A Cyanobacterial ATPase Distinct from the Coupling Factor of Photophosphorylation

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A particle-bound, Mg^{2+}-dependent ATPase activity is investigated in cell-free extracts of the cyanobacterium *Anabaena variabilis*. The enzyme can be clearly distinguished from the cyanobacterial coupling factor of photophosphorylation and from the alkaline phosphatase. It requires low concentrations of Ca^{2+} for maximal activity and is inhibited by *ortho*-vanadate, indicating that the enzyme may form a phosphorylated intermediate in its catalytic cycle. The distribution of the ATPase in sucrose density gradients does not follow that of thylakoids. It is concluded from these characteristics that the enzyme is bound to the plasma membrane. The cytochrome oxidase activity of the extracts appears to be restricted to the thylakoids.

With one exception known [1], cyanobacteria possess three kinds of membranes, intracytoplasmically located thylakoids, a plasma membrane and an outer membrane. Their cell envelope, in general, resembles that of other gram-negative bacteria [2]. The intramembrane particles of the cyanobacterial plasma membrane are different from those of the thylakoids [3]. In contrast to the thylakoids, however, this membrane has not been characterized biochemically. Pinevich [4] isolated a membrane fraction from *Anabaena variabilis* with a low chlorophyll content which he, on morphological grounds, concluded to consist of plasma membranes. Enzymic activities were not reported. Murata *et al.* [5] characterized a preparation of the cell envelope from *Anacystis nidulans* which contained mainly outer membranes associated with a peptidoglycan layer, but apparently not the plasma membrane.

Since all organisms seem to have some sort of ATPase bound to their plasma membranes, we attempted to detect such an ATPase in cell-free extracts of the cyanobacterium *Anabaena variabilis* separated by sucrose density gradient centrifugation. The ATPase activity observed is neither identical with the only cyanobacterial ATPase described so far, the coupling factor of photophosphorylation [6, 7], nor is it restricted to the thylakoid-containing fractions. It is, in all probability, bound to the plasma membrane. Some properties of the ATPase and of the particles it is bound to will be reported.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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Materials and Methods

Anabaena variabilis Kütz. (ATCC 29 413) was growth photoautotrophically and harvested as described [8]. For lysozyme digestion, the cells were suspended in 22.5 mM Tris-HCl pH 8.1, 0.38 M sucrose, 0.1 M NaCl, 10 mM EDTA, 2 mg/ml egg white lysozyme, to a final concentration of 0.6 to 0.8 mg/ml chlorophyll and incubated at 30 °C for 3 h in the dark. All subsequent isolation procedures were performed at 0 to 4 °C. The lysozyme-treated cells were collected by centrifugation (10 min at $1500 \times g$) and resuspended in 30 mM Tris-HCl pH 8.1 to give a concentration of about 2 mg/ml chlorophyll. Glass beads (1 g/ml, 0.5 mm diameter) were added and the cells broken in a cell homogenizer model MSK (Braun, Melsungen, FRG) at 3400 rpm for 1 min. Glass beads were allowed to settle down, the extract decanted and dialyzed for 2 h against 10 mM Tris-HCl pH 8.1. Aliquots of 3 ml of this extract were layered on linear gradients of sucrose (20 to 50%, w/w, in 10 mM Tris-HCl pH 8.1, total volume of 36 ml), and centrifuged for 15 h at 25 000 rpm in a Beckman SW 27 rotor. Intact cells, mostly heterocysts, formed a pellet at the bottom of the centrifuge tube. The gradients were fractionated and assayed for ATPase and chlorophyll (absorbance at 680 nm) distribution.

ATPase was assayed by determination of phosphate liberated. The assay mixture (1.0 ml) contained 6 mM $MgCl_2$, 6 mM ATP (neutralized sodium salt), 30 mM Tris-HCl pH 8.1. The reaction was started by addition of an appropriate amount of the fractions (usually giving 0.1 to 0.5 mg protein/ml) and stopped after 0 or 90 min with 0.5 ml of 6% (w/v) trichloroacetic acid. After centrifugation, phosphate release was determined colorimetrically [9]. ATPase activity was confirmed to be constant for 90 min. Incubation temperature was 37 °C except in the experiments with intact cells (see Table II).

