

Proteomics of *Synechocystis* sp. Strain PCC 6803

IDENTIFICATION OF PLASMA MEMBRANE PROTEINS*[§]

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Cyanobacteria are unique prokaryotes since they in addition to outer and plasma membranes contain the photosynthetic membranes (thylakoids). The plasma membranes of *Synechocystis* 6803, which can be completely purified by density centrifugation and polymer two-phase partitioning, have been found to be more complex than previously anticipated, *i.e.* they appear to be essential for assembly of the two photosystems. A proteomic approach for the characterization of cyanobacterial plasma membranes using two-dimensional gel electrophoresis and mass spectrometry analysis revealed a total of 57 different membrane proteins of which 17 are integral membrane spanning proteins. Among the 40 peripheral proteins 20 are located on the periplasmic side of the membrane, while 20 are on the cytoplasmic side. Among the proteins identified are subunits of the two photosystems as well as Vipp1, which has been suggested to be involved in vesicular transport between plasma and thylakoid membranes and is thus relevant to the possibility that plasma membranes are the initial site for photosystem biogenesis. Four subunits of the Pilus complex responsible for cell motility were also identified as well as several subunits of the TolC and TonB transport systems. Several periplasmic and ATP-binding proteins of ATP-binding cassette transporters were also identified as were two subunits of the F_o membrane part of the ATP synthase. *Molecular & Cellular Proteomics* 1:956–966, 2002.

Cyanobacteria are unique prokaryotes due to the presence of a differentiated membrane system. Similar to other Gram-negative bacteria, cyanobacteria have an envelope consisting of an outer membrane, a peptidoglycan layer, and a plasma membrane (1, 2). In addition, these organisms have a distinct

intracellular membrane system, the thylakoids, which are energy-transducing membranes and the site for both photosynthesis and respiration (2, 3). The role of the cyanobacterial plasma membrane as an energy-transducing membrane is also crucial since a large number of biological processes are coupled to transmembrane ion potentials or dependent on membrane-bound ATPases as well as the respiratory chain (4–6). The motility of the cell, nutrient uptake, and efflux pumps are also energy-dependent activities associated with the plasma membrane.

The unicellular, naturally transformable cyanobacterium *Synechocystis* sp. PCC 6803 (henceforth referred to as *Synechocystis*) has been widely used for genetic and biochemical studies of photosynthesis and other metabolic processes (7). It is the first photosynthetic organism for which the complete genome sequence is known (8). The genome contains 3168 open reading frames (ORFs).¹ However, for about 40% of these ORFs it is not known whether they are transcriptionally active, and the potential gene products are known only as hypothetical proteins. Furthermore the cellular localization of these hypothetical proteins is not established, and this is also the case for many of the other proteins with a suggested function deduced from homology comparisons.

Proteomic studies require, apart from the total genome knowledge, that each cellular compartment can be isolated without cross-contamination. As a crucial first step toward the analysis of the composition and function of the cyanobacterial plasma membrane, a procedure was developed for its complete biochemical purification (9–11). In this procedure, two separation techniques, one based on surface properties (aqueous polymer two-phase partitioning) and the other on densities (gradient centrifugation) of the membrane vesicles, are combined to yield completely pure plasma and thylakoid membranes from *Synechocystis* cells.

In the present work we have used the purified plasma membrane in proteome studies based on two-dimensional gel

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¹ The abbreviations used are: ORF, open reading frame; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; ASB-14, tetradecanoylamidopropyl dimethylammoniopropanesulfonate; PSI, photosystem I; PSII, photosystem II; Vipp1, vesicle-inducing protein in plastids; TAT, twin arginine translocation; EF-Tu, elongation factor Tu; Som, *Synechococcus* outer membrane; ABC, ATP-binding cassette.

electrophoresis, trypsin treatment of excised spots, and MALDI-TOF analysis with powerful database identification. The identified plasma membrane proteins (a total of 57) show the following predicted membrane topology: 17 integral membrane proteins and 40 peripheral proteins of which 20 are located on the periplasmic side and 20 are on the cytoplasmic side of the membrane.

EXPERIMENTAL PROCEDURES

Bacterial and Culture Conditions—Cells of *Synechocystis* 6803 were grown at 30 °C under 50 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ of white light in BG-11 medium (12). Liquid cultures were grown with vigorous bubbling with air. The cells were harvested at a density of $1.6\text{--}2.0 \times 10^8$ cells ml^{-1} .

Plasma Membrane Preparation—Plasma membranes from *Synechocystis* were purified according to Norling *et al.* (10) with the modification that sucrose density centrifugation was the first step and aqueous two-phase partitioning was the second step. Cells were broken with glass beads, and the total membranes were collected from the cell homogenate by centrifugation at $103,000 \times g$ for 30 min. The pellet was homogenized in 20 mM potassium phosphate (pH 7.8), and solid sucrose was added to a concentration of 42% (w/w). A discontinuous sucrose gradient containing 20 mM potassium phosphate (pH 7.8) was made consisting of 5 ml of 60% (w/w), 5 ml of 50% (w/w), 7 ml of the sample in 42% (w/w), 6 ml of 38% (w/w), 6 ml of 35% (w/w), 6 ml of 30% (w/w), and 1 ml of 10% (w/w) sucrose. The gradient was centrifuged at $131,500 \times g$ for 15 h at 4 °C. The membrane fraction from 38 to 42% sucrose density was collected and diluted 3-fold with 20 mM potassium phosphate (pH 7.8) followed by a centrifugation at $187,000 \times g$ for 45 min at 4 °C. The pelleted membranes were resuspended in a small volume of 0.25 M sucrose and 5 mM potassium phosphate (pH 7.8), and the plasma membrane was isolated by aqueous two-phase partitioning (10). Pure plasma membranes were recovered in the fifth top phase after three initial partitionings in a two-phase system consisting of 5.8% dextran T-500 and 5.8% polyethylene glycol 3350 and two more partitionings in a system containing 6.2% of both polymers (10).

