A NOVEL RNA-primed POLYNUCLEOTIDEPYROPHOSPHORYLASE FROM *E. COLI*

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1. Introduction

In the course of the preparation of termination factor ρ by a slight modification of the procedure of Roberts [1] we isolated in almost homogeneous state, a RNA-primed polyribonucleotidepyrophosphorylase which proved to be nonidentical with the poly A polymerase first described by August et al. [2]. The fact, that under proper conditions not only ATP, but, to a considerable extent, also UTP and CTP are utilized as substrates and the striking differences of the efficiencies of various primers justify a brief report of the isolation procedure and the properties of this novel enzyme.

2. Experimental

2.1. *Preparation*

2 Kg of *E. coli* (wet weight) were homogenised, after addition of 2 l of the buffer used by Roberts [1] as described previously [3]. The crude extract was treated with DNAase (5 μg/ml) for 15 min at 0°. The debris was removed by centrifugation for 30 min in rotor R 19 of the Beckman preparative ultracentrifuge at 19,000 rpm. A protein fraction containing the enzyme was precipitated from the supernatant by the addition of 334 g/l of solid ammonium sulfate. The precipitate was collected by centrifugation, redissolved in 0.025 M potassium phosphate buffer (pH 7.5) and thoroughly dialysed against this buffer. The dialysed fraction was chromatographed over a 500 ml phosphocellulose column with a linear gradient of 2 × 600 ml ranging from 0.05 to 0.5 M phosphate. The enzyme containing fractions were eluted at the end of the gradient (around 0.5 M phosphate), precipitated by the addition of 371 g/l of solid ammonium sulfate, redissolved and thoroughly dialyzed against 0.01 M potassium phosphate (pH 7.5). This fraction was applied to a 25 ml DEAE column and subjected to chromatography in a linear gradient of 2 × 50 ml of potassium phosphate ranging from 0.02 to 0.2 M (pH 7.5). The enzyme was eluted around 0.06 M phosphate and precipitated by the addition of ammonium sulfate (371 g/l). At this stage, the total yield was about 50 mg and the purity about 30%. The redissolved protein was layered on top of a linear gradient of 10 to 30% sucrose and 5 to 10% glycerol in TMA buffer [3] containing 0.5 M NH_4Cl and centrifuged for 15 hr at 50,000 rpm in rotor SW 50.1 in a Beckman ultracentrifuge. Enzymatic activity was discovered in the top part of the gradient (with a peak in the fifth out of 44 fractions). A minor contamination still present at this stage was removed by micropreparative electrophoresis on cellulose acetate sheets (cellogel, see [4]) in 0.2 M NH_4HCO_3 buffer pH 8.0 containing 0.01 M magnesium acetate, 0.02 M β-mercaptoethanol and 5% glycerol. The enzyme migrated towards the cathode under these conditions. It was recovered from the sheet as described in [4].

2.2. *Test*

The enzyme was tested in a total volume of 0.25 m containing 0.1 M KCl, 0.02 M magnesium acetate, 0.05 M glycine-NaOH buffer (usually pH 10), 0.002 M β-mercaptoethanol, 0.001 M ^{14}C-labelled substrate (usually ATP) with excess primer RNA (usually 25 μg

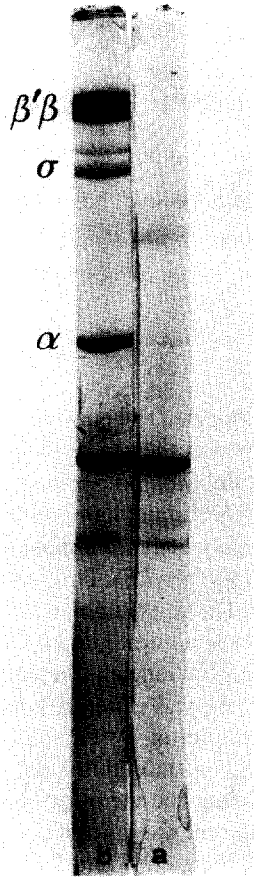

Fig. 1. Polyacrylamide disc electropherograms of the purified polynucleotidepyrophosphorylase alone (a), and with polymerase from *E. coli* (b) (see sect. 3.1).

for example t-RNA and 10 μg of the enzyme, for 30 min at 37°. Samples were prepared for counting as in the RNA polymerase assay [5].

2.3. *Analytical procedures*

Disc electrophoresis was performed according to Laemmli [6]. Analytical cellogel electrophoresis in 0.5 M borate–6 M urea buffer, and protein determinations were performed as in [4]. The molecular weight of the native enzyme (in TMA buffer containing 0.1 M KCl) was determined in a Beckman analytical ultracentrifuge equipped with a scanner by equilibrium centrifugation [7] at 17,000 rpm in a six channel cell.

