2D-isolation of pure plasma and thylakoid membranes from the cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract Aqueous polymer two-phase partitioning in combination with sucrose density centrifugation offered, for the first time, a 2D-separation method for the isolation of pure plasma and thylakoid membranes from the cyanobacterium *Synechocystis* 6803 without any cross-contaminations. The purity of the membrane fractions was verified by immunoblot analysis using antibodies against membrane-specific marker proteins. As an initiation of a proteomics project, two prominent proteins, which were observed only in the plasma membrane (Slr1513, a hypothetical protein, and HofG, a general secretion pathway protein), or in the thylakoid membrane (PsaE, a photosystem I protein, and NdhH, a subunit of NADH dehydrogenase), were identified.

Key words: Cyanobacterium; Membrane protein; Phase partitioning; Proteomics; *Synechocystis* 6803

1. Introduction

A cyanobacterial cell is surrounded by a double envelope consisting of an outer membrane, a peptidoglycan layer and the plasma membrane. However, cyanobacteria are unique prokaryotes, since they contain a differentiated membrane system. In addition to the plasma membrane, these cells have the intracellular chlorophyll-containing thylakoid membrane involved in oxygenic photosynthesis [1]. Recently, the complete genome sequence of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 has been determined [2]. Furthermore *Synechocystis* 6803 is used as an attractive model system in photosynthesis research since it is readily transformable, i.e. it is amenable to a wide variety of experiments based on the exchange of genetic material, and is also capable of both photoheterotrophic and mixotrophic growth [3]. Thus, mutations can readily be introduced in photosynthetic proteins, and maintained by growth of cells under non-photosynthetic conditions. During recent analysis of the complete nucleotide sequence of the *Synechocystis* 6803 genome, all open reading frames have been examined by database matching for the identification of the corresponding proteins. However, knowledge of the total genome cannot predict a specific protein's (i) subcellular location, (ii) relative concentration, (iii) post-translational modification, and (iv) its expression in response to external and internal stimuli. Proteome research, i.e. research on the total protein complement of the genome is therefore a central task in life sciences, in the near future. To initiate a proteome project of the model organism *Synechocystis* 6803, with respect to the localization of all the membrane proteins in this differentiated prokaryotic membrane system, the first challenge is to isolate the different membranes in a completely purified state. The isolated membrane fractions can then be used for the systematic identification of the proteins by 2D-electrophoresis followed by amino terminal sequencing or mass spectrometry, combined with sequence matching using the Cyanobase database [4].

In the present communication, a newly developed method is described that results in pure thylakoid and plasma membranes from *Synechocystis* 6803. The method combines aqueous polymer two-phase partitioning [5] with sucrose density centrifugation, and the purity of the membrane fractions has been verified by the use of antibodies against membrane specific marker proteins.

2. Materials and methods

2.1. Cell culture

The wild-type strain of *Synechocystis* 6803 was grown photoautotrophically in liquid BG11 medium [6] at 30°C under 60 μE/m^{2}s of white light. The cells were harvested at a density of 1.6–2.0×10^8 cells/ml (3–5 days of growth).

2.2. Preparation of total membranes

Frozen cells from one liter culture were suspended in a 20 mM potassium phosphate (pH 7.8) to a final volume of 5–6 ml. Glass beads (diameter 0.17–0.18 mm) were added to the sample tube, so that only 0.3–0.4 ml cell suspension was seen above the beads. The sample was shaken in a vortex mixer three times at the highest speed for 2 min with 1-min intervals on ice, and then centrifuged for 1 min. The cell suspension was collected at the top of the sedimented beads, and centrifuged for 10 min at 3300×*g*. The supernatant was centrifuged for 30 min at 103 000×*g*. The resultant dark blue supernatant was discarded and the pellet of total membranes was rinsed with a buffer containing 0.25 M sucrose, 5 mM potassium phosphate (pH 7.8) and homogenized in the same buffer to a volume of about 4 ml.

2.3. Aqueous polymer two-phase partitioning

The two-phase systems were prepared from stock solutions of 20% (w/w) Dextran T-500 and 40% (w/w) polyethylene glycol 3350. Total membranes (3.75 g) at a concentration of about 0.4 mg chlorophyll/ml were applied to a 6.25-g polymer mixture yielding a two-phase system of 5.8% (w/w) Dextran T-500, 5.8% (w/w) polyethylene glycol 3350, 0.25 M sucrose, and 5 mM potassium phosphate (pH 7.8). A repartitioning system (40 g) with the same final concentrations (but without membrane sample) was also prepared. In addition, a second repartitioning system (20 g) with 6.2% of both polymers in the same buffer and sucrose medium was prepared.

