Penicillin-binding Proteins in the Unicellular Cyanobacterium *Synechococcus* sp. Strain R2 (PCC 7942): Implications for Membrane Identification

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The isolation and identification of cytoplasmic membranes from cyanobacteria is complicated by the presence of multiple photosynthetic membranes (thylakoids), and by a lack of biochemical markers characteristic of the cytoplasmic membranes of heterotrophic prokaryotes. [³H]Benzylpenicillin was used to label the penicillin-binding proteins (PBPs) in the unicellular cyanobacterium *Synechococcus* sp. strain R2 (PCC 7942), in spheroplasts and in fractionated cell membranes. Spheroplasts were made with lysozyme/EDTA, and were broken by shaking with glass beads in hypo-osmotic buffer at 4 °C. Sequential glycerol density gradients were then used to purify the membrane fractions. A low-density membrane (LDM) fraction was isolated which, when compared to the thylakoid fraction, was enriched in a unique carotenoid with an absorption maximum at 390 nm and low in chlorophyll *a* absorption at 678 nm ($A_{390}/A_{678} > 10$). This fraction had a characteristic polypeptide profile on denaturing polyacrylamide gels (SDS-PAGE), and specific enrichment of PBPs, as detected by fluorography of membrane fractions incubated with [³H]benzylpenicillin followed by SDS-PAGE. The M_r values of the *Synechococcus* PBPs differed from those in *Staphylococcus aureus* or *Escherichia coli* membranes. The high content of specific PBPs in LDM suggested that this fraction was highly enriched in cytoplasmic membrane. A membrane fraction with density between that of the thylakoid and LDM fractions, previously considered to be a mixture of these membranes, showed specific enrichment of a PBP of M_r 78000. This fraction may contain contact zones between the different membrane types.

INTRODUCTION

The organization of membranes in cyanobacteria is complex, and three distinct locations are usually obvious in thin-section electron micrographs: an outer component of the cell envelope, a limiting layer to the cytoplasmic compartment, and intracytoplasmically as concentric rings of paired thylakoids in a species-specific arrangement. Procedures for separating a low-density membrane fraction from both filamentous (Lockau & Pfeffer, 1982, 1983) and unicellular cyanobacteria (Omata & Murata, 1982, 1984; Molitor *et al.*, 1987) have been described, based on density-gradient fractionation of membranes derived from broken spheroplasts. The low-density membrane fractions contain little if any chlorophyll *a*, and differ from thylakoids and from the wall fraction in lipid and polypeptide composition (Omata & Murata, 1983, 1984; Resch & Gibson, 1983; Bullerjahn & Sherman, 1986). There appear to be considerable

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Abbreviations: CWF, crude wall fraction; LDM, low-density membrane; MM, mixed membrane; PBP, penicillin-binding protein; TK, thylakoid fraction.

discrepancies between the functional components of the preparations made in different laboratories; Lockau & Pfeffer (1983) detected no cytochrome components in their preparations from *Anabaena variabilis*, but were able to demonstrate Ca^{2+} ATPase activity sensitive to vanadate. Cytochromes, predominantly aa_3 or d , but none of the b -type, have been detected spectrophotometrically in preparations from *Anacystis nidulans* (Omata & Murata, 1984), and functional evidence for aa_3 has also been reported (Molitor & Peschek, 1986). However, studies with intact cells suggest that the cytoplasmic membrane does not contribute directly to respiratory electron transport (Scherer *et al.*, 1984). Only one report contains positive evidence correlating the low-density membrane fraction with a domain external to the cytoplasm (Molitor *et al.*, 1987).

