# Polynucleotide Phosphorylase from a Cyanobacterium (*Synechococcus sp.*): Subunit Composition and Properties

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Poly(A) Synthesis, Affinity Chromatography, Isoelectric Focusing, Polyacrylamide Gel Electrophoresis, Tetrameric Structure

Polynucleotide phosphorylase from cells of the cyanobacterium *Synechococcus sp.* has been purified 1400-fold by an improved procedure. The enzyme purified to homogeneity and lacking nuclease, phosphatase and protease contaminations reveals a single band of ADP polymerizing activity upon polyacrylamide gel electrophoresis under nondenaturing conditions which corresponds to a molecular mass of about 275 000. The enzyme migrates as a single polypeptide of $M_r \approx 70\,000$ when subjected to gel electrophoresis in the presence of dodecyl sulfate indicating a composition of $\alpha_4$ for the native enzyme molecule.

The isoelectric point of the purified enzyme as determined by isoelectric focusing was found to be at $4.2 \pm 0.1$. Polynucleotide phosphorylase of *Synechococcus* is preferentially activated by $Mg^{2+}$; KCl has a significant stimulatory effect.

## Introduction

Despite of the fact that polynucleotide phosphorylase (PNPase; polyribonucleotide ribonucleotidyl transferase; EC 2.7.7.8) had been discovered 25 years ago the physiological role of this enzyme is still not ascertained. PNPase catalyzes *in vitro* the polymerization of nucleoside diphosphates *de novo* or by elongation of a primer as well as the phosphorolytic cleavage of ribopolynucleotides. During the last ten years the elucidation of the molecular structure of PNPase from various bacterial species has made great progress (*cf.* [1]).

The subunit pattern so far studied in detail yields several polypeptides; their number and molecular mass, however, differ when enzyme preparations obtained from several sources and purified by different procedures are compared. Moreover, PNPase has been isolated from *Escherichia coli* in two molecular forms exhibiting very similar catalytic properties [2]. It is not yet clear whether they represent true functional entities or artifacts resulting from the purification procedure.

Since cyanobacteria represent a further prokaryotic type of organisation it appeared of interest to compare the PNPase of these photoautotrophic organisms with that of bacteria. Properties of the partially purified enzyme from *Synechococcus sp.* (formerly *Anacystis nidulans*) have been studied by Capesius and Richter [3] as a first step in determining the involvement of PNPase in the nucleic acid metabolism. A prerequisite for investigating the possible physiological role of this enzyme as well as the structure-function relationship is its purification to homogeneity. The procedure described here answers this requirement and yields fairly large amounts of homogenous PNPase as a single pure protein free from contaminating nucleases and other enzyme activities. This communication also reports several properties of the *Synechococcus* PNPase including the subunit composition.

## Materials and Methods

### *Growth of cells*

The experiments were carried out with *Synechococcus sp.* (= *Anacystis nidulans;* [4]); stock cultures were obtained from the algal collection of the Institute for Plant Physiology Göttingen (1402-1). The cells were grown at 34 °C under sterile conditions in an inorganic salt medium [5] with the exception that $Fe^{3+}$ was added as EDTA complex [6]; air with 3% $CO_2$ was bubbled through the suspension. The vessels measuring 50 × 60 × 5 cm were made from 5 mm glassplates using autoclave-resistant silicone rubber glue [7]. Illumination up to 17000 lux of either side of the cuvette was achieved by neon tubes. After 72 h of growth the cells were harvested

*Abbreviations:* DTT, dithiothreitol; PNPase, polynucleotide phosphorylase; Poly(A), polyadenylic acid.

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yielding 40 – 45 g (fresh weight) per 10 l of culture. Cells were stored at – 80 °C.

*Buffers*

The extraction buffer contained 0.05 M Tris-HCl (pH 7.4), 0.01 mM $MgCl_2$, 0.2 M KCl, 0.1 mM DTT, 0.1 mM EDTA, 0.139 mM phenylmethylsulfonylfluoride, 5% (v/v) glycerol. For column chromatography buffer A [8] was used: 0.01 M Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM phenylmethylsulfonylfluoride, 5% (v/v) glycerol.