3. Results and discussion

3.1. *Physical properties*

The purified enzyme exhibited one homogeneous band both in disc electrophoresis in the presence of SDS and in cellogel electrophoresis (fig. 1). The molecular weight of the polypeptide chain was estimated from disc electrophoresis in a 10% polyacrylamide gel to be 20,000 ± 10% [8]. The native enzyme, in TMA–KCl buffer, sediments with a $s^0_{20,w} \cong 3.1 \pm 0.3$ S. The molecular weight of 40,000 ± 5,000 indicates that the native enzyme is a dimer.

3.2. *Enzymological characteristics*

The enzymatic reaction is neither inhibited by phosphoenolpyruvate and phosphoenolpyruvate kinase nor by phosphate. The ribonucleoside triphosphates required as substrates cannot be replaced by diphosphates. Thus, the enzyme is a polynucleotide pyrophosphorylase.

The ionic requirements were determined using a mixture of the 4 ribonucleoside triphosphates as the substrates and t-RNA as the primer. Whereas the activity proved rather insensitive against a variation of the ionic strength (KCl concentration) from 0.005 to 0.15 M it exhibited a well defined Mg^{2+}-optimum at a concentration of 0.02 M. Mg^{2+} is absolutely required, apparently as a cofactor.

Even after prolonged incubation no activity was observed in the absence of primer. Native and denatured DNA do not replace RNA. RNA fragments of an average chain length of 20 nucleotide residues obtained by mild alkaline fission of yeast RNA and thus preferentially carrying 2′ or 3′ phosphate residues at the 3′ end exhibited poor primer efficiency. After removal of the terminal phosphate by alkaline phosphatase from *E. coli* the primer efficiency increased 4-fold. t-RNA totally looses its activity after oxidation of the 3′ terminal ribose residues by periodate [9]. Thus, primer function requires a free 3′ terminal hydroxyl group. The polyribonucleotide material primed by the RNA polymerase transcription product of T7-DNA binds to T7-DNA but not to T4-DNA under hybridization conditions but avoiding the usual RNAase digestion step [12], the material primed by the transcription product of T4-DNA anneals with T4- but not with T7-DNA in-

Table 1
Primer and substrate specificity of the enzyme.

Labelled triphosphate primer	A		U		G		C	
	pH 7	pH 10	pH 7	pH 10	pH 7	pH 10	pH 7	pH 10
r-RNA	0.078	0.066	0.037	0.027	0.042	0.017	0.191	0.109
Statistic polynucleotide mixture (chain length 20)	12.057	12.11	0.398	7.198	0.036	0.025	1.205	0.402
Poly U	0.484	0.683	0.03	0.471	0.017	0.018	0.127	0.066
t-RNA	9.637	8.881	0.322	2.746	0.026	0.022	1.277	0.466
Poly A	2.697	0.906	0.047	0.338	0.043	0.029	0.191	0.075
M_{12} RNA	39.59						1.889	
Poly AU^{+1}	0.547	6.296	0.019	5.895	0.030	0.019	0.207	0.129
Poly G^{+2}	4.329	1.831	0.034	0.183	0.025	0.015	0.131	0.066
Poly C^{+3}	1.268	1.859	0.02	0.112	0.028	0.014	0.171	0.066
T4-RNA^{+}	4.666	11.398	0.103	0.204	0.147	0.020	0.736	0.965
T5-RNA^{+}	8.144	10.895	0.91	0.043	0.017	0.028	0.485	0.458
T7-RNA^{+}	6.889	5.714	0.155	0.128	0.017	0.022	0.887	0.592
	pH 8		pH 8		pH 8		pH 8	
t-RNA	7.61		0.25		0.81		0.23	
t-RNA^{+++}	1.21		0.05		0.19		0.11	

Primer and enzyme quantities as in the standard incubation mixture, at both pH 7 and pH 10 (see sect. 2.2). ^{14}C-labelled ATP, UTP, GTP and CTP each offered alone (except for $^{+1}$), where 0.1 mM unlabelled UTP and ATP are present besides 1 mM ^{14}C-labelled substrate, and $^{+2}$ where 0.1 mM unlabelled GTP and $^{+3}$ where 0.1 mM unlabelled CTP is present besides labelled substrate and $^{+}$, where all four substrates, at a concentration of 0.1 mM, and $^{+++}$, where all four substrates, at a concentration of 1 mM, are present besides the labelled one. Numbers are nmoles of labelled nucleotide incorporated in 30 min at 37°.

dicating that the material synthesized by the polynucleotide pyrophosphorylase is covalently linked to the primer, probably over its 3′ hydroxyl end.

The oligonucleotide mixture obtained by digestion of t-RNA with pancreatic RNAase and treated with alkaline phosphatase to liberate 3′terminal hydroxyls is inactive as a primer. Therefore, a minimal length of the primer appears to be required for activity.