The plasma membranes were washed with 0.1 M sodium carbonate (13). Plasma membranes containing 8 mg of protein were resuspended in 0.25 M sucrose and 5 mM potassium phosphate (pH 7.8) at a protein concentration of 50–60 mg/ml. Eight milliliters of 0.1 M sodium carbonate was added, and the membrane suspension was stirred for 30 min on ice. The mixture was centrifuged at $125,000 \times g$ for 40 min. The supernatant was removed, and the pellet was extracted with 0.1 M sodium carbonate for another 30 min. The pellet that resulted after the centrifugation was washed twice with 40 mM Tris to remove excess sodium carbonate. The final pellet of the membrane was resuspended in 0.25 M sucrose and 5 mM potassium phosphate (pH 7.8). Protein concentration was determined according to Peterson (14) using bovine serum albumin as a standard.

Two-dimensional Electrophoresis—Plasma membrane proteins were precipitated with methanol/chloroform according to the method described by Wessel (15). The material precipitated from 1–1.5 mg of membrane proteins was solubilized in 250 μl of an electrofocusing solution containing 7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 2 mM tributylphosphine, and 0.5% (v/v) immobilized pH gradient buffer, pH 3–10 (Amersham Biosciences). The mixture was incubated at room temperature for 1 h and then sonicated in the presence of protease inhibitor mixture (Sigma). After centrifugation at $9,000 \times g$ for 3 min the supernatant was applied onto a linear immobilized pH gradient strip (pH 4–7, 13 cm). The strip was rehydrated overnight at 20 °C. The isoelectric focusing was performed at the same temperature, and the running conditions were 300 V for 40 min, 500 V for 40 min, 1,000

V for 1 h, and 8,000 V until a total of 100,000 V-h was reached. The strip was equilibrated in a buffer described by Nouwens *et al.* (16) for 20 min and then loaded on the top of an SDS-polyacrylamide gel (12.5% polyacrylamide) prepared according to Laemmli (17) and sealed with 0.8% agarose. The electrophoresis was carried out at 5–6 °C and 5 mA/gel for 1 h and then 20 mA/gel using a Hoefer SE 600 apparatus (Amersham Biosciences). Proteins were detected by Coomassie Brilliant Blue G-250 according to Nouwens *et al.* (16) and then scanned using an image scanner and evaluated with the Image Master 2D Elite software (Amersham Biosciences).

MALDI-TOF Mass Spectrometry Analysis—MALDI-TOF analysis was performed in reflector mode on a Voyager-DE STR MALDI-TOF mass spectrometer from Applied Biosystems (Foster City, CA). In-gel trypsin digestion and sample preparation for MALDI-TOF analysis was done manually in a way similar to that described by Fulda *et al.* (18). Internal mass calibration was performed using trypsin autolysis products (842.5094 and 2211.1046 Da).

Database Search—The proteins were identified as the highest ranking result by searching in the National Center for Biotechnology Information (NCBI) database among all species using MS-Fit (prospector.ucsf.edu/ucsfhtml4.0u/msfit.htm). The search parameters allowed for oxidation of methionine, carbamidomethylation of cysteine, one miscleavage of trypsin, and 30 ppm mass accuracy. At least 50% of the measured masses must match the theoretical masses. Measured peptides masses could be excluded if their isotopic patterns were clearly atypical or if their masses corresponded to those of trypsin autolysis products or adjacent identified proteins on the gel. The identification of the proteins was repeated at least once using spots from different gels.

The presence of putative signal peptides and their cleavage sites were predicted using the SignalP program (www.cbs.dtu.dk/services/SignalP-2.0). The prediction of transmembrane helices in identified proteins was performed using the TMHMM program (www.cbs.dtu.dk/services/TMHMM-2.0). The lipoproteins were predicted using PROSITE (us.expasy.org/prosite).

RESULTS AND DISCUSSION

Isolation of Plasma Membrane, Two-dimensional Electrophoresis, and Protein Identification—Plasma membrane was purified from total membranes of *Synechocystis* by a combination of sucrose density centrifugation and aqueous two-phase partitioning (10). Plasma membranes collected from several cyanobacterial preparations were used in producing four two-dimensional gel maps from which spots were excised and trypsinized before subjection to MALDI-TOF analysis. In the first dimension an immobilized linear pH gradient pH 4–7 was used applying the nonionic detergent ASB-14, and in the second dimension SDS-PAGE (12.5%) was used. The four gels showed identical visual patterns after Coomassie Brilliant Blue staining revealing ~ 200 spots. One of these gels is shown in Fig. 1. From each of two of these four gels about 170 stained spots were analyzed. Proteins could be identified from about 110 spots, all together corresponding to 63 different gene products (Table I). Fig. 2 shows spectra of two identified proteins. In spectrum A (spot 28) a total of nine masses were used in the MS-Fit search and all of them fitted to SII0617, Vipp1 (vesicle-inducing protein in plastids). In spectrum B (spot 47) five of a total of eight masses fitted to SII1324, the b-subunit of the F_0 membrane part of ATP synthase.

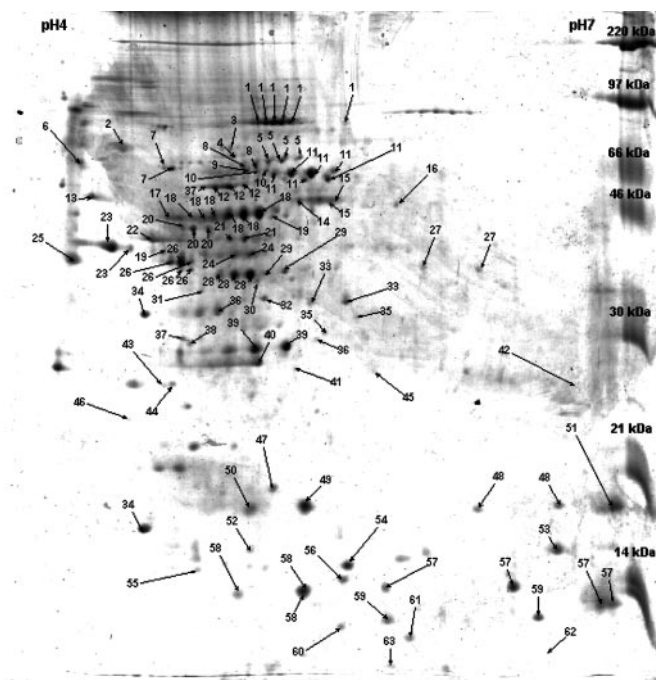


FIG. 1. Coomassie-stained two-dimensional gel map of *Synechocystis* plasma membrane proteins. The proteins were resolved by using a linear pH 4–7 immobilized pH gradient and 12.5% SDS-PAGE.