*Assay for PNPase*

PNPase activity was measured as described [3]. After 30 min at 37 °C the reaction was terminated by adding 2 ml of cold 5% (w/v) trichloroacetic acid. The mixture was allowed to stand on ice for 10 min, then it was filtered by suction through a nitrocellulose filter (0.45 µm, Sartorius, Göttingen). After washing with the same solution the filter was soaked in 5% (w/v) trichloroacetic acid containing 20 mM sodium diphosphate and 1 M KCl for 10 min, and dried at 80 °C. The radioactivity was measured in 5 ml of a standard toluene-based scintillator. 1 unit of enzyme catalyzes the incorporation of 1 nmol [$^{14}$C]AMP into ribopolynucleotide at 37 °C in 1 min. Specific activity is expressed as units/mg protein. When assaying PNPase activity enriched in a polyacrylamide gel the latter was incubated in 2 ml of the test mixture for at least 12 h at 37 °C. The subsequent determination of radioactivity incorporated has been published elsewhere [9].

*Protein determination*

Protein was measured by a modification of the Lowry procedure in a final volume of 0.5 ml [10] after precipitation with 10% perchloric acid. Alternatively, the 260 nm/280 nm ratio of absorbance was used for quantitative determination of the protein concentration.

*Column chromatography*

Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala) and DEAE-Cellulose (DE 32, Whatman) were prepared as recommended by the manufacturers. After packing the columns were equilibrated with buffer A (see above). For preparation of poly-(A)-Sepharose polyadenylic acid was immobilized to Sepharose 4B [11, 12].

*Isoelectric focusing*

Preparative technique was performed in a sucrose density gradient (50 – 20%; v/v with 1% v/v Ampholine, LKB Stockholm, carrier ampholytes; range: pH 4.0 – 6.0). The glass column used (100 ml capacity) was equipped with an outer cooling jacket and platin electrodes [13]. When focusing was complete the column contents was pumped through a flow cell for measuring the absorbance at 280 nm; fractions of 1 ml were collected and assayed for PNPase activity.

*Gel electrophoresis*

Polyacrylamide gels containing dodecyl sulfate were prepared and run as described [14] applying several modifications. The slab gel (13 × 8 × 0.1 cm) included a stacking gel of 5% acrylamide (pH 6.8). The running gel was made of 10% acrylamide (pH 8.8). Samples were electrophoresed at 30 mA for about 5 h. Then the gels were stained with Coomassie brilliant blue. – Non-denaturing polyacrylamide gels were prepared according to Davis [15]. The gel columns (6 × 0.6 cm) consisted of a 2.5% stacking gel (1 cm) and a 5% running gel. Separation was performed at 3 mA/slab for 2 h; they were stained as described above.

*Purification procedure*

All steps were performed at 0 – 4 °C unless otherwise specified. 100 g of frozen cells were suspended in an equal volume of extraction buffer. After addition of two volumes of glass beads (∅ 0.10 – 0.11 mm) the cells were disrupted in a blender (Bühler, Tübingen) under refrigeration following the procedure by Richter and Senger [16]. The homogenate was freed from the glass beads by suction through a sinter glass filter and centrifuged at 35 000 × $g$ for 35 min yielding a deep blue-green supernatant. The pH value of this crude extract (= fraction "I") was adjusted to pH 7.5 by dropwise addition of 1 N NaOH. In order to digest the nucleic acids in this extract 45 µg/100 ml of pancreatic DNase I and 1.5 mg/100 ml of intestinal phosphatase were added [8]. The mixture was kept at room temperature for 60 min, and the pH maintained at 7.5 by addition of 1 N NaOH solution. The degradation of nucleic acids resulted in a continuing drop

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in viscosity. Then the pH was raised to 8.0 and the crude cell debris precipitated by centrifugation of the extract at 105 000×$g$ for 40 min. The resulting dark blue supernatant was centrifuged at the same speed for another 10 h. The sediment was dissolved in buffer A yielding a deep blue solution (= fraction "II"). To this increasing amounts of $(NH_4)_2SO_4$ were added, and the precipitate formed between 40 and 65% saturation was collected by high-speed centrifugation (25 000×$g$ for 20 min). The sediment was resuspended in as little as possible buffer A and dialyzed overnight against 5 l of the same buffer (= fraction "III").