The Ca^{2+}-dependent ATPase activity attributed to the coupling factor of photophosphorylation [6, 7, 10] was activated by trypsin treatment. The reaction mixture was that described [11] except that the protein concentration of the membrane preparations was 0.5 mg/ml and the concentration of trypsin 0.25 mg/ml. After various times (see Fig. 2) at room temperature, digestion was stopped by addition of soybean trypsin inhibitor to 0.75 mg/ml. ATPase activity was assayed as above except that $MgCl_2$ was replaced by 6 mM $CaCl_2$, the reaction time was 40 min.

Cytochrome oxidase activity was assayed polarographically as described [8] with 15 μM cytochrome c from horse heart. The rates of oxygen uptake given are those sensitive to 100 μM KCN.

Chlorophyll was quantified in methanolic extracts [12], protein by a modified Lowry procedure [13]. Sodium dodecylsulfate gel electrophoresis in the presence of urea was carried out as in [14]. Samples with 0.5 to 1 mg of protein/ml in the final sample buffer of Laemmli [15] containing 3 or 6% (w/v) sodium dodecylsulfate were incubated for 30 min at room temperature. Electrophoresis was carried out for 1 h at 130 V followed by 2 h at 200 V, the length of the separating gels (15% in acrylamide) was 15 cm. Polypeptides were stained with Coomassie brilliant blue R-250 according to Ref. 16. The same staining procedure was used for Ouchterlony double diffusion plates.

For electron microscopy, fractions 'A' and 'B' from a sucrose gradient (see Results) were diluted 4-fold with 20 mM Tris-HCl pH 8.1, 20 mM NaCl, centrifuged for 2 h at 100 000 × *g* and the pellets resuspended in 20 mM Tris-HCl pH 8.1 to 1 to 2 mg protein/ml. After addition of 5% phosphotungstic acid (brought to pH 7.2 with NaOH) to a final concentration of 2%, aliquots were transferred to Formvar-coated grids. Excess liquid was removed with filter paper. The grids were air-dried and inspected with a Jeol electron microscope at 80 kV.

Results

The ATPase activity of cell-free extracts of lysozyme-treated *Anabaena variabilis* was broadly distributed in sucrose density gradients after 15 h of centrifugation (Fig. 1). The activity at the top of the gradient (soluble proteins) may be due to a phosphatase. It was not investigated further. From comparison with the distribution of chlorophyll ($A_{680\ nm}$), it can be seen that the bulk of the ATPase activity in the gradient was not associated with the thylakoid membranes. For further experiments, the fractions indicated by the bars 'A' (containing the thylakoids) and 'B' (high ATPase activity but low chlorophyll content) were used.

The ATPase activity of fractions 'B' appeared to be particle-associated. More than 90% of the activity was recovered in a yellowish-brown pellet after 4-fold dilution with 20 mM Tris-HCl pH 8.1, 20 mM

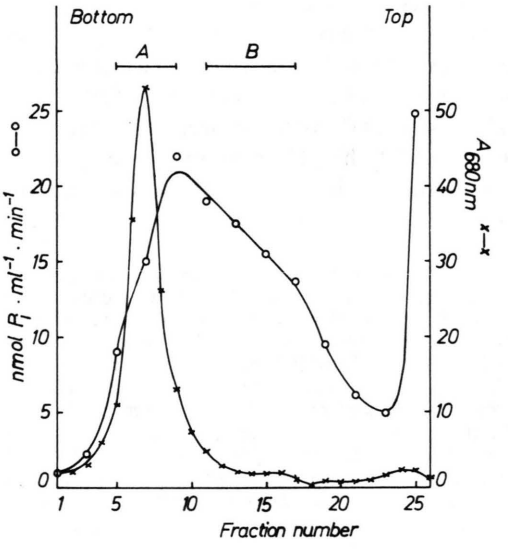

Fig. 1. Distribution of ATPase activity and chlorophyll in a sucrose gradient. Lysozyme-treated, disrupted filaments were subjected to sucrose gradient centrifugation as described under Materials and Methods. After fractionation, 0.2 ml aliquots of fractions were assayed for ATPase activity (○—○). The distribution of chlorophyll ($A_{680\ nm}$, x—x) was measured after 31-fold dilution with water. The bars 'A' and 'B' indicate the combined fractions which were used for further analysis.