The partition steps were performed by gently inverting the tubes 35 times at 3°C. Phase settling was facilitated by centrifugation for 4 min at 1000×*g*, and the upper and lower phases were collected separately (Fig. 1). The lower (B1) and upper (T1) phases were repartitioned with upper and lower phases from the first (5.8%) repartitioning system, respectively, yielding the T2 and B2 fractions. Yet another partition cycle yielded T3 and B3 fractions. The B3 fraction was repartitioned two more times with the 5.8% upper phase producing a final B5 fraction. The T3 fraction was added to a 5.8% lower phase (in total 10 g) and supplemented with 0.4 g of the Dextran (20%) stock

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solution and 0.2 g of the polyethylene glycol (40%) stock solution, resulting in a two-phase system with 6.2% of each polymer. After partitioning, the resultant upper phase (T4) was repartitioned two more times with lower phase from the second (6.2%) repartitioning system, resulting in the final T6 phase. The B5 and the T6 phases were diluted with 0.25 M sucrose, 5 mM potassium phosphate (pH 7.8) to about 60 ml and centrifuged for 1 h at 125 000 × *g*.

2.4. Sucrose density gradient centrifugation

The pelleted membranes were homogenized in 10% sucrose (w/w), 20 mM potassium phosphate (pH 7.8) to a volume of about 2.5 ml. Both fractions were supplied with solid sucrose to a final concentration of 42% sucrose (w/w). Each gradient had the following composition: 1.5 ml 50%, 3 ml membranes 42%, 1.5 ml 40%, 1.5 ml 38%, 1.5 ml 35%, 1.5 ml 30% and 1.5 ml 10% sucrose, and was centrifuged for 2.5 h at 197 000 × *g* in a Beckman SW41 Ti rotor. Fractions were collected and diluted with 20 mM potassium phosphate (pH 7.8) to a final sucrose concentration of about 10% and centrifuged for 30 min at 125 000 × *g*. The pelleted membranes were homogenized in a small volume of 0.25 M sucrose, 5 mM potassium phosphate (pH 7.8).

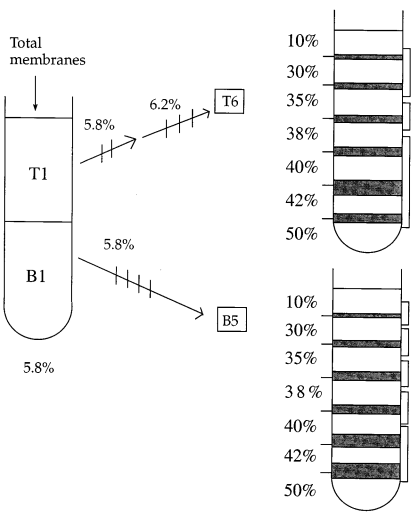
2.5. Electrophoresis, immunodetection and protein microsequencing

Membrane proteins were separated by SDS-PAGE using 12.5% polyacrylamide [7]. For immunodetection, proteins were blotted onto nitrocellulose filters, reacted with antisera and the signals were visualized by using enhanced chemiluminescence reagents (Pierce). For amino-terminal sequencing proteins were blotted onto PVDF membranes followed by the Edman degradation procedure (Patterson Laboratories, University of Texas, Austin, USA). Protein concentrations were determined using a Micro BCA protein assay reagent kit (Pierce), whereas chlorophyll concentrations were determined according to [8].

3. Results

During the subfractionation studies, the periplasmic binding protein of the nitrate transporter NrtA, known to be present exclusively in the plasma membranes of cyanobacteria [9], was used as a specific marker, whereas CP43, a chlorophyll-*a* binding protein of PSII, was used as a specific marker for the thylakoid membranes [10]. Fig. 1 illustrates the purification procedure for thylakoid and plasma membranes as well as the properties of the various isolated membrane fractions. Two-phase partitioning of the total cyanobacterial membranes resulted in a dark green lower phase (B1) due to an enrichment of the thylakoid membranes, and a yellow-green upper phase (T1) enriched in plasma membranes, but still containing thylakoid membranes. Four repartitionings of the B1 fraction resulted in the final B5 fraction which was highly enriched in thylakoid membranes as revealed by the enrichment of CP43 (Fig. 1; Fig. 2, lanes 1 and 4). However, this fraction was to some degree still contaminated by plasma membranes, as judged by the presence of the NrtA protein (Fig. 2, lane 4). Therefore, as a second dimension for purification, a sucrose density gradient was applied. The thylakoid membranes in the B5 fraction were heterogeneous with respect to density and chlorophyll/protein ratio (Fig. 1). The fractions of thylakoid vesicles denoted $B5_3$ and $B5_4$ were found to be the purest, since they were not contaminated by plasma membranes as revealed by the complete absence of the NrtA protein (Fig. 2, lanes 5 and 6). The plasma membranes contaminating the B5 thylakoid fraction were recovered in the lightest density band ($B5_1$) as well as in the heaviest density fraction ($B5_5$) (Figs. 1 and 2, lane 7).