Fraction "III" was immediately applied under pressure to a column of DEAE-cellulose previously equilibrated with chromatography buffer lacking salt. After washing the column with 200 ml of the same buffer the bound material was subsequently eluted with a linear gradient of 0.0 against 0.8 M KCl dissolved in buffer A. The enzyme containing fractions 24–42 were pooled (= fraction "IV") and their volume reduced to about 4 ml by application of immersible ultrafilters (Millipore, Bedford/Mass.).

Fraction "IV" was adsorbed to a column of Sephadex G-200 (2.5×90 cm) applying weak pressure (flow rate 4 ml/h). PNPase was then eluted with buffer A (5 ml/h). The fractions with highest specific activity (4–15) were pooled (= fraction "V"); their volume was reduced to about 2 ml (see above).

Fraction "V" was incubated with 2.5 µg/ml of intestinal phosphatase in the presence of 2.5 mg/ml of phenylmethylsulfonylfluoride for 1 h previously to affinity chromatography. At the end of the treatment 1 M sodium phosphate buffer (pH 8.0) was added to a final concentration of 0.1 M, and the mixture was further incubated for 20 min at room temperature. The autolysis mixture was then dialyzed for 1 h against buffer A supplemented with 1 mM EDTA and for 1 h against 1 l Tris-HCl buffer (0.01 M; pH 8.0).

The autolyzed PNPase preparation was applied to a poly-(A)-Sepharose column (1.5×10 cm) previously equilibrated with 300 ml of Tris-HCl buffer (0.02 M; pH 7.4) containing 10 mM $MgCl_2$, 0.1 mM DTT, 5% (v/v) glycerol. After washing the loaded column with 100 ml of buffer A lacking phenylmethylsulfonylfluoride the enzyme was eluted with a 100 ml linear gradient of 0.1 M against 4.0 M NaCl in Tris-HCl buffer (0.01 M; pH 9.0; with 10 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA) = fraction "VI".

*Materials*

[8-$^{14}$C]ADP (1.5–2.2 GBq/mM) was purchased from Amersham Buchler, Braunschweig (Germany). Unlabeled nucleoside diphosphates, DNase grade II, intestinal phosphatase grade II, polyadenylic acid and protein markers came from Boehringer, Mannheim (Germany).

Dodecyl sulfate, the substances for preparing polyacrylamide gels, bromphenol blue, Coomassie brilliant blue (R 250) were from Serva, Heidelberg (Germany). Suppliers of the other analytical grade chemicals were Merck, Darmstadt (Germany) and Riedel-de Häen, Hannover (Germany).

## Results

*Purification of polynucleotide phosphorylase*

Purification starts with the degradation of nucleic acids in the cell homogenate by added DNase I and endogenous enzymes, respectively, supported by alkaline phosphatase which removed phosphate groups from the 3′-terminus of the RNA molecules. The subsequent ultracentrifugation yielded a sediment which consisted mainly of ribosomes and contained about 70% of the initial PNPase activity. The increase in specific activity was about 4-fold. A characteristic feature of this step was the elimination of chlorophyll and yellow pigments which remained in the supernatant. When the sediment fraction was subjected to DEAE-cellulose chromatography one large peak of PNPase activity appeared between 0.3 and 0.45 M salt apart from the bulk of contaminating proteins, *i.e.* blue biliproteids (Fig. 1). During gel filtration of the active fractions PNPase was eluted in one distinct peak prior to most of the inert proteins, mainly blue biliproteids (Fig. 2). On the basis of the elution pattern the relative molecular mass of the PNPase is in the range of 250–300 000. The final affinity chromatography on poly-(A)-Sepharose removed most of the contaminating proteins still present resulting in an about 8-fold increase of the specific activity (Table I).

Since the affinity of PNPase to immobilized ribopolynucleotides is greatly influenced by nucleic acids fraction "V" had to be incubated after addition of alkaline phosphatase at 37 °C. This treatment removed phosphate moieties from the 3′-end of RNA chains thus enabling phosphorolysis by the endogenous PNPase. This autolyzed preparation was dia-

