POLYRIBOADENYLATE POLYMERASE SOLUBILIZED FROM RAT LIVER MITOCHONDRIA

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Summary

An enzyme that can synthesize polyriboadenylate chains from ATP has been solubilized from rat liver mitochondria and characterized. The enzyme incorporates AMP residues into the internucleotide linkage. It is insensitive to the usual inhibitors of RNA polymerases. In the native state, poly (A) polymerase is associated with a nuclease that degrades the product. Solubilization of this enzyme from isolated mitochondria releases active enzyme from inhibitory action of the nuclease.

Introduction

Since the discovery of poly (A) in calf thymus (1), rat liver (2) and Ehrlich ascites cells (3), there have been several reports on the function of this polynucleotide in the eukaryotic organisms (4-8). It has been observed (5-7) that polyadenylate segments containing 150 to 250 nucleotides are covalently linked to heterogeneous DNA-like nuclear RNA (HnRNA) and to mRNA in the cytoplasm. The binding of adenylate sequences to HnRNA appears to be a prerequisite for the transport of the functional mRNA to the cytoplasm (8).

Despite the rapid progress made in the characterization of an enzyme that can synthesize poly (A) in the cell nucleus (1, 3, 9-12), very little attention has been given to the identification of an enzyme that has been shown to be present in the cytoplasm (13, 14). The cytoplasmic poly (A)-forming enzymes have been obtained from the post-microsomal superna-

tants of chick embryos (13) and rat liver (14). In the course of our studies on the DNA-dependent RNA polymerase of rat liver mitochondria, we have solubilized an active enzyme that can almost exclusively synthesize poly (A) from ATP. This enzyme differs from the post-microsomal enzyme of liver (14) in some respects such as the lack of inhibition of the former enzyme in presence of remaining nucleoside triphosphates and DNA. The anamolous behaviour of certain rat liver mitochondrial RNA polymerase preparations (15, 16) may be due to their contamination with the homopolymer-forming enzyme present in this organelle.

Materials and Methods

Mitochondria from adult rat liver were isolated essentially as described by Pollak and Munn (17). The nuclei were completely removed by two centrifugations at 800 x g for 10 min. The mitochondrial pellet, obtained by centrifugation of the supernatant at 8000 x g for 10 min, was washed twice by resuspending in the buffer (0.25 M sucrose - 1 mM EDTA, pH adjusted to 7.4 with 1M tris) and centrifuging at 8000 x g for 10 min. To prepare swollen mitochondria, the washed mitochondrial pellet was resuspended in 0.1 M phosphate buffer (pH 7.4) and the suspension was incubated at 30° for 15 min. and centrifuged at 8000 x g for 10 min. The pellet containing the swollen mitochondria was resuspended in 0.25 M sucrose (0.5 ml/g wt. liver). In some experiments, the swollen mitochondria were suspended in 50 mM tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$, 0.1 mM EDTA and 1 mM dithiothreitol (TMED buffer). In other experiments, the swollen mitochondria were sonicated in TMED buffer at full output in a 20 Kcyc/sec Branson sonifier (Model W 140) for 45 sec, with a 15 sec. break for every 15

Table 1. Poly (A) Polymerase Activity of Mitochondrial Preparations

Enzyme Preparation	Enzyme activity (units/mg protein x 10^{-3})
Swollen mitochondria	0.36
Sonicated mitochondria	0.37
Solubilized preparations	13.1

The preparations of swollen and sonicated mitochondria and the solubilization of the enzyme are described in the text. The standard assay mixture contained in a volume of 0.35 ml: 10 μ moles tris-HCl (pH 8.0), 2 μ moles $MgCl_2$, 1 μ mole $MnCl_2$, 13 μ moles KCl, 3 μ moles phospheuolpyravate (calbiochem), 20 μg pyruvate kinase (Boehringer) 0.1 μ mole unlabelled ATP (9.53 Ci/mM, New England Nuclear Corp) and 0.9-1.2 mg of enzyme preparations. After incubation at 30°C for 30 min., the reaction was stopped by the addition of 0.1 μmole of cold ATP and ice cold 10% TCA (trichloroacetic acid) containing 0.04 M sodium pyrophos phate. The TCA-insoluble precipitate was collected on either Whatman GF/C filters or on millipore filters (1.2 μ pore size), washed three times with cold 5% TCA containing 0.02 M sodium pyrophosphate and finally with cold glass-distilled water. The radioactivity in the filters was determined by counting in 10 ml of a toluene based scintillation solution containing 4 g omnifluor (New England Nuclear) and 0.1 g POPOP/liter of solution. One unit of activity is that amount of enzyme which incorporates one pmole of AMP into TCA-insoluble product per 30 min.

