Oxygen-Evolving Extracts from a Thermophilic Cyanobacterium *Synechococcus* sp.

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Spheroplast membranes of a thermophilic cyanobacterium *Synechococcus* sp. have been treated with the detergent lauryldimethylamine oxide (LDAO). The resulting extracts show (1) light-induced O_2 evolution with artificial electron acceptors, (2) four-fold enhancement of the O_2 evolution relative to chlorophyll, (3) parallel increase of both the molar ratios of PS 2/Chl and cyt b_{559}/Chl in the extract, (4) dissociation of the auxiliary pigment phycocyanin upon treatment with LDAO, but still tight association of allophycocyanin to the photosystem 2 preparation.

1. Introduction

Cyanobacterial thylakoids turned out to be well apted for extraction of oxygen-evolving photosystem 2 preparations [1–3]. The method was first developed and reported [1, 2] for the thermophilic cyanobacterium *Phormidium laminosum* using LDAO as detergent (to solubilize PS 2).

Later [3], with some modifications of the method, LDAO was shown to be similarly applicable to the cyanobacteria *Anacystis nidulans* (*Synechococcus* 6301) and *Aphanocapsa.*

For another thermophilic cyanobacterium, *Synechococcus* sp. isolated from hot springs [4] extraction with digitonin and chromatographic separations of photosystems 1 and 2 have been reported [5]. These latter preparations of reference [5] however, showed electron transport only in the presence of artificial donors, but no O_2 evolution. In the present work we report the extraction of an oxygen-evolving preparation from this cyanobacterium with use of LDAO as detergent.

Abbreviations. APC, allophycocyanin; Chl, chlorophyll; Cyt, cytochrome; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; HMP-buffer, buffer containing 20 mM HEPES-NaOH, 10 mM magnesium chloride and 2 mM Na-K-phosphate; HMPM-buffer, HMP-buffer containing 0.5 M mannitol; LDAO, lauryldimethylamine oxide; MES, 2-(N-morpholino)ethane sulfonic acid; PBS, phycobilisomes; PMS, phenazine methosulfate; PS, photosystem.

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2. Materials and Methods

2.1 Culture

A *Synechococcus* sp. thermophilic cyanobacterium (gift from Prof. S. Katoh, University of Tokyo and described in [4]) was cultivated at 50 °C for 48 h in the medium of [6], supported with 5% carbon dioxide and illuminated with fluorescent lamps (Osram "L-daylight 5000"). Cells were harvested by centrifugation and washed twice in 20 mM HEPES-NaOH (pH = 7.8), 10 mM $MgCl_2$ and 2 mM Na-K-phosphate (HMP-buffer).

2.2 Preparation of spheroplast membranes and extracts

The washed cells were resuspended in 20 mM HEPES-NaOH (pH = 7.8), 10 mM MgCl, 2 mM Na-K-phosphate and 500 mM mannitol (HMPM-buffer) in a concentration of 1 mg Chl/ml and treated with 0.2% (w/w) egg lysozyme (Serva) at 50 °C for 1 h in the dark and under gentle agitation. The lysed cells were centrifuged for 5 min at $12000 \times g$ and osmotically shocked by subsequent resuspension in HMP-buffer. After two washing cycles in that buffer, spheroplast membranes were formed which were suspended in a buffer of 80% (v/v) HMPM plus 20% glycerol at a concentration of 1 mg Chl/ml. After addition of 0.2% LDAO (Fluka AG), the mixture was stirred in the dark at 4 °C for 30 min, then centrifuged at $110000 \times g$ for 60 min. The supernatant was used for further studies.