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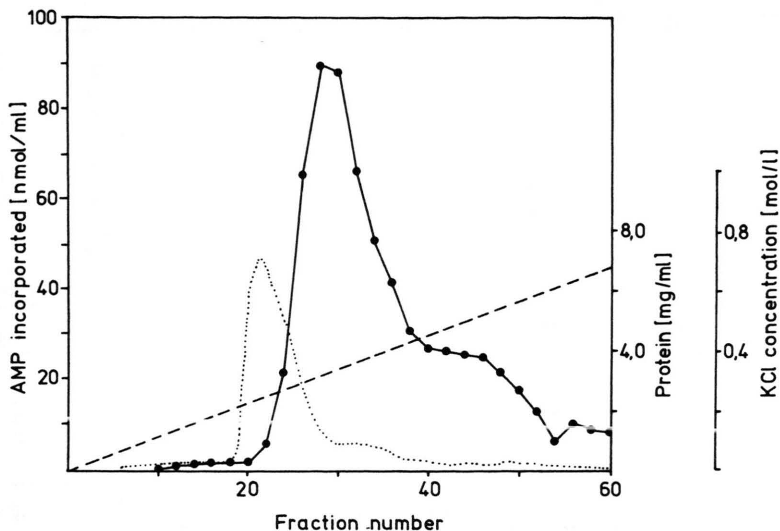


Fig. 1. DEAE-cellulose chromatography of *Synechococcus* PNPase. Fraction III (0.24 g of protein) was applied to a 2.5×15 cm column and eluted as described. 3-ml portions were collected. The contents of tubes 24–42 were pooled (= fraction IV).●——●, PNPase activity; ·····, protein (mg/ml); – – – – –, KCl concentration.

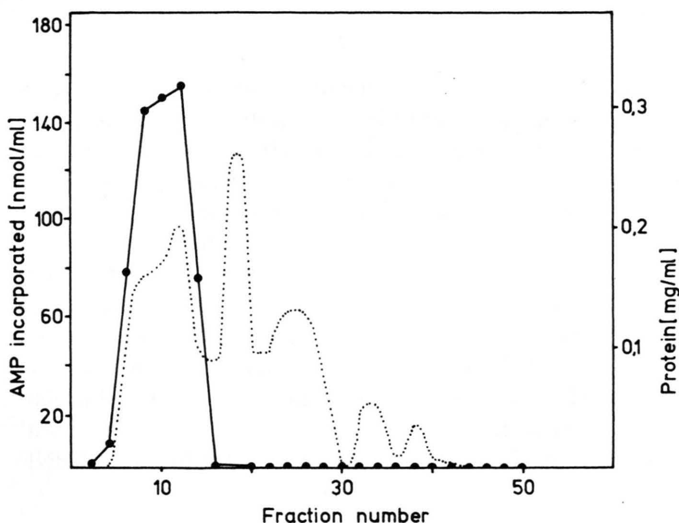


Fig. 2. Sephadex chromatography of PNPase from *Synechococcus*. Fraction IV (60 mg of protein) was applied to a 2.5×90 cm column of Sephadex G 200 and eluted with buffer A. 5-ml fractions were collected. Tubes 4–15 contained PNPase activity (= fraction V).

Table I. Purification of *Synechococcus* polynucleotide phosphorylase. The table summerizes the purification of the enzyme from about 100 g of deep-frozen cells. 1 unit of PNPase activity is defined as the amount catalyzing the incorporation of 1 nmol [8-$^{14}$C]AMP into ribopolynucleotide at 37 °C in 1 min.

| Fraction | Total protein [mg] | Total activity [units] | Spec. activity [units/mg protein] | Yield [%] | Purification factor |
|---|---|---|---|---|---|
| Crude extract (I) | 14 850 | 2 079 | 0.14[a] | 100 | 1 |
| Ultracentrifugation sediment (II) | 2 633 | 1 422 | 0.54 | 69.4 | 3.9 |
| Ammonium sulfate precipitate (III) | 240 | 1 132 | 4.72 | 54.4 | 34.0 |
| DEAE-cellulose eluate (IV) | 60 | 665 | 11.08 | 32.0 | 79.2 |
| Gel filtration eluate (V) | 18 | 437 | 24.30 | 21.0 | 173.6 |
| Affinity chromatography (VI) | 1.9 | 378 | 199.00 | 18.2 | 1 421.4 |

[a] Activity determined in samples which were diluted to optimal protein concentration.

