Purification and Characterization of Polynucleotide Phosphorylase from Escherichia coli

PROBE FOR THE ANALYSIS OF 3' SEQUENCES OF RNA*

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A simple procedure for purifying polynucleotide phosphorylase from Escherichia coli cells by means of affinity chromatography on an RNA-Sepharose column is described. The purified enzyme preparation has a specific activity 3500-fold that of the crude extract and is essentially homogeneous, as determined by ultracentrifugation, polyacrylamide gel electrophoresis under denaturing conditions, isoelectric focusing and serological assays. It is virtually free of nuclease contamination, a property which permits its use in the synchronous phosphorolysis of RNA chains.

The enzyme molecule is composed of three identical subunits of \( M_r = 84,000 \). Each subunit contains three cysteine residues, one of which reacts with 5,5'-dithiobis(2-nitrobenzoic acid) whereas the two other groups are only exposed on denaturation of the protein. All three enzyme subunits participate in the processive phosphorolysis of the poly(A) tail of each globin mRNA chain. An advantageous method was developed for synchronous phosphorylation of RNA molecules using a molar excess of polynucleotide phosphorylase immobilized onto Sepharose.

The enzyme polynucleotide phosphorylase (EC 2.7.7.8) was purified from a variety of sources and has been widely used in nucleic acids research (cf. Ref. 1). Recently, we have developed a method for the removal of the poly(A) tail from mRNA chains, based on synchronous processive phosphorolysis of mRNA using a molar excess of polynucleotide phosphorylase at 0°C (2). This method enabled us to determine the location, length, and the cytoplasmic role of the poly(A) tail of globin mRNA (3-6). The molar amounts of enzyme used in such reaction mixtures are much higher than the catalytic quantities normally used for RNA phosphorolysis and require, therefore, a more stringent removal of nuclease contamination. The purification procedure described here answers this requirement: it is relatively simple and is easily scaled up, so that large quantities of the enzyme can be obtained for physical and immunological characterization.

EXPERIMENTAL PROCEDURES

The experimental procedure, including materials and methods and comments on the purification of polynucleotide phosphorylase, is described in the adjacent miniprint. Figures and tables in the supplementary miniprint are identified by "S."

RESULTS

Several procedures for the purification of polynucleotide phosphorylase from Escherichia coli cells have been described (7-12). Our aim was to develop a rapid and efficient purification procedure, which could be easily adapted to large scale operation and yield a single pure protein. As can be seen from the data summarized in Table I, which are outlined in detail in the miniprint, the enzyme was purified about 3500-fold (RNA-Sepharose fraction), with a recovery of 44% and a final specific activity of 415 units/mg.

Enzyme Properties

Homogeneity - The molecular weight of the purified enzyme was determined by gel electrophoresis under denaturing conditions. The denatured enzyme migrates as one main band of 84,000 ± 5,000 with a hardly detectable contaminant of 41,000 (Fig. 1 S). Ultracentrifugation of the denatured enzyme revealed an \( s_{20, w} \) value of 8.98 ± 0.1 for the enzyme molecule and equilibrium sedimentation analysis indicated a molecular weight of about 230,000 ± 20,000 and a composition of \( \alpha_2 \) for the native enzyme molecule. The isoelectric point of the purified enzyme was found to be at pH 6.1 ± 0.1, as determined by isoelectric focusing followed by activity and protein staining of the electrophoretically separated protein. Purified polynucleotide phosphorylase also reveals one major band of ADP polymerizing activity upon electrophoresis under nondenaturing conditions. The amino acid composition was distinguished by a low cysteine content, which was also found by reaction...
with DTNB followed by spectral assay (Table 1). The immunological purity of the enzyme was ascertained by immunodiffusion assays. Antibodies to polynucleotide phosphorylase were elicited in rabbits by two injections of 0.3 mg of pure enzyme. The antisera obtained 1, 2, 3, and 4 weeks after the second injection were examined for specific immunodiffusion reactions with polynucleotide phosphorylase. Fig. 3 reveals only one precipitation line, the intensity of which increases with time after the injection, indicating that the major immunodeterminant is a single protein species. Immuno-photophoresis was used as a sensitive method to detect contaminating proteins. As shown in Fig. 4 the enzyme preparation contained a minor contamination of S, ribosomal protein which could not be observed using other analytical methods.