2.3 Analytical Methods

Chlorophyll-a extracted from membranes or PS 2 preparations with 80% acetone was determined from

the absorbance at 663 nm [7]. C-phycocyanin in situ was estimated from the absorbances at 620 nm and 678 nm using equations of Arnon *et al.* [8]. O_2-evolving activity was measured with a Clark-type electrode under conditions as elaborated in [4]: at 40 °C in 50 mM MES-NaOH buffer (pH = 5.5) containing 500 mM sucrose, 20 mM sodium chloride, 10 mM magnesium chloride and 1 mM $K_3(Fe(CN)_6)$ (potassium ferricyanide) as an electron acceptor. Illumination was provided by light from a 250 W halogen lamp through a water filter at an intensity of 80 mW/cm². PS 2 activity was also estimated by measuring the absorption decrease of $K_3(Fe(CN)_6)$ at 420 nm at 40 °C in the above buffer. In either case the LDAO concentration in the assay medium did not exceed 0.01%.

Content of photosystem 1 was determined by two methods: a) chemically recording reduced minus oxidized difference spectra on a Beckman UV 5260 spectrophotometer with 2 mM ascorbate as reductant and 1 mM $K_3(Fe(CN)_6)$ as oxidant [9] and calculating with a molar extinction coefficient $\varepsilon = 64\,000$ $M^{-1}\,cm^{-1}$ at 700 nm [10]; b) monitoring the flash – induced absorbance changes at 702 nm as described in [11] in HMPM-buffer supplied with PMS (20 µM), sodium ascorbate (0.2 mM) and methylviologen (0.1 mM). Content of active photosystem 2 was determined by measuring the average oxygen yield in repetitive single turnover flashes as described in [12] and under the following conditions: HMPM-buffer pH = 6.5 plus 0.1 mM $K_3(Fe(CN)_6)$, averaging 100 flashes, half maximal flash duration ~ 20 µs, flash repetition frequency 2 s^{-1}.

Cytochrome b_{559} was estimated from chemically reduced minus oxidized difference spectra with dithionite as reductant and $K_3(Fe(CN)_6)$ as oxidant using $\varepsilon = 20\,000$ $M^{-1}\,cm^{-1}$ [13].

Fluorescence spectra were measured with an SLM 4800 S spectrometer. The samples were frozen in liquid nitrogen and connected with the spectrometer by means of a bifurcated fiberoptic. Thus, emission was measured in reflection perpendicular to the surface of the sample. Intensities of the irradiation arriving at the surface were determined by means of a Hewlett-Packard radiant flux meter 8330 A.

3. Results and Discussion

Fig. 1 shows the effect of LDAO concentration on PS 2 extraction. With increasing concentration of the detergent, an increasing amount of chlorophyll was solubilized in a form which remained in the supernatant. Concomitantly, a decrease of the water-splitting activity of the pellet (closed circles) was observed. Usually, after extraction with about 0.2% LDAO the supernatants showed maximal oxygen evolving activity per chlorophyll. Under such conditions about 5% of the total amount of chlorophyll were solubilized. Higher detergent concentrations for extraction caused a decrease of the specific activity of the supernatants (open circles).

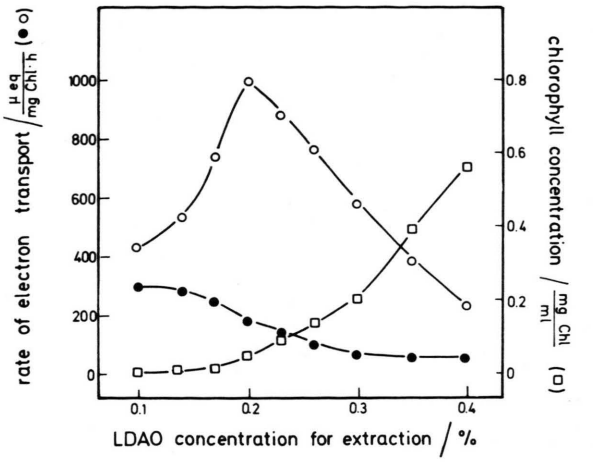

Fig. 1. Effect of LDAO concentration on extraction of chlorophyll and PS 2 activity. $K_3(Fe(CN)_6)$ reduction activity of supernatant (○), of pellet (●). Chlorophyll-a concentration in supernatant (□).