General Characterization of Identified Proteins—Proteins of the cyanobacterial plasma membrane can be of three types: integral hydrophobic membrane proteins and peripheral (extrinsic) hydrophilic proteins located on the outer (periplasmic) or inner (cytoplasmic) surface of the membrane. Peripheral proteins located on the inner side do not need an amino-terminal signal sequence for their cellular targeting, whereas peripheral proteins located at the outer side contain such a signal (19). In addition some integral membrane proteins have a signal peptide. Fig. 3 illustrates the distribution of the identified proteins according to membrane topology as will be discussed below.

About two-thirds of the proteins identified in the present work have known functions as judged either by genetic or biochemical studies or annotated from high sequence similarity. For many of these proteins their cellular localization has not been previously established. About one-third of the proteins are annotated as hypothetical proteins due to lack of sequence similarity with any other protein with known function, thus their functional significance in the plasma membrane remains to be elucidated. In the present work the expression and the localization of these hypothetical proteins are determined.

As can be seen in the gel map of Fig. 1 several proteins produced multiple spots probably due to post-translational modifications. In most cases the shift in position is horizontal, suggesting that the modification influences only the pI and leaves the molecular mass substantially unchanged. Thus, the

modifications are in the side chains of the amino acids rather than due to differential processing of the precursor molecules. These types of protein modifications are currently under investigation.

It is well known that there are limitations in the present technology for resolution and identification of integral membrane proteins by two-dimensional gel electrophoresis and MALDI-TOF analysis. Hydrophobic proteins are not easily solubilized in the nonionic detergents used for isoelectric focusing, and the hydrophobic fragments obtained after trypsin treatment are difficult to ionize for mass spectrometer analysis. By using the TMHMM program (20), currently the best performing transmembrane prediction program (21), only 17 integral membrane proteins could be identified in the isolated plasma membranes (Table II). Notably as many as 12 of these proteins only possess one single transmembrane helix, and the rest have two or three transmembrane helices. This means that a majority of the resolved and identified proteins are peripheral plasma membrane proteins (Fig. 3).

By applying the SignalP program for Gram-negative bacteria (22) all identified proteins were analyzed, and for 25 of them a signal peptide could be predicted (Table III). Only two of the identified integral membrane proteins have a signal peptide, cytochrome c oxidase subunit II (CoxB) and photosystem I subunit III (PsaF). As summarized in Table III typical Sec signal amino-termini (23, 24) were found in 13 of the identified proteins, while three proteins have the twin arginine signal peptide motif TAT (25, 26). For one of the hypothetical proteins (Slr1506) we suggest that the methionine which is in position 46 is the actual start of the protein since then a typical signal peptide is clearly predicted in the N terminus. For another hypothetical protein (Slr0431) a 41-amino acid, or a 30-amino acid, signal peptide could be predicted by the SignalP program (22). Among the three proteins with TAT signals, two (Slr0447 and Slr0513) have a “Sec-avoidance” positive charge in the carboxyl-terminal region just upstream of the signal peptidase cleavage site (27, 28). We found earlier that the periplasmic proteins of *Synechocystis* contain both Sec and TAT signals (18).

Leader peptidase type I is the processing enzyme for proteins with Sec and TAT signals (29). In *Escherichia coli* there is only one copy of this enzyme. The genome of *Synechocystis* contains two predicted leader peptidases type I with similar size and a high homology, indicating that a gene duplication has occurred. However, only one of these two genes seems to be expressed since only the gene product of *slr1377* (spot 35) was found in the present work. In *E. coli* the leader peptidase type I has two transmembrane helices, whereas in the Gram-positive bacterium *Bacillus subtilis* (30) this protease is considerably smaller and contains only one single transmembrane helix. Thus, the *Synechocystis* leader peptidase type I resembles the enzyme in Gram-positive bacteria in this respect.

Nine of the identified proteins have an N terminus with a

TABLE I
 Proteins identified in plasma membranes of *Synechocystis*

No., spot number in Fig. 1; predict., predicted; exper., experimental; theor., theoretical.

