti-Tien, H. (1978) Biochem. Biophys. Res. Commun. 81, 212–216.
[9] French, C. S., Brown, J. S. and Lawrence, M. C. (1972) Plant Physiol. 49, 421–429.
[10] Radmer, R. and Kok, B. (1973) Biochim. Biophys. Acta 314, 28–41.
[11] Forbush, B., Kok, B. and McGloin, M. (1971) Photochem. Photobiol. 14, 307–321.