This can be due to an increased solubilization of such chlorophyll-protein-complexes which do not contribute to PS 2 activity (*e.g.* light harvesting- or PS 1-complexes). The supernatant with maximal oxygen evolving activity was applied to an Ultrogel A 4 column for gel-filtration. The resulting elution patterns of oxygen evolving activity, chlorophyll concentration, C-phycocyanin-concentration, and OD_{280} nm (for protein concentration) are illustrated in Fig. 2. Chlorophyll is eluted in the first peak and C-phycocyanin (possibly from a remainder of phycobilisomes) can be separated from the PS 2 preparation without loss of activity. The peak fractions for chlorophyll will be termed purified PS 2 preparations in this paper.

The activities for the spheroplast membranes, the supernatant after 0.2% LDAO extraction and a purified PS 2 preparation are compared in Table I.

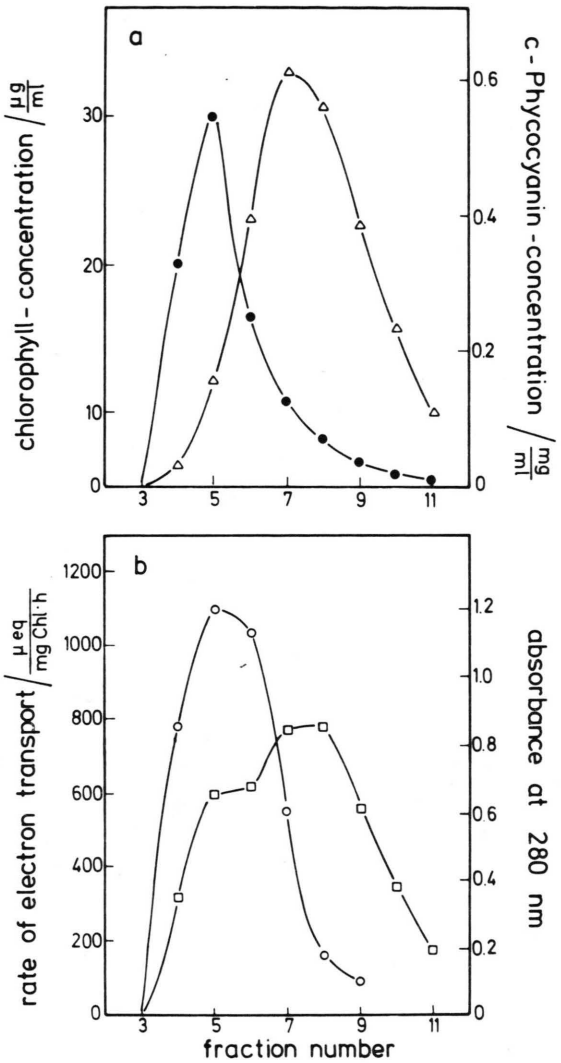

Fig. 2. Chromatographical fractionation of the PS 2 preparation: 1.5 ml of supernatant was applied on a Ultrogel A 4 (LKB) column (1 cm × 17 cm) and eluted with 90% (v/v) HMPM plus 10% glycerol, 60 drops per fraction. a) ●—●: chlorophyll-a concentration, △—△ C-phycocyanin concentration, b) □—□: absorbance at 280 nm. ○—○: $K_3(Fe(CN)_6)$ reduction activity.

Comparison between spheroplast membranes and the supernatant shows a four-fold increase of activity. Oxygen-yields in single turnover flashes show that this corresponds with an increase of the number of active PS 2 centers per chlorophyll. The content of Cyt b_{559}/Chl, also an indicator of PS 2 reaction centers, was increased with a similar ratio as that for enhancement of activity. Simultaneously, the content of P 700/Chl decreased upon extraction. Thus, the method is shown to cause a rather specific extraction of PS 2 components without desactivation.

The absorption spectra of spheroplast membranes, supernatant and purified PS 2 preparations are shown in Fig. 3. They indicate that the treatment with LDAO does solubilize most of the phycocyanin ($\lambda_{max} = 620$ nm), but only part of the chlorophyll (about 5% of total chlorophyll according to Fig. 1).

Furthermore, the shoulder at about 650 nm in the absorption spectrum of the supernatant indicates that allophycocyanin is also extracted.