No.	ORF	Gene product	Matched peptides/total	Mature protein	
				pI predict./exper.	Mass theor./exper.
1	SII1021	Hypothetical	7/11	5.1/5.2–5.4	74.4/80
2	Slr1841	Putative porin	4/7	4.4/4.5	65.0/70
3	SII0416	60-kDa chaperonin 2 GroEL-2	4/6	4.9/5.1	57.8/66
4	SII1053	Membrane fusion protein MtrC	6/7	4.9/5.1	56.3/64
5	SII1699	Periplasmic oligopeptide-binding protein	7/11	5.2/5.3–5.6	64.5/63
6	Slr1624	Hypothetical	8/14	4.2/4.3	46.2/60
7	Slr1270	TolC	10/14	4.7/4.7,4.8	53.6/60
8	SII1326	ATP synthase α chain	5/7	5.0/5.1,5.2	54.0/60
9	SII0141	Membrane fusion protein	9/14	5.0/5.2	47.9/60
10	Slr1506	Hypothetical	8/10	5.1/5.2	61.1/60
11	SII0180	Membrane fusion protein	10/12	5.3/5.3–5.6	52.0/59
12	Slr1908	Putative porin	8/10	4.9/4.9–5.2	61.6/55
13	Slr1220	Hypothetical	5/6	4.2/4.4	36.0/54
14	Slr0872	Hypothetical	3/4	5.3/5.3	41.5/54
15	SII1099	EF-Tu	11/15	5.2/5.5	43.7/54
16	SII0606	Hypothetical	6/7	5.8/5.9	50.7/50
17	Slr0447	Putative periplasmic binding protein	4/7	4.7/4.8	45.5/46
18	Slr0040	Bicarbonate transporter substrate-binding protein CmpA	6/9	5.1/4.9–5.2	46.8/46
19	Slr1751	Carboxyl-terminal protease CtpC	16/17	4.9/5.2	44.4/46
20	SII0752	Hypothetical	7/9	4.8/4.8–5.0	31.4/45
21	Slr1897	SrrA protein	7/7	5.0/5.0,5.1	43.7/42
22	Slr1274	Membrane protein PilM	10/11	4.5/4.7	40.9/42
23	Slr1275	PilN	3/4	4.5/4.5	30.0/41
24	Slr0151	Hypothetical	6/7	5.0/5.1	34.9/41
25	Slr1276	PilO	6/7	4.2/4.3	30.1/39
26	Slr1295	Periplasmic iron-binding protein FutA1	6/7	4.8/4.8	36.3/39
27	SII0034	Hypothetical	4/6	5.8/6.0,6.3	28.6/39
28	SII0617	Vipp1 Im30	9/9	5.0/5.0–5.1	28.9/38
29	Slr0513	Periplasmic iron-binding protein FutA2	8/9	5.2/5.2,5.3	34.9/38
30	SII1363	Ketol-acid reductoisomerase IlvC	3/4	5.0/5.1	40.0/37
31	Slr1319	Iron(III) dicitrate periplasmic binding protein FecB	4/5	4.8/4.9	33.0/36
32	Slr1106	Prohibitin	6/9	5.2/5.2	30.6/35
33	Slr1768	Hypothetical	7/9	5.6/5.0,5.2	31.8/34
34	Slr0875	Large conductance mechanosensitive channel MscL	3/5	4.7/4.6	15.8/33
34	Slr0875		4/5	4.7/4.6	15.8/17
35	Slr1377	Leader peptidase type I LepB	6/8	5.4/5.2,5.3	24.7/33
36	SII1041	ATP-binding protein of ABC transporter	6/8	5.4/5.2	28.7/30
37	SII0427	PsbO	3/4	4.7/4.8	26.9/30
38	Slr0431	Hypothetical	7/8	4.8/4.8	22.5/30
39	Slr1258	Hypothetical	4/7	4.8/5.2,5.4	23.7/29
40	Slr0677	ExbB protein, TolQ	3/5	5.1/5.2	22.1/28
41	Slr1128	Stomatin-like protein	4/6	5.7/5.4	33.8/28
42	SII0947	Light-repressed protein, LrtA	6/6	6.1/6.6	21.9/27
43	SII1835	Hypothetical	3/5	4.7/4.8	25.0/27
44	Slr1730	Potassium-transporting ATPase C chain, KdpC	4/6	4.5/4.8	17.8/27
45	Slr1881	High affinity branched-chain amino acid transport ATP-binding protein BraG (LivF)	6/7	5.7/5.8	25.7/27
46	SII0749	Hypothetical	4/4	4.5/4.6	17.9/25
47	SII1324	ATP synthase subunit b AtpF	5/8	5.1/5.2	19.8/19
48	SII0813	Cytochrome c oxidase subunit II CoxB	4/7	6.0/6.2,6.6	29.5/19
49	Slr0695	Hypothetical lipoprotein	5/10	5.5/5.4	16.2/18
50	SII1578	Phycocyanin α chain	4/5	5.3/5.2	17.6/18
51	Slr0737	Photosystem I subunit II PsaD	4/7	8.9/7.0	15.7/19
52	SII1323	ATP synthase subunit b' AtpG	5/8	5.2/5.1	16.2/16
53	SII0819	Photosystem I subunit III PsaF	5/8	6.1/6.6	15.7/16
54	Slr0013	Hypothetical	8/9	6.5/5.6	13.7/15
55	SII0565	Hypothetical	3/5	4.6/4.9	15.2/15
56	SII1405	ExbD protein, TolR	3/4	9.0/5.6	15.6/14
57	Slr1513	Hypothetical	4/6	6.6/5.7–7.0	12.0/14
58	SII1638	Hypothetical lipoprotein	4/5	5.6/5.1,5.4	14.2/14
59	Ssl0707	Nitrogen regulatory protein P-II GlnB	6/7	6.6/5.7,6.5	12.4/12
60	SII1028	Carbon dioxide-concentrating mechanism protein CcmK	4/7	5.3/5.6	11.1/12
61	SII1118	Hypothetical	6/6	5.6/5.9	11.5/12
62	SII1029	Carbon dioxide-concentrating mechanism protein CcmK	5/6	6.1/6.5	12.1/10
63	Ssl0563	PsaC	2/3	5.7/6.0	8.8/9

FIG. 2. MALDI-TOF mass spectra of spot numbers 28 and 47 from the two-dimensional gel map of *Synechocystis* plasma membrane proteins. A, the MALDI-TOF mass spectrum of peptides generated by tryptic digestion of protein spot 28. B, the MALDI-TOF mass spectrum of peptides generated by tryptic digestion of protein spot 47. The spectra were internally calibrated using the trypsin-autodigested peptides 842.5094 and 2211.1046. Spot 28 matched Vipp1, and spot 47 matched the b-subunit of the F₀ membrane part of ATP synthase.

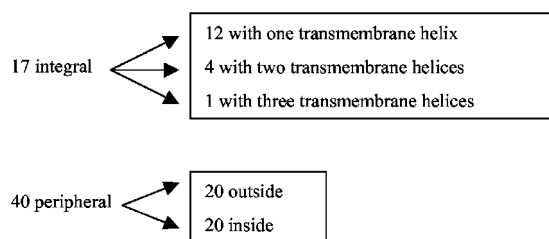
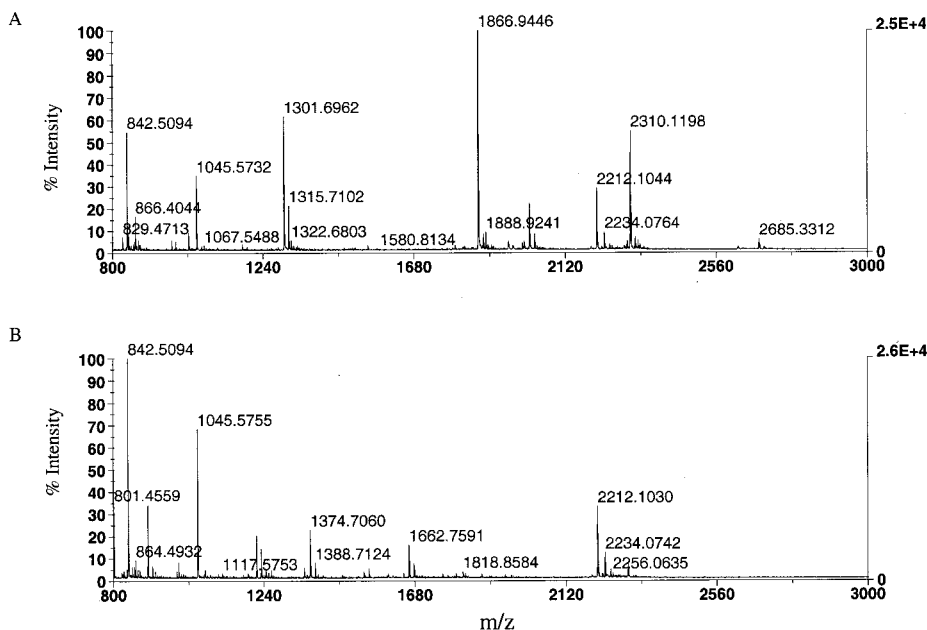