It remains unclear whether these discrepancies reflect major variations in cellular organization among cyanobacteria, or are to be attributed, at least in part, to differences in the membrane fractionation procedures employed or the physiological condition of the fractionated cells. An entirely different criterion for identifying cytoplasmic membrane is provided by localizing penicillin-binding proteins (PBPs), which are components of the peptidoglycan-synthesizing system (Spratt, 1983; Waxman & Strominger, 1983). These have been clearly shown to be largely restricted to the cytoplasmic membrane and outer envelope in Gram-negative heterotrophic bacteria (Barbas *et al.*, 1986), and to be excluded from developing intracytoplasmic membranes during photodifferentiation of *Rhodospseudomonas sphaeroides* (Shepherd *et al.*, 1981). Accordingly, we have examined membrane fractions obtained in a procedure somewhat modified from those published previously, which in our hands gives more reproducible yields. The specific content of proteins which bind radioactively labelled penicillin is highest in a low-density membrane fraction whose pigment and polypeptide composition correspond closely with those described by Omata & Murata (1984). The location of PBPs thus confirms the identification of this fraction as cytoplasmic membrane by an independent characteristic.

METHODS

Culture conditions. *Synechococcus* sp. strain R2 (PCC 7942: obtained from J. G. K. Williams) was grown axenically in BG-11 medium (Rippka *et al.*, 1979) with 10 mM- NaNO_3 , and 10 mM-sodium bicarbonate as buffer, in an illuminated water bath at 30 °C, sparged with air/ CO_2 (95:5, v/v), 75 ml min⁻¹ (1 culture)⁻¹. Cells were harvested by centrifugation when the OD_{750} was 0.7–1.1, equivalent to 80–120 µg protein ml⁻¹.

Fractionation protocol. Cells were converted to spheroplasts after concentrating the cell suspension to approximately 10% (wet wt/vol.) in STNE buffer (0.4M-sucrose, 20 mM-Tricine, 10 mM-NaCl, 10 mM-EDTA, pH 8), by digestion with 0.5 mg lysozyme ml⁻¹. The mixture was incubated at 37 °C with occasional gentle mixing for 3–4 h. Cells were checked microscopically for spheroplasts until 80–90% were sensitive to (lysed by) dilution with distilled water. After centrifugation the integrity of the spheroplasts was verified by a lack of measurable phycocyanin (A_{621}) in the supernatant. The temperature in all remaining steps was kept between 0 and 4 °C.

Spheroplasts were pelleted and diluted into hypo-osmotic TN buffer (20 mM-Tricine, 10 mM-NaCl, pH 8) at 5% wet wt/vol.; DNAase I and RNAase A were added to 5 and 10 µg ml⁻¹, respectively. The mixture was shaken in a Braun shaking homogenizer, with an equal weight of glass beads (0.25–0.35 mm), for 4–6 min. The serine-protease inhibitor phenylmethylsulphonyl fluoride was omitted from the preparation to prevent possible interactions with the active sites of the PBPs (Hayes & Ward, 1986).

The homogenate was separated from the glass beads by decanting after allowing the beads to settle, centrifuged at low speed (1250 g_{av}) to remove unbroken cells, and the supernatant recentrifuged (185000 g_{av} , 30 min) to yield the crude membrane fraction. This fraction was carefully resuspended, on ice, in TN buffer, with the aid of a ground-glass homogenizer.

The homogenate was layered onto a 40–50–60–80% glycerol (w/w in TN) step gradient, and spun overnight (16 h) in a swinging-bucket rotor (Beckman SW41 Ti) at 190000 g_{av} , yielding the pattern in Fig. 1(a). From this primary gradient the upper yellow, light green, and thylakoid bands were drawn off, diluted with TN, and pelleted at 185000 g_{av} . The surface of the pellet was carefully rinsed.

Following resuspension, each of the primary fractions was layered onto a secondary glycerol gradient, yielding the pattern in Fig. 1(b). From these bands the low-density membrane (LDM), mixed membrane (MM), purified thylakoid (TK) and crude wall fractions (CWF) were isolated.

Fractions were resuspended in small volumes of TN and stored at –70 °C. Protein concentrations ranged from 0.2–0.4 mg ml⁻¹ for LDM, to 10 mg ml⁻¹ for TK.

Fig. 1. Schematic representation of the primary (a) and secondary (b) glycerol step gradients. Glycerol concentrations are given as % (w/w) made up in TN buffer, pH 8 (20 mM-Tricine, 10 mM-NaCl). Gradients were centrifuged in a Beckman SW41 Ti rotor for 16 h at 190000 g_{av} .