Table I. Substrate specificity of the ATPase activity of fractions 'B'. In part I, the substrate concentration was 6 mM in each case. In part II, the concentrations of triphosphates and of $MgCl_2$ was 3 mM. All rates are given in nmol P_i liberated · mg protein^{-1} · min^{-1}.

I. Substrate	Rate	
	minus $MgCl_2$	+6 mM $MgCl_2$
ATP	1	40
ADP	2	4
p-Nitrophenyl-phosphate	0.5	4
Glucose-6-phos-phate	0	0.5

II. Substrate	Rate plus $MgCl_2$
ATP	56
GTP	15
ITP	18
CTP	19
UTP	27

NaCl (to reduce the sucrose concentration) and 2 h of centrifugation at 100 000×*g*. Table I shows the substrate specificity of the ATPase. ADP, *p*-nitrophenylphosphate and glucose-6-phosphate as potential substrates for an unspecific phosphatase were only split at a low rate compared to ATP. Hydrolysis of ATP depended on Mg^{2+}. Highest rates required equimolar concentrations of ATP and Mg^{2+}; half-maximal rates of ATP hydrolysis were obtained with 0.8 mM Mg^{2+}-ATP, the pH optimum was at pH 8 to 8.3 (data not shown). In the presence of Mg^{2+}, all nucleotide triphosphates were hydrolyzed, but at a lower rate than ATP (Table I).

Table II. Hydrolysis of phosphate-containing compounds by intact filaments of *Anabaena variabilis*. Cultures in the late logarithmic phase of growth were harvested by centrifugation, washed once with distilled water and resuspended in 30 mM Tris-HCl pH 8.1. Assays contained 30 mM Tris-HCl pH 8.1, algae with 1.6 mg protein/ml, and the substrates at a concentration of 3 mM. After 0 or 60 min at 30 °C, the filaments were spun down and the supernatants assayed for phosphate liberated. No significant phosphate release occurred in the absence of substrates. Rates are given in nmol P_i liberated · mg protein^{-1} · min^{-1}.

Substrate	Rate	
	minus $MgCl_2$	+3 mM $MgCl_2$
ATP	1.0	3.1
ADP	1.5	2.9
p-Nitrophenyl-phosphate	1.7	4.0

Table II shows that intact filaments of *A. variabilis* hydrolyzed phosphate-containing compounds with a different substrate specificity than fractions 'B'. Since *p*-nitrophenylphosphate was hydrolyzed faster than ATP, this activity can be attributed to the alkaline phosphatase located in the periplasmic space of cyanobacteria [17, 18]. Enhancement of the activity of this enzyme by Mg^{2+} (Table II) has been observed before [19].

Several lines of evidence indicate that the ATPase contained in fractions 'B' is different from the coupling factor of photophosphorylation: As Fig. 2 shows, limited digestion with trypsin which activates the latent ATPase activity of the cyanobacterial coupling factor and converts it to a Ca^{2+}-dependent ATPase [6, 7] had little effect on fractions 'B'. It should also be noted that Ca^{2+} could not replace Mg^{2+} in support of the ATPase activity of fractions 'B' (compare the rates in Table I). Under the same conditions, the Ca^{2+}-dependent ATPase activity of the thylakoid-containing