FIG. 3. Predicted topology of identified plasma membrane proteins of *Synechocystis*. Integral membrane proteins were predicted using the program TMHMM (www.cbs.dtu.dk/services/TMHMM-2.0), and peripheral proteins were predicted by the SignalP program (www.cbs.dtu.dk/services/SignalP-2.0).

consensus pattern for lipoproteins (31), and four of these have a twin arginine motif (Table III). After cleavage of the amino-terminal signal peptide, lipoproteins are anchored to the plasma or outer membrane through fatty acyl chains covalently attached to the amino-terminal cysteine of the mature protein (31). For *E. coli*, aspartate at position 2 has been proposed to function as a lipoprotein sorting signal that retains the lipoprotein to the plasma membrane (32). Also the amino acid at position 3 is important for retention of the lipoprotein in the plasma membrane. The strongest plasma membrane retention signals in *E. coli* are Asp-Asp, Asp-Glu, and Asp-Gln. This lipoprotein sorting signal does not seem to be valid in *Synechocystis* since none of the eight proteins containing a consensus lipoprotein signal peptide has aspartate in position 2. Among the nine proteins six have alanine, serine, or glycine, which are amino acids with a small side chain. By analyzing the whole *Synechocystis* genome for the consensus lipoprotein signal peptide (31) 39 predicted lipoproteins can be found (not shown). None of these proteins has aspartate at position 2. Glycine is at position 2 in 11 of

these proteins; alanine, asparagine, and leucine are each in five proteins; valine is in four proteins; and serine and glutamine are each in three proteins. In *E. coli*, two proteins have been identified that appear to be involved in localization of lipoproteins to the outer membrane (33). A periplasmic chaperone, LolA, is suggested to transport lipoproteins to the outer membrane, and a second protein, LolB, is suggested to be involved in the incorporation of murein lipoprotein into the outer membrane. No LolA or LolB sequence homologues are present in the genome of the *Synechocystis* or *Anabaena* sp. PCC 7120 for which the genome is also sequenced (34). Thus, cyanobacteria do not have the same mechanism for the sorting of lipoproteins to the plasma and outer membranes as inferred for *E. coli*.

The isolated plasma membrane fraction is composed of closed vesicles of the same orientation as in the cyanobacterial cell.² After sodium carbonate washing proteins captured inside the vesicles are released (13). Proteins such as EF-Tu (spot 15), phycocyanin (spot 50), and GroEL-2 (spot 3), all of which are known to be present in high abundance in the cytoplasm, were detected in the plasma membrane preparation. No other cytoplasmic protein with known function was detected in the plasma membrane preparation. This indicates that the hypothetical proteins, with no predicted signal peptide, identified in this work represent peripheral proteins attached to the inside of the plasma membrane.

Subunits of Photosystem I and II—It is well established that the site of photosynthetic electron transport in cyanobacteria is in the thylakoid membrane (1, 2). Both PSI and PSII consist of numerous protein components in addition to the reaction

² B. Norling, L. Zak, Y. Migashita, B. Andersson, and H. B. Pakrasi, unpublished results.

TABLE II
Integral membrane proteins identified in the plasma membrane of *Synechocystis*

Position of transmembrane helices predicted by the TMHMM program (20).

ORF	Gene product	Position of transmembrane helices
Slr0677	ExbB protein, TolQ	13–35, 111–135, 153–175
Slr0875	Large conductance mechanosensitive channel MscL	30–52, 72–94
Slr1106	Prohibitin	13–35
Slr1275	PilN	39–61
Slr1276	PilO	37–59
Slr1377	Leader peptidase type I LepB	21–43
Slr1730	Potassium-transporting ATPase C chain KdpC	13–35
Slr1768	Hypothetical	1–20, 31–52
SII0034	Hypothetical	40–59
SII0606	Hypothetical	19–41
SII0813	Cytochrome c oxidase subunit II CoxB	48–70, 91–113
SII0819	Photosystem I subunit III PsaF	88–110, 123–145
SII1021	Hypothetical	60–82
SII1053	Membrane fusion protein MtrC	40–59
SII1323	ATP synthase subunit b' AtpG	10–27
SII1324	ATP synthase subunit b AtpF	30–50
SII1405	ExbD protein, TolR	21–43

center subunits containing the functionally important cofactors (35, 36). Unexpectedly we have recently demonstrated (37), using immunoblotting analysis, that the plasma membrane from *Synechocystis* contains a number of proteins that are intimately associated with the reaction center of PSI (PsaA, PsaB, PsaC, and PsaD) or PSII (D1, D2, cytb559 α , cytb559 β , PsbO, and CtpA), thereby corroborating an earlier study by Smith and Howe (38) who demonstrated the presence of D1, D2, and PsbO in a cyanobacterial plasma membrane fraction. Other subunits (e.g. CP43 and CP47) were located exclusively in the thylakoid membrane (37). Moreover, these photosynthetic subunits present in the plasma membrane were assembled as chlorophyll-containing multiprotein complexes, verified from non-denaturing green gels and low temperature fluorescence spectrometry data. Electron paramagnetic resonance spectroscopic analysis also showed that the partial assembled PSI complex was able to perform light-induced charge separation. Based on these results it has been proposed that the plasma membrane is the primary site for early steps in the biogenesis of the photosynthetic reaction center complexes in cyanobacteria (37, 38). Subsequently these reaction center assemblies were suggested to be translocated to the thylakoid membrane, possibly by membrane vesicle transport or by lateral movement through connecting membranes. In this work we verify the presence of the extrinsic subunits PsaC (spot 63) and PsaD (spot 51) of PSI and PsbO (spot 37) of PSII in the plasma membrane. In addition PsaF (spot 53), with two transmembrane helices, was identified in the plasma membrane. Other subunits (D1, D2, PsaA, and PsaB), which were previously detected by immunoblotting of one-dimensional SDS-PAGE (37), contain several transmembrane helices and are therefore difficult to resolve in the present two-dimensional gel system.