Protein determination. The method of Bradford (1976) was followed, using reagent purchased from Bio-Rad, with bovine serum albumin as a standard.

Absorption spectra. Spectra were run on a Beckman DU-7 spectrophotometer, at 4 °C.

SDS-PAGE. Samples were thawed and kept on ice. Protein samples were precipitated with 4 vols ice-cold acetone (see PBP assay below), and suspended in 20 μ l 1 $\times$ electrophoresis sample buffer [0.4 M-sucrose, 0.01 M-Tris-base, 0.02 M-glycine, 2% (w/v) SDS, 2.2 mM-EDTA, 0.05% (w/v) bromophenol blue, 2% (v/v) β -mercaptoethanol] using a Teflon mini-pestle. Solubilization was at 90 °C for 3 min. Solubilization of membranes at different temperatures (60 min at 30 °C; 30 min at 70 °C) did not affect the apparent M_r values of PBPs estimated from fluorograms, although some minor changes in the high- M_r Coomassie-blue stained polypeptides were detected. Gels (12.5%, w/v, acrylamide) were prepared according to Laemmli (1970).

PBP assay. Samples containing 40–400 μ g protein were diluted with TN buffer to 250 μ l; 962 kBq (2 μ l) [3 H]benzylpenicillin (a gift from Merck, Sharp & Dohme) was added to each tube (final concentration 3.5 μ M), and the tubes were then incubated at 37 °C for 10 min. The reaction was stopped by addition of unlabelled benzylpenicillin (3.5 mM) and further incubation at room temperature for 5 min.

Unlabelled benzylpenicillin (to 0.35 mM) was added to control samples of membrane for 10 min before treatment with [3 H]benzylpenicillin.

After labelling, proteins were precipitated by adding 4 vols ice-cold acetone, left on ice for 10 min, and collected by centrifuging for 7.5 min in an Eppendorf microfuge (15000 g_{av}) at 4 °C (Amanuma & Strominger, 1980). The pellets were homogenized in 50 μ l 1 $\times$ electrophoresis sample buffer using a Teflon mini-pestle, and heated at 90 °C for 3 min. The entire sample was loaded in a well of a 14 cm $\times$ 16 cm polyacrylamide gel. After electrophoresis, gels were soaked in fluor (Autofluor, National Diagnostics), dried, and placed on X-ray film at –70 °C for the desired exposure (1–6 weeks). Relative concentrations of PBPs in different fractions were estimated by scanning densitometry, and normalized to total protein loaded.

RESULTS

The fractionation protocol developed here differed in two major ways from the methods reported previously (Omata & Murata, 1983; Molitor & Peschek, 1986). Gentle breakage of the

Fig. 2

Fig. 2. Absorption spectra of LDM(---) and TK (—) fractions, prepared as described in the text, and determined at 4 °C.

Fig. 3

Fig. 3. SDS-PAGE banding patterns of LDM and TK fractions run on a 12.5% acrylamide gel. M_r standards are in the left lane. Asterisks mark bands which are unique to each fraction.

Table 1. *Pigmentation and density differences of membrane fractions*

Fraction	A_{390}/A_{678} *	Density (g cm ⁻³)†
Low density membrane	12.3 ± 1.0 (12)	1.104–1.130
Mixed membrane	1.5 (1)	1.130–1.156
Thylakoid	1.02 ± 0.05 (11)	1.156–1.208

* Values are the mean $\pm$ standard error of the mean, with the number of determinations in parentheses.

† Densities were determined from the densities of the corresponding glycerol concentrations.

spheroplasts with a shaking glass-bead homogenizer minimized intermixing of membrane types and improved the yield of LDM (estimated yields of LDM protein, as a percentage of whole cell protein, were 0.05–0.15%). The use of glycerol as a density support produced sharper banding of membranes in the step gradients than did sucrose.