As seen from table 1, efficiencies for poly A synthesis (measured in excess of primer) are strikingly different for various RNA's and polyribonucleotides. Thus, r-RNA with its strong secondary structure is almost inactive. On the other hand, t-RNA, a statistic mixture of polynucleotides of the average chain length 20 (see above), the RNA polymerase transcription products of T4-, T5- and T7-DNA, the RNA of bacteriophage M12 and poly AU (alternating), are efficient primers. Homopolymers possess weak to intermediate primer activities.

Besides ATP, UTP and CTP but scarcely GTP are utilised as substrates. Depending on the conditions (pH, primer), incorporation of CTP in the absence of other triphosphates, varies around 10% of that of ATP alone. Incorporation of UTP alone, which is always weak at pH 7, at pH 10 is in the range of that of ATP on the statistic polynucleotide mixture, on poly AU and on poly U, is intermediate on t-RNA but weak on all other, especially the natural primers.

The reaction is strongly influenced by the pH of the incubation mixture. The pH effects depend both on substrate and primer. Thus, CTP, when offered singly, is generally incorporated stronger at pH 7 than at pH 10, except for the "natural" RNA's. UTP, on the other hand, is utilised much better at pH 10 than at pH 7, again except for the "natural" primers. For ATP, the direction of the effect varies with the primer. Thus, poly A and poly G are better primers at pH 7 than at pH 10. The contrary is true for poly AU and T4-RNA. No major difference is

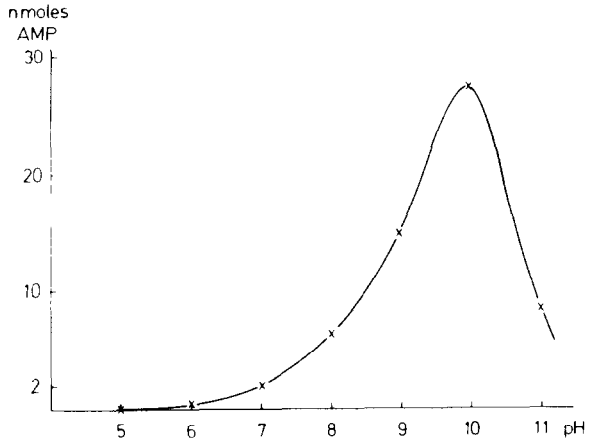

Fig. 2. pH Dependence of the utilization of ^{14}C-ATP, in the presence of unlabelled UTP, GTP and CTP, by the enzyme. Standard assay conditions but with varying pH (see sect. 2.2).

observed on the other primers. The pH effect for one substrate may be greatly changed by the presence of other substrates. Thus, on t-RNA, incorporation of ATP alone is almost the same at pH 7 as at pH 10. In the presence of the three other triphosphates, however, it is almost 14-fold higher at pH 10 than at pH 7 (fig. 2). The presence of other triphosphates strongly inhibits the utilization of each of the labelled substrates.

Though the primer clearly directs the substrate specificity of the enzyme in some way, the product is neither complementary to it (the primer is not a template) nor is the nature of the product in another evident way directly related to the structure of the primer. Thus it appears (see also the inactivity of r-RNA) that the secondary structure is of importance. On the other hand the difference of pH effects both on different substrates and different primers indicates the significance of the dissociation status of base residues for their interaction with the enzyme.

The pH dependence of ATP incorporation, the fact that other substrates are utilized efficiently by the enzyme described here and finally the physical data clearly distinguish this enzyme from the well known poly A-polymerase which is almost inactive at pH 7, which almost exclusively utilises ATP and, which, in SDS polyacrylamide disc electrophoresis, exhibits 2 main bands with higher molecular weights [10].

Under standard conditions, polynucleotide synthesis by this enzyme proceeds linearly for at least 70 min. The chain length of the product may grow up to several thousand nucleotide residues or more. For the saturation of a given amount of primer, a much larger than stoichiometric amount of enzyme is required. The K_m for the binding of enzyme to t-RNA is about 8×10^{-5} M. This is in agreement with the finding that the enzyme does not stay fixed to the chain which it has first started but even in large excess of primer remains able to use a second primer added after the onset of synthesis (start on excess t-RNA, T5-RNA added later, and vice versa, product analysed by hybridization to T5-DNA).

The enzyme is thermostable and active at least up to 45°. This and its substrate utilization pattern distinguish it from a similar activity enriched from *Acetobacter vinelandii* by Burma [11]. It is not inhibited by rifampicin and proflavine but strongly inhibited by heparin, actinomycin and at high ionic strength (above 0.2). It is also blocked by pyrophosphate which, however, does not cause detectable pyrophosphorolysis of the product.

In spite of the rather high efficiencies of "natural" RNA's as primers, the physiological role of this enzyme as that of poly A polymerase remains obscure.

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