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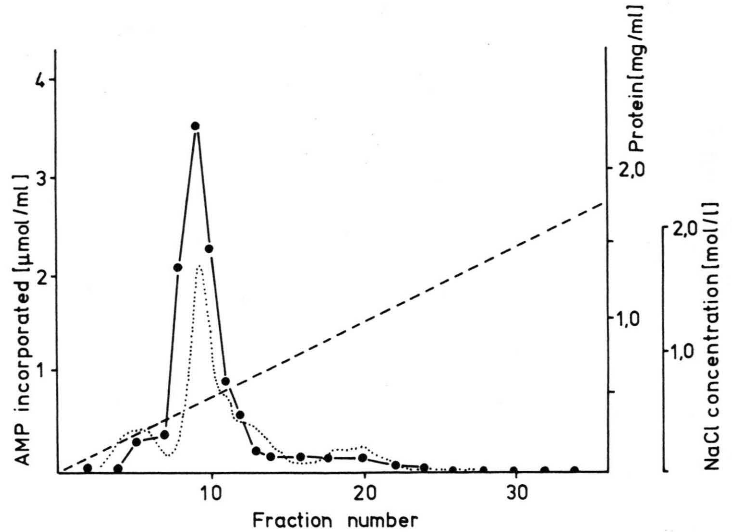


Fig. 3. Affinity chromatography of PNPase from *Synechococcus*. Fraction V (18 mg of protein) was adsorbed to a column (1.5×10 cm) of poly-(A)-Sepharose and eluted with a 0.1 – 4.0 M linear gradient of NaCl in buffer (see text). 1-ml fractions were collected. The contents of tubes 8 – 11 containing the main activity of PNPase were pooled (= fraction VI).

lyzed and then adsorbed to a poly-(A)-Sepharose column. The flow through material contained only traces of PNPase activity indicating a complete binding of the enzyme to the column under the conditions applied. Upon elution with a linear gradient of NaCl one peak of PNPase activity was separated (Fig. 3) being eluted at about 0.6 M NaCl.

Table I summerizes the purification steps of PNPase from *Synechococcus sp.*

*Isoelectric focusing*

Alternatively, an attempt has been made to achieve a time-saving fractionation and a concentration of PNPase at an earlier stage of the purification procedure. Accordingly, samples of fraction "III" (= ammonium sulfate precipitate; 10 mg protein) were subjected to isoelectric focusing in a sucrose density gradient with a pH gradient of 4 – 6 (Fig. 4).

Most of the PNPase activity formed a major band in the pH region of about 4.2. A minor fraction banded at a slightly higher pH value at the trailing edge of the main protein peak. This fraction could result from associated nucleic acids which may change the isoelectric point of the separated protein molecules. Due to this disadvantage correlated with a relatively low specific activity purification of PNP-

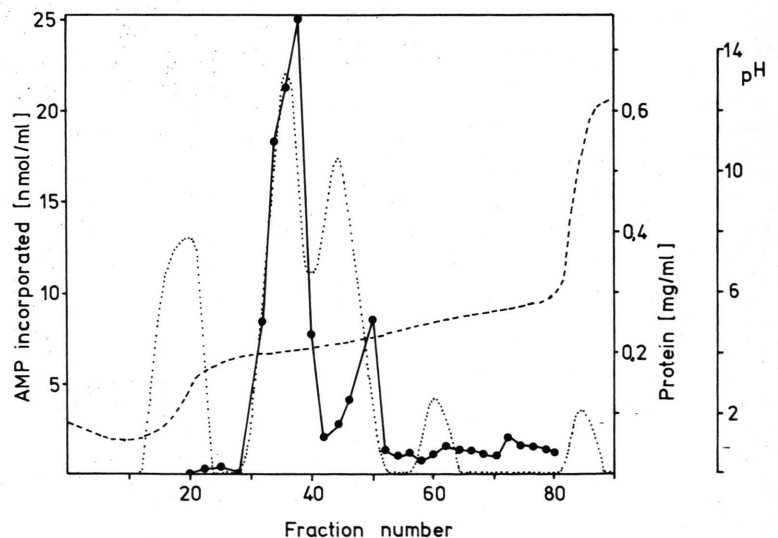


Fig. 4. Preparative isoelectric focusing of partially purified PNPase (fraction III; about 10 mg of protein) from *Synechococcus* in a sucrose density gradient (50% – 20%; w/v) applying a pH-gradient of 4 – 6. 1-ml fractions were collected and assayed for PNPase activity. ●—●, PNPase activity; · · · · ·, protein (mg/ml); – – – – –, pH-gradient.

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ase by isoelectric focusing had to be restricted to relative pure preparations.