**How Many Enzyme Subunits Participate in Phosphorolysis of Each RNA Chain?**—Although we have shown that the enzyme contains three subunits, this does not necessarily imply that the same number participates in the phosphorolysis of RNA chains. Polynucleotide phosphorylase has been shown to phosphorolyse RNA by a processive mechanism, in which the enzyme does not dissociate from the polynucleotide molecule until the length of the chain decreases below 20 nucleoside residues (13, 14). This mechanism implies that when the number of enzyme molecules is lower than that of the RNA chains, initially only part of the molecules will be phosphorolyzed, the rest remaining intact. When the number of enzyme molecules equals that of the RNA chains, all the molecules will be phosphorolyzed synchronously from the onset of the reaction. We have utilized this feature to titrate the number of enzyme subunits attached to each RNA chain by analysis of the phosphorolysis products initiated at various enzyme concentrations.

Rabbit globin mRNA, as a substrate, was incubated at 0°C with increasing amounts of Sepharose-bound enzyme. Under the conditions employed, only the poly(A) tail of the mRNA is removed, the rest of the RNA chains remaining intact with the enzyme still attached (2). The proportion of poly(A)-free mRNA obtained depends on the amount of enzyme employed. At the end of the reaction, the RNA was extracted and analyzed by polyacrylamide-urea gel electrophoresis, stained, and photographed. The transparencies of the different slots were scanned using a Gilford 2400 spectrophotometer with a scanning attachment (Fig. 1A). This enabled us to distinguish between the phosphorolyzed and intact mRNA chains. The amount of poly(A)-free mRNA obtained from each reaction mixture was calculated by integration of the scanned peaks (Fig. 1B). Fig. 1A shows that at low enzyme concentrations (Scans 1 and 2) there remains a substantial amount of more slowly migrating, native poly(A)-containing RNA molecules.

The proportion of which decreases with increasing enzyme concentration (Scan 3) until all the RNA has a mobility identical with that of poly(A)-free mRNA (Scan 4). Further increase in the enzyme concentration does not change the mobility (since we have chosen reaction conditions under which only the poly(A) tail is removed (2)); similar results were obtained with a soluble enzyme preparation. It is clear from this experiment that polynucleotide phosphorylase indeed degrades RNA by a processive mechanism, and that a molar ratio of three subunits of the enzyme to 1 RNA molecule is required to remove the poly(A) tail from globin mRNA. The active form of the purified enzyme is therefore α3. This conclusion rests on the assumption that substantial inactivation of the enzyme did not take place during its purification. The apparent Kᵣ of the enzyme molecule for binding to the RNA substrate, derived from this binding curve, was 1.66 × 10⁻¹⁵ M, which is identical with previous estimates (15).
These results also indicate that the apparent decrease observed in the rate of phosphorolysis with Sepharose-bound enzyme (16) is not due to enzyme inactivation, since the number of subunits actively engaged in the phosphorolysis reaction (1.08 pg of enzyme protein/pg of RNA) was identical with that obtained with a soluble enzyme.

Acknowledgments—We would like to acknowledge the helpful suggestions of Drs. Hiroshi Inouye and Zvi Bohak.

REFERENCES


Supplementary Notes

The P-fraction and characterization of polyuridylic acid polymerase with Sepharose affinity chromatography. P-primed RNA synthesis. The enzyme purified from the P-primed RNA was resolved into two subunits by gel filtration and native polyacrylamide gel electrophoresis. The subunits were identified by their molecular weights and relative mobilities in the gel. The enzyme activity was found to be stable for at least 1 year.