Considering the possibility of a two-membrane biogenesis

mechanism it is interesting to note that the Vipp1 protein was found in the plasma membrane (spot 28). Fig. 2A shows the MALDI-TOF mass spectrum of trypsinized Vipp1. Vipp1 has been suggested (39, 40) to be involved in membrane vesicle transport from the plasma membrane of cyanobacteria, or from the inner envelope membrane of chloroplasts, during biogenesis of thylakoid membranes.

A carboxyl-terminal protease, Slr1751 (spot 19), is present in the plasma membrane. This protease is highly homologous (4e-90) with the D1 carboxyl-terminal processing protease CtpA (37, 41) and also with another putative carboxyl-terminal protease, CtpB (7e-96), in *Synechocystis*. Slr1751 is therefore annotated as CtpC (42, 43).

ATP Synthase and Respiratory Chain—There are reports on the presence of ATP synthase and respiratory chain components in plasma membrane of different cyanobacterial species (4, 5). However, in those previous studies the purity of the plasma membrane preparation can be questioned since separation of plasma and thylakoid membrane vesicles using only sucrose density centrifugation is not complete as initially demonstrated by Norling *et al.* (9) and later confirmed by other groups (44, 45). In our plasma membrane preparation, which is completely free of contaminating thylakoid membranes (10), we found subunits b (SII1324, spot 47) and b' (SII1323, spot 52), which are integral F₀ membrane proteins of the ATP synthase. Fig. 2B shows the MALDI-TOF mass spectrum of trypsinized protein from spot 47. Although the plasma membranes were washed with 0.1 M sodium carbonate the α -subunit of the peripheral F₁ complex was also found (spot 8). In gels from samples that were not washed both the α - and β -subunits were found (not shown).

The presence of a cytochrome oxidase in the cyanobacterial plasma membrane demonstrates the presence of the respiratory chain in this membrane. Cytochrome oxidase activity

TABLE III
Prediction of signal peptides in proteins identified in the plasma membrane of *Synechocystis*

aa represents the number of amino acids in the signal peptide, hydrophobic regions are underlined, cleavage sites are represented by -, twin arginines are in bold, and Sec-avoidance is in italic.

ORF	Gene product	Signal sequence	aa
		Sec signal	
Slr0013	Hypothetical	MKLIDSRGRIFGIVSLLDLGAALILMVAVGIFVLP <u>GGSSGKSILAQANA</u> -AS	49
Slr0431	Hypothetical	MRPKFFSRRT	41
		M*GISKLSKFSASVLLSGAILTTLPPSPLWA-NE	30 ^a
Slr1258	Hypothetical	MSTIKALLPPKFPQLLTGLALLSLVSTAIA-AK	33
Slr1270	ToIC	MKSIHPLKFWSSSTLLLLLSTSVGVFLPGFSGGQGAIVA-QS	40
Slr1624	Hypothetical	MLDRHWHNQNNCRPSYWSHVTTVLTICLLAIAMGLGGCQSLSA-SS	43
Slr1751	Carboxyl-terminal protease CtpC	MLKQKRSLILGTTALLTTVAVT-GV	23
Slr1841	Putative porin	MLKLSWKSLLVSPAVIGAAALVAGAASA-AP	27
Slr1908	Putative porin	MNKLTSHLLKLFPLALGSSLAIVPGAMA-QS	28
SII0427	PsbO	MRFRPSIVALLSVCFGLLTFLYSGSAFA-VD	28
SII0749	Hypothetical	MEKIMSEQKSSSSTGFALAAALMVALVGTGFVAF-WT	32
SII0813	Cytochrome c oxidase subunit II CoxB	MSRKNLILLAVYIVFTVVGASLWLGQRAYQWLPPAAA-QE	36
SII0819	PsaF	MKHLALLLAFTLWFNFAPSASA-DD	23
SII1835	Hypothetical	MATHNLDRAAPLISKLFPPFLVLAGMFSGLTAAQA-QG	36
		TAT signal	
Slr0447	Putative periplasmic binding protein	MTNPFGRRKFLLYGSATLGASLLKA-CG	26
Slr0513	Periplasmic iron-binding protein FutA2	MTTKISRRTFFVGGTALTALVWANLPRRASA-QS	31
Slr1506	Hypothetical (Met-46 as start)	MVTFPLNLRRLQSVCLGALTAIA-VQ	24
		Lipoprotein	
Slr0695	Hypothetical	MRKRLTRFLSLALVLGLLWFGTAA-CASQP	24
Slr1295	Periplasmic iron-binding protein FutA1	MVQKLSRRFLSIGTAFTVVVGSQQLSS-CGQSP	28
Slr1319	Iron(III) dicitrate periplasmic binding protein FecB	MKSKLIIFTFCLVLFQ-CAKQV	16
Slr1897	SrrA	MVSWCRWRSPRRWFLFACLGLLSGLIS-CQSNS	28
SII0141	Membrane fusion protein	MNKYIPHQRLRRQLSLLGLLSFLMG-CSDLW	26
SII0180	Membrane fusion protein	MVRKRSQFPVIGSMVALALLNTA-CGGDK	25
Slr0040	Bicarbonate transporter substrate-binding protein CmpA	MGSFNRRKFLLLSAATATGALFLKG-CAGNP	25
SII1638	Hypothetical	MSRLRSLLSLILVLTTLVLS-CSSPQ	22
SII1699	Periplasmic oligopeptide-binding protein	MRWGNKVAM*SRVAGQRKTAIAREKNPGQQNYLSGRSWGQKLIS- ALLCCLALTFSLGG-CFSPE	57 49 ^a

^a Assuming that the second methionine represents the real translational starting point.

was previously detected in pure plasma membranes of *Phormidium laminosum* (9), corroborating results from less pure plasma membrane preparations (4). In the present work we found subunit II (SII0813, spot 48), one of the two cofactor-binding subunits of cytochrome oxidase. Subunit II has two transmembrane helices, whereas subunits I and III of the oxidase have 12 and 5, respectively, hampering their resolution in two-dimensional gels.