*Extent of purification, yield*

The specific activity of the final preparation (fraction "VI") is comparable with that reported for purified PNPase from other prokaryotic organisms, *e.g. Escherichia coli* [8]. On the basis of the activity of fraction "I" measured with an optimal protein concentration (see below) the purification procedure described here resulted in the recovery of about 18% of the original PNPase activity with an overall purification of about 1400-fold.

The PNPase activity in less pure fractions from earlier steps of purification showed a dependency from the protein concentration which has been described previously for *Synechococcus* [3, 17]. With samples from fraction "III" upon dilution up to 0.4 mg protein in the standard assay a steady increase of the PNPase activity was observed (Fig. 5). Further lowering of the protein content lead to a steady decrease in activity. The protein concentration per se seemed not to interfer since addition of bovine serum albumin to the enzyme preparations did not suppress the PNPase activity.

On the contrary: a slight stimulation of the latter was observed following addition of the inert protein. The implication of these data is that the stimulation observed after dilution originates from diluting out endogenous degrading enzymes like nucleases, phosphatases and proteases which interfer with PNPase in less pure preparations. They are eliminated during the further course of purification as is evident from the results of the following experiments. Incubation of highly purified PNPase (fraction "VI") at 37 °C with [$^{14}$C]labeled poly(A) or ribopolynucleotides in the presence of inorganic phosphat (3.2 μM) which inhibits PNPase completely did not yield significant amounts of acid-soluble [$^{14}$C]ribonucleotides thus proving the absence of nucleases detectable in less pure preparations of the enzyme (see above).

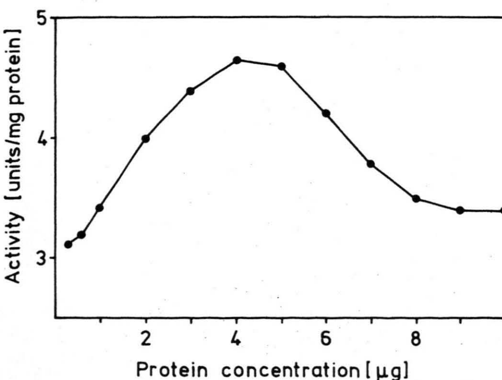


Fig. 5. Influence of protein concentration on the activity of partially purified PNPase (fraction III) from *Synechococcus.* Various amounts of protein obtained by diluting the original enzyme preparation were added to the standard test assay and incubated as described.

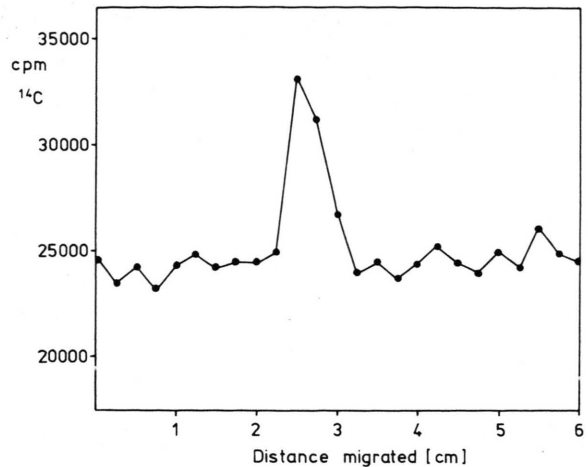


Fig. 6. Separation by polyacrylamide gel electrophoresis under non-denaturing conditions of PNPase (fraction V). For localization of the enzym activity the entire gel was incubated at 37 °C for 12 h with the test mixture containing [8-$^{14}$C]ADP. Migrating direction is from left to right. Abscissa: Length of gel in cm; ordinate: radioactivity (cpm) incorporated into AMP-polymer.

Samples of fraction "VI" preincubated at 37 °C for various periods of time in the assay mixture lacking [$^{14}$C]ADP had the same PNPase activity after addition of the substrate as the controls; moreover, the molecular structure of the enzyme as revealed by gel electrophoresis under nondenaturing and denaturing conditions remained unchanged (see below). These findings are strong evidence against the presence of degrading protease activities in the highly purified enzyme fraction "VI".