After gel filtration, separating the phycocyanin containing proteins from the PS 2 preparation (Fig. 2), the absorption spectrum reveals that large amounts of the auxiliary pigment allophycocyanin are still tightly bound to the PS 2 preparation. This has not been observed in the previously reported PS 2 preparations from cyanobacteria [1–3].

Our findings can be understood within the present model [14] for the organization of auxiliary pigments in cyanobacteria. Phycocyanin (absorbing at $\lambda_{max} \approx 620$ nm) localized in the peripheral rods of phycobilisomes transfers its excitation energy to allophycocyanin ($\lambda_{max} \approx 650$ nm) and allophycocyanin-B ($\lambda_{max} \approx 670$ nm) localized in the core of the PBS [14]. From there it is channeled to the chlorophylls of the PS 2. The efficiency of the latter energy transfer is facilitated by attachment of the core of a PBS to chlorophyll proteins within the membrane (near or at PS 2). Dissociation of complete phycobilisomes from chlorophyll usually is achieved with 1% Triton X-100 as detergent and under high ionic strength (0.75 M K_2HPO_4 [14]); therefore it could be possible that treatment of our spheroplast membranes with LDAO in low salt buffer solubilizes a membrane fragment from which the phycocyanin containing part of the PBS has been detached, while parts of the core complex remain still associated.

Fluorescence emission spectra recorded at 77 K from spheroplast membranes and from a purified PS 2 preparation are presented in Fig. 4. Upon excitation of chlorophyll (at 430 nm) spheroplast membranes show the well known emission spectrum [5, 15], with peaks termed F 685 and F 695, attributed to PS 2 [15, 16] and an intense peak of F 730 due to PS 1 [15, 16]. Fluorescence from purified PS 2 preparations shows peak values at 687.5 nm and 694 nm and only a very weak emission from PS 1. This is in qualitative accordance with the results of Table I, reflecting the increase of the molar ratio PS 2/PS 1.

Table I. Properties of preparations from *Synechococcus* sp.

	Rate of electron transport	PS 1		PS 2	cyt b_{559}	PS 1
	μmol O_2 · (mg Chl)$^{-1}$ · h^{-1}	1000 Chl		1000 Chl	1000 Chl	PS 2
		a	b			
Spheroplast membranes	75	4.1	3.6	1	2.6	3.6
Supernatant	246	2.3	2.2	3.8	11.2	~0.6
Purified PS 2 preparation	251	1.6	1.6	not analyzed[c]	13.7	<0.5[c]

[a] From chemically, [b] from flash-induced absorbance changes (see analytical methods); [c] PS 2 not determined because of technical problems arising from the low chlorophyll-concentration of the sample; therefore, the PS 1/PS 2 ratio is estimated on the assumption that the PS 2/Chl ratio of purified PS 2 preparations is at least that of the supernatant. This assumption is justified by the results obtained for cyt b_{559} and the rate of electron transport. Values give molar ratios.

In [5] and [17] the 77 K emission spectra of cyanobacterial PS 2 complexes inactive in oxygen-evolution have been reported. Those spectra are very similar to that of the oxygen-evolving PS 2 preparation shown in Fig. 4.

Interestingly, upon irradiation at 650 nm, and thus exciting mainly the auxiliary pigment allophycocyanin, we observe a significantly altered emission spectrum. Fluorescence then has a peak maximum at 685 nm (not shown) and also fluorescence yields are different: the determination of the exciting irradiation flux revealed that the excitation at 650 nm gives about twice the yield compared to excitation at 430 nm. The emission cannot arise from allophycocyanin which fluoresces at 655 nm [16]. Therefore, pigments to be discussed are allophycocyanin B, for which an emission at 680 nm is reported [16, 18, 19] and chlorophyll. An intensive emission at 683 nm was reported [5] from a chlorophyll-protein complex tentatively interpreted as antenna complex. Both