Pilus Proteins—Several species of both filamentous and unicellular cyanobacteria are motile. The filamentous cyanobacteria show gliding motility, whereas the unicellular *Synechocystis* shows sporadic motility of twitching (46). In the Gram-negative pathogen *Pseudomonas aeruginosa*, the type IV pilus has been shown to be responsible for the twitching motility (47). In *Synechocystis* there is an operon, *pilMNOQ*, of *pil* genes and two other *pil* genes, *pilA1* and *-B1*, that are homologous to the type IV pilus genes in *P. aeruginosa* (48). We found four of the *pil* gene products in the plasma membrane, namely PilM (spot 22), PilN (spot 23), PilO (spot 25), and PilA1 (SII1694). The latter was previously identified by Norling *et al.* (10) in plasma membranes of *Synechocystis* by

SDS-PAGE using amino-terminal sequencing and is also found in one of the four two-dimensional gels investigated in the present work (not shown). Furthermore, we have also identified in outer membranes of *Synechocystis* the PilQ protein, Slr1277.³ Mutation in *pilA1* causes loss of motility (49), and mutation in any of the genes of the *pilMNOQ* operon leads to loss of motility as well as competence for DNA uptake (48).

ToIC Efflux Pump and Porins—In Gram-negative bacteria small molecules up to large proteins can be exported directly across both the plasma and outer membranes. The efflux is brought about by reversible interaction of substrate-specific plasma membrane proteins with an outer membrane protein of the ToIC family, thus bypassing the intervening periplasm (50). The ToIC homologue Slr1270 of *Synechocystis* (51) is found in three compartments, the periplasm (18), the plasma membrane (this work, spot 7), and the outer membrane.³ The crystal structure of ToIC from *E. coli* has been resolved to

³ F. Huang, E. Hedman, C. Funk, T. Kieselbach, W. Schröder, and B. Norling, unpublished results.

2.1-Å resolution (50). Three TolC monomers assemble to a continuous channel that is suggested to span both the outer membrane as a β -barrel and the periplasmic space as an α -helical barrel, a novel substructure. In *E. coli* association of the periplasmic end of the TolC channel to the integral plasma membrane translocase is mediated by a membrane fusion protein that is either anchored in the plasma membrane with one transmembrane helix or as a lipoprotein (51, 52). Four ORFs coding for membrane fusion proteins (or periplasmic efflux proteins) are predicted in the genome of *Synechocystis* (52). We detected three of these in the plasma membrane: SII0141 (spot 9) and SII0180 (spot 11), which are both lipoproteins (Table III), and SII1053 (spot 4), which has one single transmembrane helix (Table II).

The presence of TolC in the periplasmic fraction and the plasma membranes could be a result of transient translocation of newly synthesized TolC from the cytoplasm to the outer membrane. The presence in the plasma membrane of two putative outer membrane porins (Slr1841, spot 2 and Slr1908, spot 12) may be explained in a similar manner. Slr1841 and Slr1908 were found to have a significant homology with the sequences of SomA/SomB (*Synechococcus* outer membrane) proteins (53). SomA and SomB have been isolated from the outer membrane of *Synechococcus* 6301 and characterized as pore-forming proteins (54). The presence of these outer membrane porins in the plasma membrane preparation cannot simply be explained by contamination of outer membranes since four of the most abundant proteins in outer membranes are not found in the plasma membrane preparation.³

TonB Exb and Tol-Pal Systems—The TonB Exb and Tol-Pal systems are able to couple the cytoplasmic membrane proton gradient to energy-requiring processes (55, 56) and thus energize active transport across the outer membrane. In *E. coli* and related Gram-negative bacteria both systems, which are organized in operons, contain three homologous integral plasma membrane proteins, TonB/TolA, ExbB/TolQ, and ExbD/TolR (55). In the present preparation of plasma membranes we found putative homologues of ExbB/TolQ (Slr0677, spot 40) and of ExbD/TolR (SII1405, spot 56). ExbB/TolQ has three predicted transmembrane helices, and ExbD/TolR has one (Table II), which is the same membrane topology as the corresponding *E. coli* proteins. Interestingly *sll1405* is part of one operon (*sll1404/sll1405/sll1406*) coding for the ExbB and ExbD proteins and the FhuA protein, which is the outer membrane part of the TonB Exb system, whereas *slr0677* is part of another operon *slr0677/slr0678*, consisting of genes coding for ExbB- and ExbD-like proteins. Thus, we demonstrate that both operons are expressed in *Synechocystis*. No TonB/TolA homologue is found in the genome of *Synechocystis*. Studies on the organization and evolution of the *tol-pal* gene cluster (57) showed that gene pairs homologous to *tolQ* and *tolR* are more widespread throughout the eubacteria than the entire *tol-pal* cluster. However, it was also found that there is a lack

of conservation of the TolA sequence, which could explain why there is no TonB/TolA homologue found in the *Synechocystis* genome.

ABC Transporters, Cation ATPases, and Ion Channel—Several binding proteins of ABC transporters were found in the plasma membrane. Five of them have earlier been identified only by sequence homology with binding proteins in eubacteria. Thus, the present work demonstrates, for the first time, that these proteins are present in the plasma membrane of *Synechocystis*. The proteins are: SII1699 (spot 5), periplasmic oligopeptide-binding protein; Slr1881 (spot 45), high affinity branched-chain amino acid transport ATP-binding protein, BraG; Slr1319 (spot 31), periplasmic binding protein of citrate-dependent iron(III) transport system, FecB (58); SII1041 (spot 36), putative ABC transporter ATP-binding protein; Slr0447 (spot 17), putative periplasmic binding protein. The physiological functions of these newly experimentally defined proteins remain to be elucidated.

In *Synechocystis* genes essential to iron transport have been studied (58). Two of these gene products, FutA1 and FutA2, both periplasmic binding proteins, were found in the plasma membrane (spots 26 and 29). CmpA, the substrate binding subunit of the bicarbonate transporter (59) was also identified (spot 18).

Four subunits of the putative K⁺-transporting P-type ATPase are found as an operon, *kdpABCD*, in the *Synechocystis* genome. In *E. coli* a similar operon is present (60), and it has been shown that KdpA is involved in binding and transport of K⁺ and that KdpB is the catalytic subunit. KdpA and KdpB of *Synechocystis* have the same predicted secondary transmembrane structure as the *E. coli* subunits. KdpC (Slr1730), which is present in the plasma membrane (spot 44) of *Synechocystis*, also has the same predicted secondary structure as the *E. coli* counterpart with one transmembrane helix in the amino-terminal end anchored in the plasma membrane exposing the C terminus to the cytoplasm (61).