Several criteria were used to evaluate the membrane fractions. Fig. 2 shows absorption spectra from LDM and TK fractions from a typical preparation. The chlorophyll *a* absorption maximum at 678 nm was used as an index of thylakoid contamination, whereas a characteristic carotenoid absorption peak at 390 nm was associated with LDM. These differences are quantified in Table 1, where the densities of the membranes are also described.

The protein profiles of the LDM and TK fractions are shown in Fig. 3. Major proteins which are obviously different in each fraction have been marked with asterisks. In addition, there are a number of less intense bands in each fraction which emphasize the overall difference between the two fractions.

The PBP profiles of the LDM, MM, TK and CWF fractions are shown in Fig. 4. Specific binding of [³H]benzylpenicillin was distinguished from nonspecific binding by comparison with

Fig. 4. PBP profiles of different membrane fractions. Samples were prepared as described in Methods, and run on a 12.5% acrylamide gel according to Laemmli (1970). Gels were soaked in fluor and X-ray film was exposed to them at -70°C for 4 weeks. '1 $\times$ ' loading represents 40 μg protein per lane. Lanes marked with an asterisk represent samples which were blocked from binding the [^3H]benzylpenicillin label by the prior addition of unlabelled benzylpenicillin, as described in Methods.

samples pretreated with excess unlabelled benzylpenicillin (lanes 2, 4, 6 and 10). LDM had two PBPs, of M_r 51 000 and 26 000 (lane 5), with respective relative concentrations of 1:8. When TK was overloaded relative to LDM, by 4 $\times$ (lane 8) or 10 $\times$ (lane 9), four PBPs were apparent of M_r 78 000, 63 000, 51 000 and 26 000, and occasionally a fifth at M_r 44 000. The M_r 51 000 PBP was found at only half the specific concentration found in LDM. The M_r 26 000 PBP occurred with an estimated relative concentration of approximately 10^{-1} that of LDM. The CWF fraction (lane 1) showed the four PBPs also found with the TK fraction, but the specific concentration of the M_r 51 000 PBP was much higher, approximating that found in LDM material. MM (lane 3) contained both LDM-type PBPs (M_r 51 000 and 26 000) and CWF-types; however, the specific concentration of the M_r 78 000 PBP was three to fourfold greater than in either CWF or TK fractions.

DISCUSSION

Prokaryotes with peptidoglycan-containing cell envelopes contain a number of polypeptides which bind penicillin covalently. These proteins are thought to be enzymes involved in the building and modification of cross-links in the murein sacculus as the cell grows, although precise enzymic function has been assigned to only a few. In Gram-positive organisms, these proteins are located in the cytoplasmic membrane, and this is also substantially the case in Gram-negative organisms (Spratt, 1983; Waxman & Strominger, 1983), although there are recent reports that suggest some may be found in the outer envelope as well (Barbas *et al.*, 1986).

It is especially relevant to the results presented here that PBPs were excluded from developing intracytoplasmic membranes in a purple phototrophic bacterium (Shepherd *et al.*, 1981).

Most cyanobacteria are obligate phototrophs and, although their respiratory activities have been extensively investigated, especially by Peschek and his group (Molitor & Peschek, 1986; Peschek, 1983, 1984), these are always low compared with photosynthetic rates. Consequently, highly active and convenient cytoplasmic enzyme markers are not found in any membrane fraction derived from these organisms, and there has been disagreement between different laboratories about distribution of cytochrome components. Localization of PBPs afforded an independent marker for identifying membranes from different cell locations following their fractionation by isopycnic density gradient separation.

The conventional numbering of PBPs is in order of decreasing molecular size, and thus the overall PBP profile from *Synechococcus* R2, detected with [³H]benzylpenicillin, is 1, 2, 3, 4 and 5, with respective M_r values of 78 000, 63 000, 51 000, 44 000 and 26 000. Our methods detected all of the PBPs from *Escherichia coli* which bind [³H]benzylpenicillin (Hayes & Ward, 1986), namely 1a (M_r 91 000), 1b (81 500–86 500), 2 (66 000), 3 (60 000), 4 (49 000), 5 (42 000) and 6 (40 000), and all but one of the PBPs from *Staphylococcus aureus* (Wyke, 1984), namely 1 (83 000), 2 (76 000), 3 (73 000), and 4 (45 000); PBP 3' (70 000) was not detected by us nor by Hayes & Ward (1986) (data not shown). Estimates from fluorograms showed that *Synechococcus* membranes contained less than $0.025 \times$ the amount of specific penicillin-binding activity per μ g membrane protein found in *E. coli* or *S. aureus*.