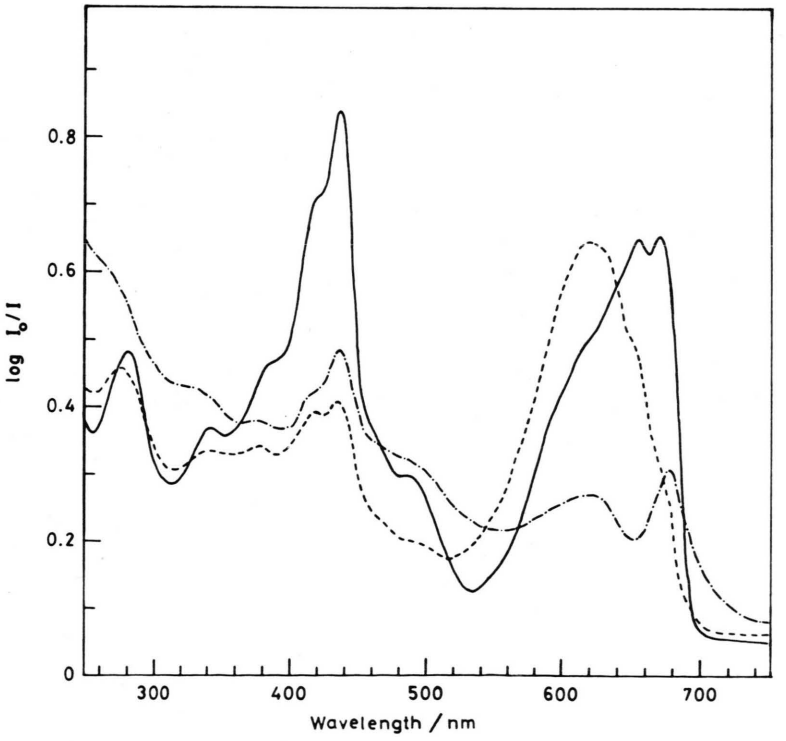

Fig. 3. Absorption spectra of spheroplast membranes (–·–·–), supernatant (– – –) and purified PS 2 preparation (——). Spectra were recorded in HMPM buffer at 298 K. Samples were diluted arbitrarily.

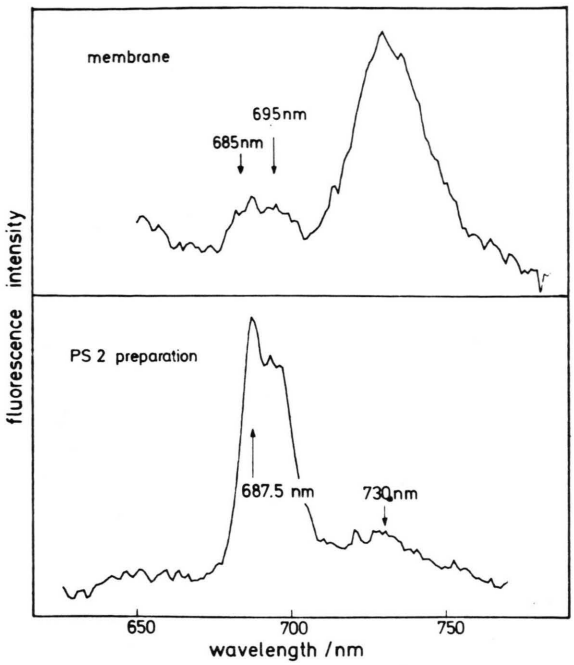

Fig. 4. Fluorescence emission spectra of spheroplast membranes (top) and of purified PS 2 preparations (bottom) upon excitation with light of 430 ± 8 nm. Resolution of emission wavelength: 1 nm, chlorophyll concentration 5 µg/ml. Spectra were recorded in HMPM buffer at 77 K.

emissions could contribute to the observed spectrum if the pigments were linked to APC, but no longer efficient in energy transfer towards the reaction center.

At present, a clear discrimination between the possible contributions is not possible. Therefore, further experiments are required in order to clarify the question about the origin of the strong emission at 685 nm excited by APC.

Hence, the preparations described in this paper may be of great use for future studies on the energy-transfer mechanisms in cyanobacteria as well as on the donor sites of PS 2 and on the water splitting reactions in general.

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