Enzyme Purification

The first two steps of the enzyme purification procedures are based on precipitation of RNA with 5 M guanidium chloride. All operations were performed at 0-4°C unless otherwise stated.

Preparation of Poly(dU)poly(dA)-Primed RNA

1. The standard reaction mixture (50 ml) contained 50 mM Tris-HCl (pH 7.8 at 0°C), 100 mM NaCl, 2.5 mM MgCl2, 0.1 mM dithiothreitol, 0.05 mg/ml 14C-ATP (specific activity, 500-1000 Ci/mmol), and 0.05 μg/ml Poly (dA)poly(dT) (100,000 cpm). The mixture was incubated for 10 min at 0°C and then in a water bath at 37°C for 20 min. After the incubation, the reaction mixture was diluted with 500 ml of 1% Triton X-100 and 25 ml of 2 M NaCl, followed by 2 ml of 1% Nonidet P-40. The mixture was then extracted three times with an equal volume of phenol containing 0.01% NaCl and 0.01% Triton X-100. The following buffer solution was added to the above mixture: 200 ml of 10 mM Tris-HCl (pH 7.8), 100 mM NaCl, 2.5 mM MgCl2, and 2 ml of 2 M NaCl. The mixture was then applied to the Sepharose-primed RNA column. The column was eluted with 2 M NaCl and the nucleic acid was eluted with 0.1 M NaCl.

Electrophoresis on Polyacrylamide Gels

5. The electrophoresis was performed on 12.5% polyacrylamide gels in borate buffer (pH 8.3). The gel was run in a horizontal electrophoresis apparatus at 5 mA/cm2 for 2 hr. The gel was stained with Coomassie blue and analyzed for polyuridylic acid content.

6. In order to separate the RNA from the protein, the gel was stained with Coomassie blue and analyzed for polyuridylic acid content. The gel was stained with Coomassie blue and analyzed for polyuridylic acid content.
Polynucleotide Phosphorylase

Fig. 1. A. Polynucleotide phosphorylase from E. coli crude extract. Purification of the enzyme was carried out on a column of DEAE-Sephadex A-25 (Fig. 1A). The enzyme was eluted with a linear gradient of 0-0.5 M NaCl. The active fractions were pooled and dialyzed against 0.01 M Tris HCI, pH 7.5. The dialyzed enzyme was then concentrated by ultrafiltration and used for further studies.

Fig. 1. B. SDS-PAGE analysis of purified polynucleotide phosphorylase. A 12.5% SDS-PAGE gel was run under reducing conditions. The enzyme was stained with Coomassie Blue R-250.

Table 1. Activity of various polynucleotide phosphorylase preparations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Number of total units</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>1.5</td>
<td>300</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.0</td>
<td>200</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>3.5</td>
<td>150</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.0</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 2. Spectral analysis of purified polynucleotide phosphorylase. Absorbance was measured at 280 nm. The enzyme was dialyzed against 0.01 M Tris-HCl, pH 7.5, and then concentrated by ultrafiltration.

Fig. 3. Immunoblot analysis of purified polynucleotide phosphorylase. Blots were probed with polyclonal antibodies raised against the purified enzyme.

Fig. 4. Immunoblot analysis of purified polynucleotide phosphorylase. Blots were probed with monoclonal antibodies raised against the purified enzyme.

Fig. 5. UV-visible absorption spectrum of purified polynucleotide phosphorylase. The enzyme was dialyzed against 0.01 M Tris-HCl, pH 7.5, and then concentrated by ultrafiltration.

Fig. 6. Electrophoretic analysis of purified polynucleotide phosphorylase. A 12.5% SDS-PAGE gel was run under reducing conditions. The enzyme was stained with Coomassie Blue R-250.

REFERENCES

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