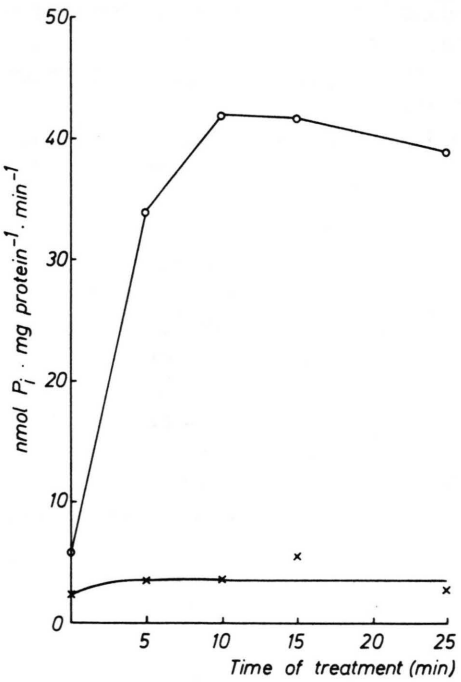

Fig. 2. Effect of trypsin digestion on the Ca^{2+}-dependent ATPase activities of fractions 'A' (o–o) and 'B' (x–x). Note that the assays did not contain $MgCl_2$ but ATP and $CaCl_2$ in equimolar amounts. Details are described under Materials and Methods.

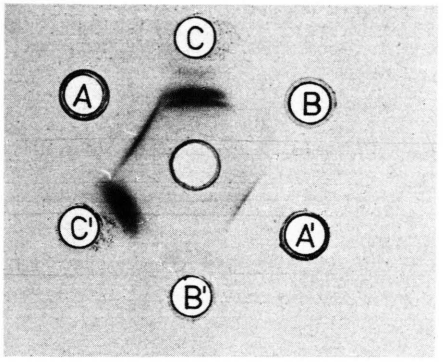

Fig. 3. Cross-reaction of Triton-solubilized thylakoids (fractions 'A' from a sucrose gradient) with an antiserum against the plastidic coupling factor CF_1 from spinach. Wells A and A′ received 40 and 25 µl, respectively, of fractions 'A' dissolved in 1% (v/v) Triton-X 100, wells B and B′ 40 and 25 µl of fractions 'B' dissolved in 1% Triton, wells C and C′ 10 and 6 µl of a crude preparation of spinach CF_1 (kindly provided by Dr. G. Hauska, Regensburg), and the center well 35 µl of the antiserum against spinach CF_1 (kindly donated by Dr. R. G. Berzborn, Bochum, FRG). Diffusion was at 4 °C for 36 h in 0.8% (w/v) agarose, 10 mM Tris-HCl pH 8.1, 100 mM NaCl, 0.1% Triton-X 100. Fractions 'A' and 'B' had, on a volume basis, identical Mg^{2+}-dependent ATPase activity.

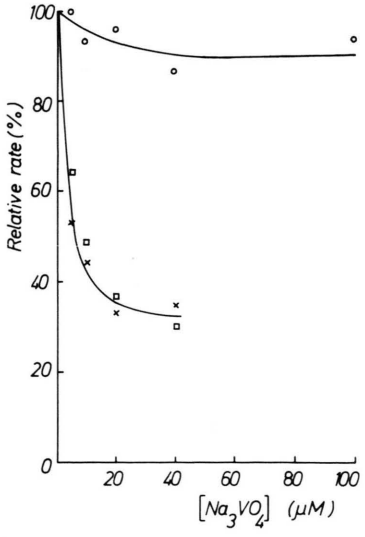

Fig. 4. Effects of ortho-vanadate on the Mg^{2+}-dependent ATPase activities of fractions 'A' (□−□) and 'B' (x−x) and on the trypsin-activated, Ca^{2+}-dependent ATPase activity of fractions 'A' (○−○). Mg^{2+}-dependent ATPase activity was assayed with 6 mM ATP and 6 mM $MgCl_2$. For the Ca^{2+}-dependent ATPase activity, an aliquot of fractions 'A' was digested with trypsin for 10 min (compare Fig. 2) and assayed with 6 mM ATP and 6 mM $CaCl_2$. Details are described under Materials and Methods.

fractions 'A' was strongly enhanced by trypsin treatment (Fig. 2). An antiserum against the plastidic coupling factor from spinach cross-reacted partially with the green fractions 'A' but not with fractions 'B' (Fig. 3). Ortho-vanadate, an inhibitor of most cation-translocating ATPases which has, as far as investigated, no effect on coupling factors [20, 21], inhibited the Mg^{2+}-dependent ATPase of fractions 'A' and 'B' at µM concentrations (Fig. 4). It had little effect on the Ca^{2+}-dependent ATPase activity of trypsin-treated fractions 'A' which is attributed to the coupling factor. N,N′-dicyclohexyl carbodiimide, an efficient inhibitor of the ATPase activity of coupling factors, inhibited the ATPase activity of fractions 'B' only at high concentrations. At a concentration of 0.125 mM about 10% inhibition, at 1 mM about 30% inhibition were obtained. This may be an unspecific inhibition since carbodiimides are very reactive substances with a broad specificity [22].