For the purification of plasma membranes, the initial upper phase (T1) was repartitioned twice at the 5.8% polymer concentration followed by three times at the 6.2% polymer concentration (Fig. 1). The final T6 fraction, consisting of yellow membranes, was practically devoid of the CP43 protein (Fig. 2, lane 2), demonstrating little, if any, contamination by thylakoid membranes. Subsequent subfractionation of the T6 fraction on a sucrose density gradient revealed that also the plasma membrane fractions consisted of membranes with different densities, most of which had a density corresponding to 38–42% sucrose (Fig. 1). This plasma membrane fraction



Membrane fraction	Protein (mg)	CP43 (arb)	NrtA (arb)	chl/protein (mg/mg)	chl/protein (%)
Tot membr	21	+	+++	0.07	50
T6	2.5	(+)	+++	0.01	7
$T6_1$	0.2	nd	nd	0.009	6
$T6_2$	0.2	nd	nd	0.008	6
$T6_3$	0.8	-	+++++	0.012	9
B5	5.1	++++	++	0.08	57
$B5_1$					
$B5_2$					
$B5_3$	0.5	+++	-	nd	nd
$B5_4$	0.5	+++	-	0.08	57
$B5_5$	2.4	++	+	0.14	100

Fig. 1. Schematic presentation of the procedure for the 2D-purification of thylakoid and plasma membranes from *Synechocystis* 6803 and characterization of the different membrane fractions. +/−, Arbitrary units for the amount of protein as determined by immunoblot analysis using antibodies against CP43, a chlorophyll-*a* binding protein of PS II, and NrtA, the periplasmic binding protein component of a nitrate transporter; nd: not determined.

($T6_3$) was also highly enriched in the NrtA protein as compared to the total plasma membrane fraction in T6 (Fig. 2, lanes 2 and 3) and contained no detectable level of the CP43 protein. As discussed below the residual amount of 'chlorophyll' (6–9%) may therefore represent a precursor form of the pigment as previously suggested [11].

As seen in Fig. 3, there is a distinct difference in the polypeptide patterns of the purest thylakoid membranes of fraction $B5_3$ (lane B) and the purest plasma membranes of fraction $T6_3$ (lane C). To exemplify the usefulness of the pure plasma and thylakoid membranes from *Synechocystis* 6803 for a proteomic approach, two distinct proteins present only in the plasma membrane (1 and 2) and two present in thylakoid membrane (3 and 4) were blotted onto PVDF membrane and their amino terminal sequences were determined (Fig. 3). The N-terminal 12 amino acid residues of protein 1 (Fig. 3) exhibited 100% match to amino acids 2–13 of a putative protein encoded by the open reading frame of slr1513 in the *Synechocystis* 6803 genome [4]. This hypothetical protein has no homologue in any other existing genomic database. For protein 2 of the plasma membranes, a sequence of 14 N-terminal amino acids showed 100% match to amino acids 23–36 of the protein encoded by the sll1694 open reading frame of the *Synechocystis* 6803 genome. This protein is a general secretion pathway protein G (HofG), which has homologous counterparts in several other organisms. The presence of this protein in plasma membranes is expected, but has not been demonstrated previously. Two proteins present in the thylakoid membrane were identified as the subunit PsaE of photosystem I (protein 3) and subunit 7 of NADH dehydrogenase (protein 4), respectively.

4. Discussion

Thylakoid and plasma membranes of the widely used cyanobacterium *Synechocystis* 6803 have previously been isolated by sucrose density gradient centrifugation [12–14]. By using antibodies against NrtA, a protein localized only in the plasma membrane, Sonoda et al. [14] have determined that the thylakoid membranes isolated using such an approach were up to 38% contaminated by plasma membranes. Moreover, based on the chlorophyll content of isolated thylakoid and plasma membranes, Ogawa [12] has calculated that the plasma membranes were about 25% contaminated by thylakoid membranes. Interestingly, Hinterstoisser et al. [13] found no detectable levels of chlorophyll in isolated plasma membranes. However, the cross-contamination of plasma membranes in the isolated thylakoid membranes was not determined.