The LDM fraction obtained in our experiments corresponded in absorption spectrum and in major polypeptide composition to the fraction which Omata & Murata (1983) identified as cytoplasmic membrane. Our LDM contained two PBPs, with M_r 51 000 and 26 000, in greater concentration relative to total protein than was found in the thylakoid membranes. This is consistent with the identification made by Omata & Murata (1983), and subsequently by Molitor *et al.* (1987), using different criteria.

In this study, as well as those of others (Omata & Murata, 1983, 1984; Molitor *et al.*, 1987), the recovery of LDM appears poor. Assuming that the M_r 51 000 and 26 000 PBPs are characteristically confined to the LDM/cytoplasmic membrane fraction, then their presence in other fractions can be taken as a measure of contamination with membrane from a different location. Whether the TK fraction, even after two gradient separations, still contains a mixture of vesicles derived from two locations in the cells, or whether some membrane fusion or trapping has occurred during cell breakage, is at present uncertain. However, [³H]benzylpenicillin bound to PBPs did appear in the TK fraction from externally-labelled spheroplasts (data not shown). This is inconsistent with PBPs being endogenous to the thylakoids, since cell membranes are not permeable to benzylpenicillin (Blumberg & Strominger, 1974). In the same way, the very dense CWF fraction contains not only these two components but also easily detectable quantities of the M_r 78 000 protein found in the MM fraction. While the crude walls undoubtedly still contain some unbroken cells, we have observed repeatedly that good yields of LDM depended on transformation of a high proportion of cells to spheroplasts during lysozyme treatment, minimizing the extent to which membranes were trapped within the wall fraction. This implies an association of the M_r 78 000 PBP with the wall structure of this bacterium.

An unexpected finding was that membranes recovered from a region of the glycerol gradients lying between the well-defined cytoplasmic and thylakoid membrane bands (the MM fraction) contained a uniquely high quantity of a larger PBP, with M_r 78 000. This suggests that this fraction is not, as previously thought, simply a mixture of the two major membrane types, but has specific components and, presumably, specific functions. It is always possible that membrane scrambling can occur during cell breakage and fractionation procedures, but the specific enrichment found here implies a unique character to this fraction. The components may represent material from regions where thylakoid, cytoplasmic, and even outer envelope membranes come into contact. Since only a small fraction of all PBPs in a cell may be involved at any one time in the synthesis of new cell wall material (Tuomanen, 1986), it is possible, even probable, that the PBPs are not uniformly distributed throughout classically defined cellular fractions.

Although contact zones appear uncommon in many unicellular cyanobacteria (Stanier & Cohen-Bazire, 1977), they have been reported (Allen, 1969), and in a strain now identified as a *Synechococcus* a regular arrangement of contact zones has been demonstrated (Nierzwicki-Bauer *et al.*, 1983). Orderly enlargement of growing cells must also involve communication with the outer regions of the envelope, as shown by Bayer (1968) and Cook *et al.* (1986). Specific enzyme activities have not been assigned to the cyanobacterial PBPs, which to our knowledge have not been described previously, but it is perhaps significant that the M_r 78000 PBP described here is closest in M_r to the PBP 1b (81 500–86 500) of *E. coli*, which is a bifunctional transpeptidase/transglycosylase thought to be involved in cell expansion (Hayes & Ward, 1986).

We propose that the MM fraction of *Synechococcus* R2 is specifically enriched in membrane components involved in cell envelope expansion, analogous to a similar fraction from enteric bacteria described by Ishidate *et al.* (1986), and that the M_r 78000 PBP plays a key role in this process.

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