Addition of 20 mM KCl or NaCl, or a combination of both, stimulated the ATPase tested with the Tris

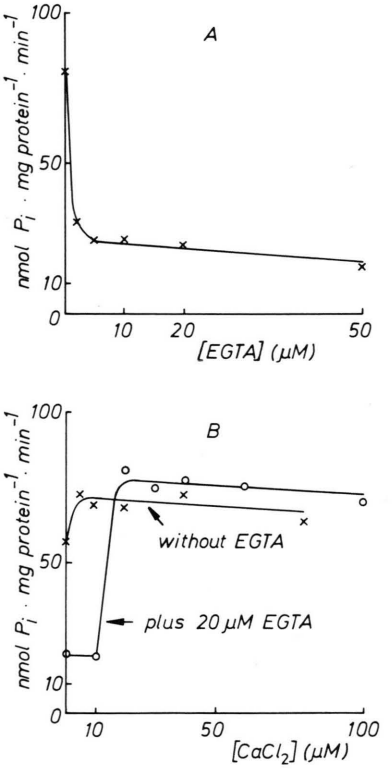

Fig. 5. Inhibition, by EGTA, of the Mg^{2+}-dependent ATPase activity of fractions 'B' and reversal of the inhibition by low concentrations of $CaCl_2$.

salt of ATP plus equimolar concentrations of $MgCl_2$ only slightly (0 to 20%). Inconsistent results were obtained with $CaCl_2$. Addition of 5 µM $CaCl_2$ stimulated the Mg^{2+}-dependent ATPase activity of some preparations of fractions 'B' up to 150%, whereas in others only a slight stimulation was observed (as in Fig. 5B), indicating that the preparations may have contained variable amounts of endogenous Ca^{2+}. It was consistently found (Fig. 5A) that very low concentrations of the Ca^{2+} chelator, EGTA, inhibited the ATPase activity. This inhibition was reversed by $CaCl_2$ (Fig. 5B). It is concluded that the Mg^{2+}-dependent ATPase requires low concentrations of Ca^{2+} for maximal activity.

The polypeptide profiles of fractions 'A' (which contain the thylakoids) and fractions 'B' were rather different (Fig. 6). Most important, polypeptides in the region around M_r=45 kDa which are prominent in preparations of the outer membrane of gram-negative bacteria and of the cyanobacterium *Anacystis nidulans* [23, 24] were absent in fractions 'B'. The polypeptide profile of fractions 'A' is typical for cyanobacterial thylakoids (see *e. g.* [25, 26]).

In electron micrographs of negatively stained samples of fractions 'A' particles with a diameter of about 0.3 to 0.5 µM were seen, presumably the thylakoids. Fractions 'B' contained smaller particles, frequently with a rough surface (Fig. 7).

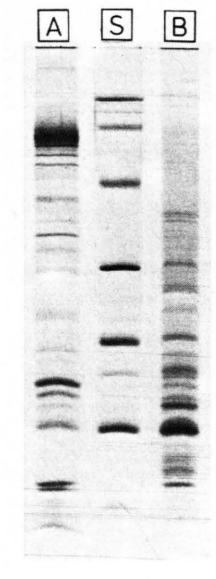

Fig. 6. Polypeptide patterns of fractions 'A' (containing the thylakoids) and fractions 'B'. Lane A, fractions 'A'; lane B, fractions 'B'; lane S, molecular weight standards from Biorad Laboratories (from top to bottom: 92.5, 66.5, 45, 31, 21.5, 14.3 kDa). 13 µg of protein were applied to lane A, 15 µg to lane B.