In our gel map of plasma membrane proteins we found the gene product of *mscL* (*slr0875*), the large conductance mechanosensitive channel, which responds to mechanical stress applied to the cell membrane (62). It is present as two spots (spots 34) with the same isoelectric point but two different molecular masses, 17 and 33 kDa, respectively. The theoretical mass of the gene product of *slr0875* is 15.8 kDa. The best characterized member of these kind of channels is the prokaryotic large conductance mechanosensitive channel (MscL) that was originally identified, isolated, and characterized in *E. coli* (62). The crystal structure of MscL from *Mycobacterium tuberculosis* shows that the protein is organized as a homopentamer (63). Each monomer contains two transmembrane helices, and in the three dimensional structure it has been found that there are substantial intermolecular contacts between the first transmembrane helix in one monomer and the second transmembrane helix in the adjacent subunit. Of our two spots of MscL, the lower molecular mass correlates

well with the theoretical molecular mass of the monomer, and the experimentally determined pI (4.6) correlates with the predicted pI value (4.7). The higher molecular weight protein may represent a dimer (with the same isoelectric point), which is not dissociated in the SDS solubilizing buffer.

Regulatory Proteins—The family of PII signal transduction proteins contains the most highly conserved signaling proteins in nature (64). The cyanobacterial PII homologue transmits signals of the cellular nitrogen and carbon status through phosphorylation of a serine residue (65). In the present work we found that *Synechocystis* PII (Ssl0707) is bound to the plasma membrane (spot 59). The PII protein is present in the gel as two spots with equal staining, one corresponding to a pI value of 6.5 and one to pI 5.7. The predicted pI value from the amino acid sequence is 6.6. The two spots may represent the unphosphorylated (pI 6.5) and phosphorylated (pI 5.7) forms, respectively. Phosphorylation of PII and binding of 2-oxoglutarate have been proposed to be the main switch for the short term control of nitrate/nitrite uptake in *Synechococcus* sp PCC 7942 (66). The direct target(s) of PII regulation of the nitrate/nitrite uptake system is currently unknown. It has, however, been shown (67) that deletion of the carboxyl-terminal part of NrtC, the ATP-binding subunit of the nitrate/nitrite ABC-transporter, partially relieves the inhibition by ammonium, which is thought to be transmitted by PII. Our results, demonstrating that PII is bound to the plasma membrane, support the idea that NrtC is the target for PII.

The transcriptional regulator gene *srrA* has been studied in *Synechococcus* 7942 (68). An SrrA homologue in *Synechocystis* was found in the plasma membrane (spot 21). Although SrrA resembles the OmpR class response regulators of the typical bacterial two-component system, it differs in several aspects (68). It is unusually large, it is a membrane-anchored lipoprotein according to sequence prediction, and it lacks the conserved aspartate, the usual site for phosphorylation by a histidine kinase. However, similar to the OmpR class response regulators, it has the DNA-binding domain in the carboxyl terminus. It is speculated (68) that SrrA via the putative membrane attachment site in the amino-terminus is placed in the plasma membrane in a position to directly sense an environmental signal. Our present localization of SrrA to the plasma membrane supports such a functional assignment of SrrA suggested from the protein structure (68).

Miscellaneous Proteins—In *Synechococcus* sp. strain PCC 7942 there are three genes in sequence, which have been named *ccm* genes (*ccmK*, *ccmL*, and *ccmM*) on the basis that insertional mutagenesis of each gene produces a phenotype requiring a high level of CO₂ (69). In the genome of *Synechocystis* the gene arrangement is as follows: two gene copies of *ccmK* (*sll1028* and *sll1029*) and one each of *ccmL* (*sll1030*) and *ccmM* (*sll1031*). The gene product of both copies of *ccmK* were found in the plasma membrane (spots 60 and 62).

Prohibitins, stomatins, and a group of plant defense genes have been demonstrated to belong to a novel protein super-

family (70). In the plasma membrane of *Synechocystis* we found a hypothetical protein with a stomatin-like structure (Slr1128, spot 41) and prohibitin (Slr1106, spot 32). Members of this superfamily are involved in diverse functions, but their structural similarity suggests a conserved molecular mechanism, which has been proposed to be ion channel regulation (70).

Final Remarks—From systematic hydropathy analyses of all ORFs in the genome of *Synechocystis* it has been proposed that 35% of the predicted proteins are integral membrane proteins with one or more transmembrane helices (71). Thus if expressed, about 1,000 integral proteins should be distributed among the three membranes of *Synechocystis* cells. In another study a bimodality was found when calculated molecular weight was plotted against predicted pI values for all proteins in the *E. coli* genome. Two peaks were centered around pI 5.5 and pI 9, respectively (72). Furthermore, when predicted pI values were correlated to subcellular localization, it was found that most integral proteins have high pI values, whereas cytoplasmic proteins have low pI values (73). Hence it was suggested that the two peaks represent soluble and integral membrane proteins, respectively. In the present investigation we have identified 17 integral membrane proteins in the pI range of 4–7. The low number is, as discussed earlier, partly due to well established problems in solubilizing the integral membrane proteins in detergents suitable for isoelectric focusing and difficulties in ionizing hydrophobic peptides for mass spectroscopy (74–76). In the light of the observation of Schwartz *et al.* (73) it can, however, be expected that many additional integral membrane proteins will be resolved in two-dimensional maps encompassing pI values higher than pH 7, which was the upper limit in the present two-dimensional electrophoresis system. Furthermore, SDS-PAGE separation of hydrophobic proteins extracted by organic solvents in combination with electrospray ionization and tandem mass spectrometry analysis seems at present to be the best method to identify integral membrane proteins. In a recent study on chloroplast inner envelope 27 proteins with multiple α -helical transmembrane regions were identified with such an approach (77). We have initiated investigations using this procedure for identification of integral membrane proteins of the plasma membrane of *Synechocystis* 6803.

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§ The on-line version of this article (available at <http://www.mcponline.org>) contains supplemental data showing MALDI MS spectra of all identified proteins as pdf-files.

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