sec sonication.

To solubilize the enzyme, the mitochondria were sonicated for 1 min in TMED buffer (pH 9.0) containing 20 mM KCl. The sonicate was mixed with glycerol (final concentration, 20% v/v), incubated at 37° for 30 min and centrifuged at 105,000 x g for 1 hr. The supernatant, containing the enzyme activity, was dialysed against several volumes of TMED buffer (pH 7.5) containing glycerol (20% v/v) to deplete the nucleotides. In some experiments, the enzyme was further purified by precipitation with ammonium sulfate (40-60% saturation) with significant loss of activity. The enzyme preparation usually contained 4-5 mg of protein/ml.

RESULTS AND DISCUSSION

Enzyme activity of the mitochondrial preparations

The solubilized enzyme preparations incorporated 13 x 10^3 picomoles of AMP/mg protein/30 min (Table 1). The activity of swollen or sonicated mitochondrial preparations was in no way comparable to the activity present in the solubilized or partially purified enzyme. The residue left after the extraction of the enzyme did not show any significant activity.

Product of the enzyme

Based on the following properties (Table 2), it can be concluded that the product is made up of adenylate sequences: (a) Deletion of the remaining three nucleotides does not inhibit incorporation of labelled ATP (b) the acid-insoluble product can be retained on the millipore filters in presence of 0.5 M KCl which does not bind other RNA preparations to the filters (5) (c) Pancreatic RNase does not inhibit poly (A) synthesis (5). The reaction was usually linear up to 30 min. If labeled ATP had been reversibly bound to a protein, cold ATP added at the end of the reaction, would have diluted the label considerably by exchange reaction. The authenticity of the product was further determined by analysis of the radioactivity in AMP, obtained by hydrolysis of the product with 0.3 N KOH. About 90% of the radioactivity was recovered in AMP. The recovery of most radioactivity in AMP clearly shows that AMP is incorporated into the internucleotide linkage.

Substrate Specificity

The substrate specificity of the enzyme was determined by measuring the rates of incorporation of the four labelled nucleotides individually into TCA-insoluble product. The enzyme was specific in catalyzing the incorporation of ^{3}H-ATP. The specific activity of the enzyme as measured by picomoles AMP incorporated/mg protein was 13 x 10^3, whereas the activity

Table 2. Properties of the enzyme catalyzing the incorporation of ^{3}H-ATP

	Enzyme activity (units/mg protein x 10^{-3})
Complete	13.0
+DNase (100 μg/ml)	12.7
+RNase (100 μg/ml)	12.9
+CTP, GTP, UTP	12.7
+Rifampicin (75 μg/ml)	11.8
+Ethidium bromide (50/μg/ml)	13.1
+DNA (50 μg/ml)	13.2
+Poly AU (10 μg/ml)	14.0
+0.5 M KCl, added prior to filtration	13.1
-PEP, Pyruvate kinase	10.9

Incubation conditions were as described in Table 1. The enzyme was pre-incubated for 30 min at 0°C with inhibitors (DNase, RNase, Rifimpicin and Ethidium bromide), prior to the addition of the co-factors and ^{3}H-ATP. In studies on the effect of addition of unlabelled nucleotides, 0.2 μmole each of the nucleotide was added. In some experiments, the product was collected in presence of 0.5 M KCl to test the synthesis of poly A.

expressed as picomoles UMP or CMP incorporated/mg protein was about 0.5×10^{3}. The ability of the enzyme to make poly (G) was negligible. The specific activity of the enzyme as measured by picomoles UMP incorporated/mg protein was remarkably identical to that reported for the partially purified rat liver mitochondrial RNA polymerase (15). The varying degrees of response of some liver mitochondrial RNA polymerase preparations (as measured with labeled UTP) to actinomycin D (15, 16), RNase (16) and rifampicin (15) may be the result of contamination of the polymerase with poly U-synthesizing enzyme activity.