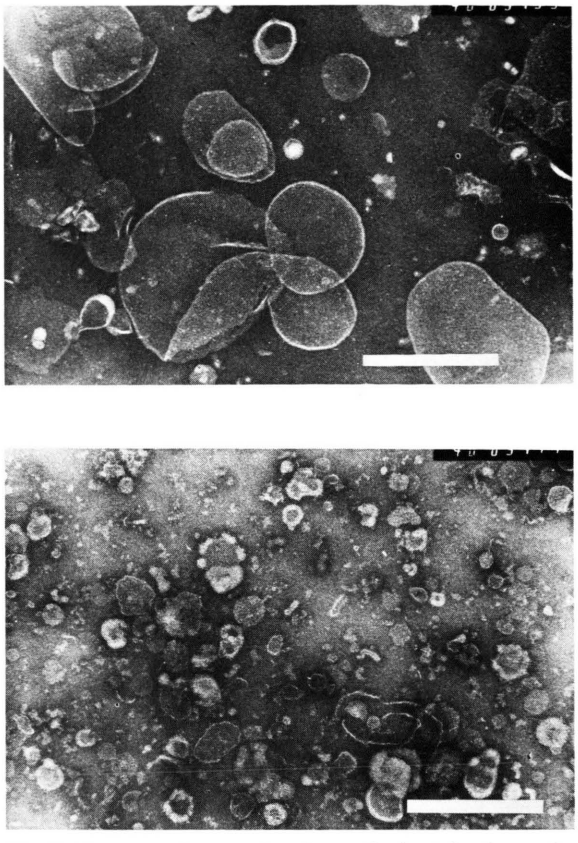

Fig. 7. Electron micrographs of negatively stained samples of fractions 'A' (top) and fractions 'B' (bottom). The bars equal 0.5 µm.

The protein and chlorophyll content and the specific activities of the ATPase and the cytochrome oxidase of the fractions are listed in Table III. For different preparations, the protein/chlorophyll ratio of the thylakoid-containing fractions was rather constant (4.8 to 6.7), whereas that of fractions 'B' was quite variable (40 to 300). When calculated on a protein or on a chlorophyll basis, the specific activity of the ATPase of fractions 'B' was several-fold higher than that of 'A'. A different situation was found for the cytochrome oxidase activity. The thylakoid-containing fractions, in this experiment, had a 9-fold higher specific activity on a protein basis. On a chlorophyll basis, however, the activity of the oxidase in both fractions 'A' and 'B' was nearly identical (Table III). In preparations of fractions 'B' which had much higher protein/chlorophyll ratio than the one

Table III. Distribution of Mg^{2+}-dependent ATPase and cytochrome oxidase activities in fractions 'A' and 'B' of a sucrose gradient. All assays are described under Materials and Methods.

		Fraction	
		'A'	'B'
Protein concentration	$\frac{mg}{ml}$	2.1	0.45
Chlorophyll concentration	$\frac{mg}{ml}$	0.44	0.01
Mg^{2+}-ATPase activity	$\frac{nmol\ P_i\ released}{mg\ protein \cdot min}$	14	65
	$\frac{nmol\ P_i\ released}{mg\ chlorophyll \cdot min}$	67	2925
Cytochrome oxidase activity	$\frac{nmol\ O_2\ taken\ up}{mg\ protein \cdot min}$	17	1.9
	$\frac{nmol\ O_2\ taken\ up}{mg\ chlorophyll \cdot min}$	82	86

used in the experiments of Table III, the activity of the cytochrome oxidase was too low to be measured accurately with the technique used.

Discussion

The cyanobacterial ATPase described here is certainly different from the coupling factor of photophosphorylation. It is not converted to a Ca^{2+}-dependent ATPase by trypsin treatment, does not cross-react with an antiserum against such a coupling factor and requires μM concentrations of Ca^{2+} (in addition to much higher concentrations of Mg^{2+}) for maximal activity (Figs. 2, 3, 5). It is inhibited by vanadate (Fig. 4), a compound which is believed to affect, amongst other enzymes, ATPases forming a phosphorylated intermediate in their catalytic cycle only [20, 21] and is insensitive to N,N′-dicyclohexyl carbodiimide. Although the enzyme shows an alkaline pH optimum around pH 8, substrate specificity and complete dependence on Mg^{2+} for activity (Table I) distinguish it from the alkaline phosphatase of cyanobacteria (Table II; see [18, 27–29]).