*Properties of polynucleotide phosphorylase from Synechococcus*

*Criteria of purity.* Enzyme-containing fractions from various stages of purification were separated by polyacrylamide gel electrophoresis under non-denaturing conditions. After incubation *in situ* (see

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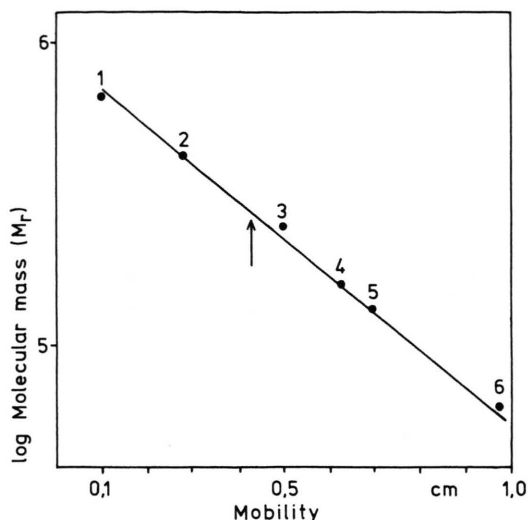


Fig. 7. Molecular mass determination of purified PNPase (arrow). Samples of fraction "VI" were electrophoretically separated on 5% polyacrylamide gels under nondenaturing conditions. Reference proteins (circles): 1, thyroglobulin (669 000); 2, ferritin (440 000); 3, catalase (232 000); 4, lactate dehydrogenase (140 000); 5, bovine serum albumin (67 000); 6, ovalbumin (45 000). Abscissa: Distance migrated; Ordinate: log molecular mass.

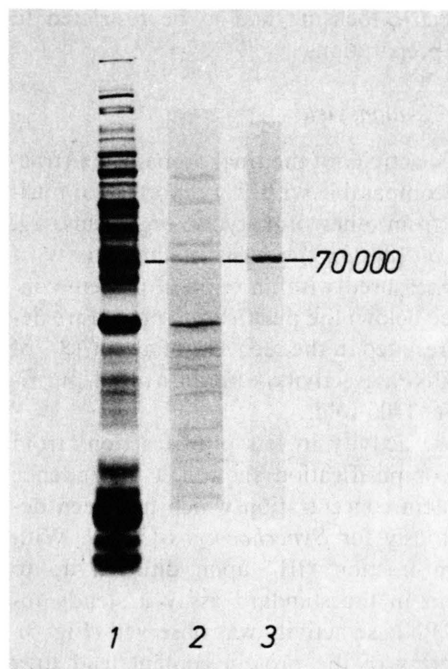


Fig. 8. Sodium dodecyl sulfate gel electrophoresis in polyacrylamide (10%) of fractions resulting from various steps of the PNPase purification procedure. 3–5 µg of the following samples were applied to the slots of the gel slab: 1, fraction III; 2, fraction IV; 3, fraction VI. Electrophoresis was for 5 h at 30 mA/slab gel. The gel was stained with Coomassie brilliant blue as described.

Materials and Methods) with substrate only one band of ADP polymerizing activity could be located within the gel (Fig. 6) which always coincided with one single protein band of identical molecular mass. Apparently, the homogeneity of the nondenatured enzyme is maintained throughout the purification procedure. Molecular mass analysis of purified PNPase (Fraction "VI") using nondenatured polyacrylamide gels and various reference proteins revealed one band with an apparent molecular mass of about 275 000 (Fig. 7).

*Subunit composition.* From electrophoresis in polyacrylamide gels (10%) under denaturing conditions, *i.e.* in the presence of 0.1% SDS, it is evident that the DEAE-cellulose step removed most of the contaminating high and low molecular mass proteins (Fig. 8, slot 2). The passage through the poly-(A)-Sepharose column purified the enzyme further which exhibits now one single band of about $M_r$ = 70 000 in the SDS gel (Fig. 8, slot 3; Fig. 9). Since estimation of the molecular mass as described gave the value 275 000 it is logical to conclude that the native enzyme molecule is composed of four identical subunits.