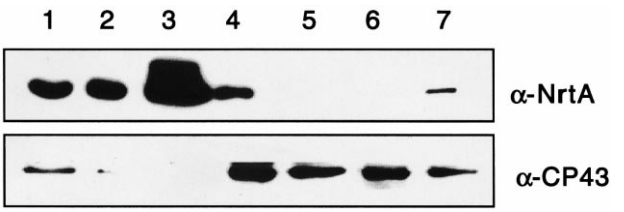


Fig. 2. Immunodetection of CP43 and NrtA proteins in different membrane fractions. Lane 1: Total membranes; lane 2: fraction T6; lane 3: isolated plasma membranes (fraction $T6_3$); lane 4: fraction B5; lane 5: isolated thylakoid membranes (fraction $B5_3$); lane 6: isolated thylakoid membranes (fraction $B5_4$); lane 7: isolated thylakoid membranes (fraction $B5_5$).

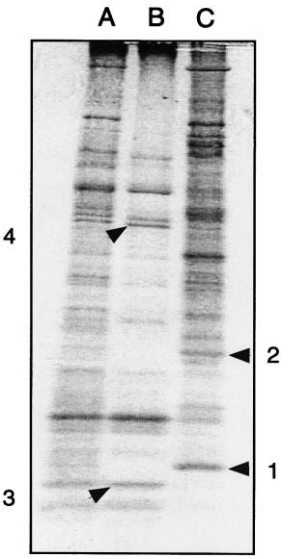


Fig. 3. SDS-PAGE analysis of the polypeptide compositions of various membrane fractions. Lane A: Total membranes; lane B: isolated thylakoid membranes (fraction $B5_3$); lane C: isolated plasma membranes (fraction $T6_3$). The arrows and numbers indicate polypeptides whose N-terminal sequences were determined by protein microsequencing.

Plasma membranes from the cyanobacterium *Phormidium laminosum* have been isolated using aqueous two-phase partitioning [15,16], a procedure that separates according to the surface properties of the membrane vesicles, such as charge and hydrophobicity rather than their size and densities [5]. The plasma membranes were virtually free of chlorophyll, demonstrating no cross-contamination by thylakoid membranes. Upon subsequent density gradient centrifugation, it was found that the majority of the plasma membranes had the same density as thylakoid membranes.

In the present work, both the thylakoid and plasma membranes from *Synechocystis* 6803 were isolated in pure form by a 2D-combination of aqueous polymer two-phase partitioning and sucrose density centrifugation. After the initial partitioning steps, practically pure plasma membranes were obtained in the final upper phase (T6) while thylakoid membranes were highly enriched in the final lower phase (B5). Subsequent sucrose gradient centrifugation of the plasma membranes (T6) and the enriched thylakoid membranes (B5) showed that both types of membranes contained subspecies of a variety of densities. Moreover, each plasma membrane fraction had a corresponding thylakoid membrane fraction of identical density, illustrating the experimental limitations in purifying cyanobacterial membranes by density gradient centrifugation only. However, by a combination of density centrifugation with the two-phase procedure, which has the ability to separate membrane vesicles according to their surface properties, pure plasma membrane (fraction $T6_3$) and thylakoid membranes (fractions $B5_3$ and $B5_4$) could be obtained (Fig. 1).

The plasma membranes which contained no detectable CP43 protein, and by that criterium were not contaminated by thylakoid membranes, did however contain small amounts of 'chlorophyll', the origin of which is unclear. Peschek et al. [11] have suggested that the residual 'chlorophyll' present in plasma membranes isolated from *Anacystis nidulans* could be

due to the presence of chlorophyll precursors. The thylakoid membranes of different densities isolated in this work from *Synechocystis* 6803 had a variation in their chlorophyll contents between 0.08 and 0.14 mg chlorophyll/mg protein (Fig. 1). Since this difference cannot be explained by contamination of plasma membrane, this observation may indicate a compositional heterogeneity within the thylakoid membrane. This suggestion is also supported by the fact that the thylakoid membrane fraction ($B5_4$) with no plasma membrane contamination (i.e. no detectable NrtA protein present) had a lower chlorophyll/protein ratio than the thylakoid fraction containing some residual NrtA ($B5_5$).

Synechocystis 6803 is an organism of significant genetic potential. However, biochemical and biophysical studies of this cyanobacterium have suffered due to a lack of reliable procedures for the purification of various subcellular components. The present work shows that by a combination of aqueous polymer two-phase partitioning and sucrose density centrifugation, plasma and thylakoid membranes from *Synechocystis* 6803 can be isolated in a highly pure state, and of a quality which allows for a proteomic approach, currently in progress, for the systematic localization of all putative membrane proteins. Such a protein-gene linkage map of the plasma and thylakoid membranes will have a great potential in further studies of various aspects related to cyanobacterial function and organization as well as biogenesis.

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