The distribution of the enzyme activity in sucrose density gradients overlaps, but does not coincide, with the distribution of thylakoids (Fig. 1, see also the identical concentration dependence of inhibition by vanadate of the Mg^{2+}-dependent ATPase in the thylakoid-containing fractions 'A' and in fractions 'B', Fig. 4). It seems that the enzyme is bound to another membrane or particle and, if at all, only to a minor extent also to the thylakoids. Phosphate-containing compounds added to intact cells of *Anabaena variabilis* are split by what appears to be an unspecific phosphatase (Table II), an enzyme which has been shown to be located in the periplasmic space of the cyanobacterium *Plectonema boryanum* [17]. Since the ATPase described here can be distinguished from a phosphatase, one is left with the plasma membrane as the site of its most probable intracellular localization. Further support comes from the polypeptide pattern of the fractions with the highest specific ATPase activity, fractions 'B' (Fig. 6), which differs from the patterns of outer membranes from bacteria and from a cyanobacterium [23, 24]. It should also be mentioned that outer membranes of bacteria do apparently not contain an ATPase [23]. Superficially, the polypeptide pattern of fractions 'B' shows little similarity to the polypeptide pattern Pinevich [4] reported for his plasma membrane preparation from another strain of *Anabaena variabilis*, where many bands were common to both the thylakoid and the plasma membrane fractions. The polypeptides, however, which he found to be specific for the plasma membrane all had molecular weights smaller than 36 kDa, and this is the molecular weight region where almost all polypeptides of fractions 'B' are located (Fig. 6). Pinevich's plasma membrane preparation may have been more heavily contaminated with thylakoids than fractions 'B'.

It is startling that ATPase activity is so broadly distributed in the sucrose gradient (Fig. 1). Possible reasons could be that the thylakoid membranes also contain the enzyme in addition to the coupling factor of photophosphorylation, or that the membranes carrying the enzyme partially stick to the thylakoids. The last-mentioned possibility is not unlikely since EDTA was omitted, which is usually included into such gradients to improve separation by removal of divalent cations. EDTA was omitted on purpose because it is known [6, 7] to solubilize the cyanobacterial coupling factor, thus making the comparison of different fractions of the gradient more complicated. A third possibility could be a heterogenous density of the particles to which the ATPase is bound, *e.g.* because the small fragments generated by the disruption of sphaeroplasts (Fig. 7) contain variable amounts of cell wall material. This problem, however, can not be resolved with certainty at present.

In view of recent evidence [30–33] that the respiratory chain of cyanobacteria may not be restricted to the thylakoids, as thought before [34], but may partly also be associated with the plasma membrane, the distribution of cytochrome oxidase activity in the thylakoid-containing fractions and the fractions presumably enriched in plasma membranes (*i. e.* fractions 'B') is of interest. This enzyme is one, of possibly two, terminal oxidases of cyanobacteria (compare [35]). Only a low activity of the oxidase was recovered in fractions 'B' (Table III). Since this activity, when calculated on a chlorophyll basis, equals that found with thylakoids, it may be associated with thylakoids contaminating fractions 'B'.

The physiological role of the ATPase is, at the present stage, open to speculation. Its sensitivity to vanadate and its possible localization on the plasma membrane are suggestive of a proton-pumping ATPase like the one recently identified in fungi (see Malpartida and Serrano [36] and references therein). However, the requirement for low concentrations of Ca^{2+} for maximal activity suggests that the enzyme may be a Ca^{2+}-translocating ATPase. Such an enzyme is well-known from eukaryotic cells but has been found in bacteria only once [37]; it is sensitive to vanadate [38] as is the ATPase described here. Outwardly-directed Ca^{2+} transport is a general feature of plasma membranes and has been proposed to be involved in the phototactic movement of a cyanobacterium [39], but this may also be secondary active transport. We currently attempt to characterize the function of the cyanobacterial ATPase described here.

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