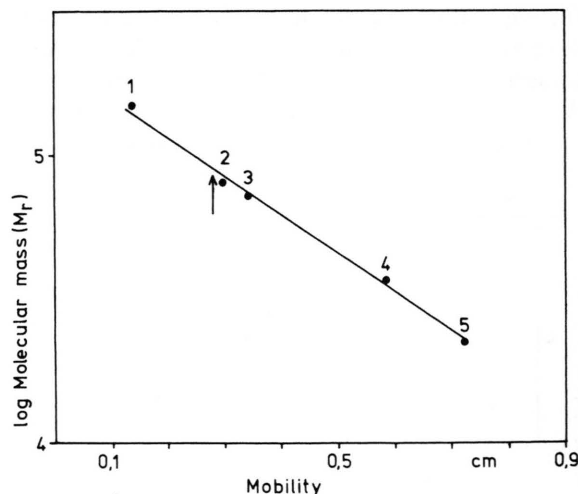


Fig. 9. Determination of the apparent molecular mass of the subunit of highly purified PNPase from *Synechococcus* (arrow) by electrophoresis in a 10% polyacrylamide gel containing dodecyl sulfate. The molecular masses of the proteins used as references are as follows (dark circles); 1, $\beta$ subunit of *Escherichia coli* RNA polymerase (155 000); 2, bovine serum albumin (67 000); 3, catalase (subunit of 60 000); 4, lactate dehydrogenase (35 000); 5, trypsine inhibitor (21 500).

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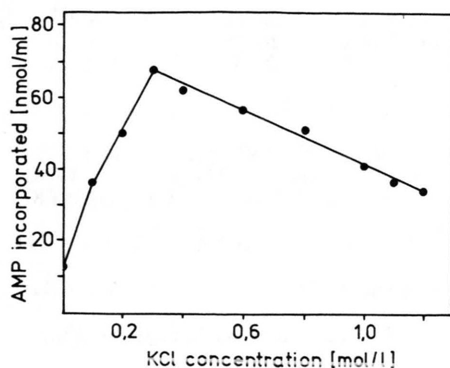


Fig. 10. Effect of KCl on the activity of highly purified PNPase (fraction VI) from *Synechococcus*. Assays were performed with various concentrations of KCl in the standard test mixture.

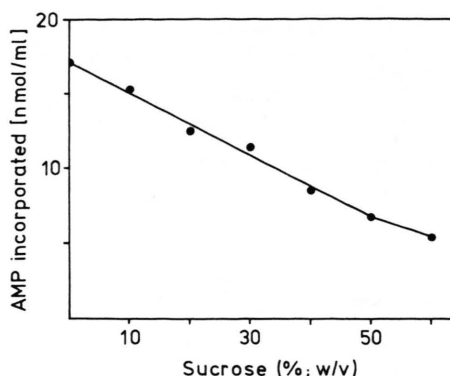


Fig. 11. Inhibitory effect of sucrose concentration on the activity of partially purified PNPase (fraction V) from *Synechococcus*. Various amounts of sucrose were added to the standard test assay.

*Isoelectric point.* Isoelectric focusing of the purified enzyme in a sucrose density gradient followed by determination of activity and protein content revealed an isoelectric point of $4.2 \pm 0.1$.

*KCl requirement.* In partial or highly purified state PNPase was preferentially activated by 0.3 M KCl (Fig. 10). We have no functional interpretation of this property so far.

*Sensitivity to inorganic phosphate and sucrose.* Through all steps of the purification did $Na_2HPO_4$ inhibit PNPase when added in a concentration of 3.2 µM. The presence of sucrose in the enzyme preparations reduced the activity significantly as shown in Fig. 11.

## Discussion

The molecular mass of PNPase from *Synechococcus sp.* compares well with preparations from *Escherichia coli* ($M_r = 250\,000$; [8]), *Micrococcus luteus* ($M_r = 270\,000$ and $M_r = 230\,000$, respectively; [18]) and *Thermus thermophilus* ($M_r = 190\,000$; [19]).

The subunit composition of PNPase, however, varies considerably among bacterial species and differs also from the algal enzyme. For *Escherichia coli* a single subunit of $M_r = 84\,000$ has been reported [2, 8, 20] indicating a composition of $\alpha_3$ for the native enzyme molecule. Occasionally traces of smaller peptides with molecular masses of 68 000 and 41 000, respectively, were found presumably resulting from proteolytic degradation of the native subunit. PNPase from *Thermus thermophilus* consists of three subunits with the following molecular masses: 92 000, 73 000 and 35 000 [19]. Four identical subunits each with a molecular mass of about 51 000 appear to form the native PNPase molecule in *Bacillus stearothermophilus* [21] indicating a tetrameric structure.

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