Table 3. Effect of addition of Solubilized enzyme to the residual fraction

	Enzyme activity (units/mg protein x 10^{-3}
Solubilized enzyme	14.5
Residual fraction	0.32
Solubilized enzyme + Residual fraction	0.50
Solubilized enzyme, incubated for 30 min, followed by incubation with residual fraction for additional 30 min.	0.56
Solubilized enzyme, incubated for 30 min., followed by further incubation for 30 min. with heated residual fraction.	13.9

The enzyme was solubilized as described in the text. The residual fraction, containing 1-1.5 mg protein, obtained after removal of the supernatant, was incubated either alone or in presence of the supernatant containing 800-900 µg protein. These experiments were carried out in presence of the remaining three nucleotides. In addition, ATP concentration was increased two-fold. The enzyme was assayed as described in Table 1. The 'heated' residual fraction was prepared by heating the residue for 3 min at 90° in TMED buffer.

The nature of the inhibitor

The lack of any significant activity in swollen or sonicated mitochondrial preparations could be due to the following reasons: (a) the enzyme combines with a factor or factors present in whole mitochondria to form a relatively inactive molecule. The modification in the enzyme structure may be due to a reversible transformation of the poly (A) polymerase to RNA polymerase which appears to be relatively inactive enzyme (15, 18) as compared to the nuclear RNA polymerases (19, 20) (b) the product formation is inhibited by a degradative enzyme present in the mitochondrial residue. The former possibility is attractive on the basis of the finding that poly (A) polymerase of E. Coli differs from RNA polymerase only in two of the five subunits (21). Preliminary experiments, however, suggested to

us that the inhibition was primarily due to the degradation of poly (A) by a nuclease, present in the residual fraction. To test this possibility, the enzyme was initially incubated with the substrate and co-factors and the product was further incubated in the presence of heated or unheated residual fraction (Table 3). The radioactivitv in the product was lost almost completely by a 30 min incubation with the unheated residue, which suggests that poly (A) is rendered acid-soluble by a nuclease associated with this fraction. Since the poly (A)-forming enzyme can be solubilized by mild treatment of the mitochondria, it is possible that the nuclease is tightly associated with the membrane fraction, whereas the poly (A) polymerase is present in the mitochondrial matrix. McMurray and Dawson (20) have shown that very vigorous sonication in phosphate buffer is required for the release of membrane-bound enzymes.

Template and primer requirements

The insensitivity of the enzyme to DNase, actinomycin D and ethidium bromide indicates that unlike DNA-dependent RNA polymerase, this enzyme does not require DNA as a template. The small stimulation of the activity in presence of poly AU (Schwarz Bioresearch) may be significant (Table 2) if the enzyme had already been associated with saturating quantities of the primer. Addition of more poly AU did not produce further stimulation of the activity. Further purification of the enzyme, without loss of enzymatic activity, is needed to establish the absolute requirement of a primer for this enzyme. The insensitivity of the reaction to pancreatic RNase suggests that bulk of the mitochondrial RNA does not act as a primer.

Conclusions

The specific activity of mitochondrial poly (A) polymerase is comparable to the highest values reported for the purified enzymes obtained from E. Coli (21) and mammalian nuclei (3). In view of the large amounts of ATP in the mitochondria, it is not surprising to detect an enzyme that can make poly (A) in this organelle. It is possible that poly (A) is a storage form of mitochondrial ATP and that the nuclease ultimately controls the amount of ATP present in the mitochondria. The relationship of mitochondrial poly (A) polymerase to the poly (A) segments detected in nuclear RNA is not very clear. It is conceivable that mitochondrial poly (A) has a unique function, that is different from that of nuclear poly (A).

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