MECHANISM OF POLYADENYLATE POLYMERASE: FORMATION OF ENZYME–SUBSTRATE AND ENZYME–PRIMER COMPLEXES

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1. Introduction

Polyadenylate sequences of 100–200 nucleotides have been found at the 3′ terminus of heterogeneous nuclear and non-histone messenger RNA of eukaryotic cells [1–6]. Enzymes which catalyze the addition of poly(A) to RNA primers have been described in a variety of mammalian systems [7–16]. Poly(A) polymerase has an absolute requirement for a divalent cation, ATP and an oligo- or polyribonucleotide primer [11,12,15,16]. In order to understand the mechanism of the poly(A) polymerase reaction, a nitrocellulose membrane filter binding assay has been developed in our laboratory. Tritium-labeled substrate or primer is retained on a Millipore nitrocellulose membrane filter in the presence of poly(A) polymerase. The enzyme–substrate and enzyme–primer complexes are dissociated with increasing potassium chloride concentrations. This paper describes the assay system and the binding of poly(A) polymerase to its substrate and primers.

2. Materials and methods

2.1. *Reagents*

The tetralithium salts of ^{3}H-labeled nucleoside triphosphates, [^{3}H] poly(A), [^{3}H] poly(U) and [^{3}H]-poly(C) were purchased from Schwarz/Mann Co., Orangeburg, New York. Unlabeled nucleoside triphosphates were obtained from Worthington Biochemical Corp., Freehold, New Jersey or from P. L. Biochemicals, Inc., Milwaukee, Wisconsin. Phosphocreatine (disodium), creatine phosphokinase (rabbit skeletal muscle, EC 2.7.3.2), dithiothreitol (DTT) and tRNA from calf liver (type IV) were purchased from Sigma Chemical Co., St. Louis, Missouri. Crystalline bovine serum albumin was obtained from Miles Laboratories, Elkhart, Indiana. Nitrocellulose membrane filter discs (Cat. No. HAWP02500) were purchased from Millipore Corporation, Bedford, Mass. All other chemicals were of analytical reagent grade.

2.2. *Poly(A) polymerase assay*

The enzyme activity was determined by the incorporation of [^{3}H] AMP into an acid insoluble product. The assay mixture in a total volume of 0.10 ml contained 0.1 M Tris–HCl, pH 8.0, 1 mM $MnCl_2$, 4 mM DTT, 0.25 mM [^{3}H] ATP (10–20 cpm/pmole), 5 mM NaF, 10% (v/v) glycerol, 25 μg phosphocreatine, 2 μg creatine phosphokinase, 200 μg tRNA and enzyme. The assay mixture was incubated at 37°C for 60 min. The enzyme activity was determined as described [15,16].

2.3. *Purification and properties of poly(A) polymerase*

Poly(A) polymerase was purified from cytoplasm of 12–16 day NIH Swiss mouse embryos, by a procedure established in our laboratory [15,16]. The enzyme preparation was purified by DEAE-cellulose, phosphocellulose and tRNA-Sepharose affinity column chromatography. The specific activity of the affinity chromatography-purified enzyme was 80 nmoles AMP incorporation/mg/h. Poly(A) polymerase had an absolute requirement for an oligo- or polyribonucleotide, ATP and a divalent cation [15,16]. With tRNA, the divalent salt concentrations for optimum enzyme activity were 1 mM $MnCl_2$ or 10 mM $MgCl_2$. The enzyme activity was 10–15 fold higher with $MnCl_2$ than with $MgCl_2$. The poly(A) polymerase reaction was linear with tRNA as a primer up to two hours [15,16].

2.4. *Nitrocellulose membrane filter binding assay*

Nitrocellulose filters were soaked prior to use in binding buffer (0.05 M Tris–HCl, pH 8.0, containing 200 μg/ml bovine serum albumin). The assay mixture in a volume of 1.0 ml binding buffer contained the indicated amounts of poly(A) polymerase, radioactive substrate and/or the primer. The assay mixture was kept in ice for 10 min filtered through a nitrocellulose membrane filter in a Millipore filtration unit, and washed under gentle vacuum with 50 ml ice cold binding buffer. The membrane filters were dried, and placed in vials containing 10 ml of toluene-based PPO-POPOP scintillation fluid. The radioactivity was determined in a Packard tri-carb liquid scintillation spectrometer.

3. Results

3.1. *Formation of enzyme-primer and enzyme-substrate complexes, and their dissociation with potassium chloride*

Tritium-labeled poly(A) when filtered alone through a nitrocellulose membrane filter, was not retained. When mixed with poly(A) polymerase, [^{3}H] poly(A) was retained on the filter (fig.1), suggesting that an enzyme-primer complex was formed. This complex was dissociated at increasing KCl concentrations (fig.1), indicating that the enzyme–primer complexes are ionic in nature. On increasing poly(A) polymerase in the in the reaction mixture, a typical saturation curve was obtained (fig.1) which suggests that the enzyme binds to the primer at a specific site. The stoichiometry of this reaction could not be ascertained due to the unknown purity of the enzyme preparation and varying sizes of the commercial poly(A) preparation. When [^{3}H] poly(C) was used instead of poly(A), similar results were obtained as in fig.1 (data not shown). Tritium-labeled poly(U) showed poor binding.

Formation of a complex between poly(A) polymerase and substrate was studied in a similar manner as above. Radioactive nucleoside triphosphates of high specific activities (ATP, 27 μM, 11 150 cpm/pmole; CTP, 19 μM, 9 670 cpm/pmole; GTP, 42 μM, 3 250 cpm/pmole; UTP 21 μM 9 220 cpm/pmole) were used in the binding reaction. Labeled UTP and CTP did not bind to the enzyme in the presence or absence of poly-(A) (data not shown), but [^{3}H] ATP and [^{3}H] GTP were retained on the membrane filter (fig.2). These experiments suggest that enzyme–substrate and enzyme–substrate–primer complexes are formed and these are dissociated with increasing amounts of KCl (fig.2). The amount of purine nucleoside triphosphate bound to the enzyme–primer complex was always higher than to the enzyme alone. Despite the fact that GTP is not a substrate for poly(A) polymerase reaction [15,16], it is of interest that the amount of [^{3}H] GTP

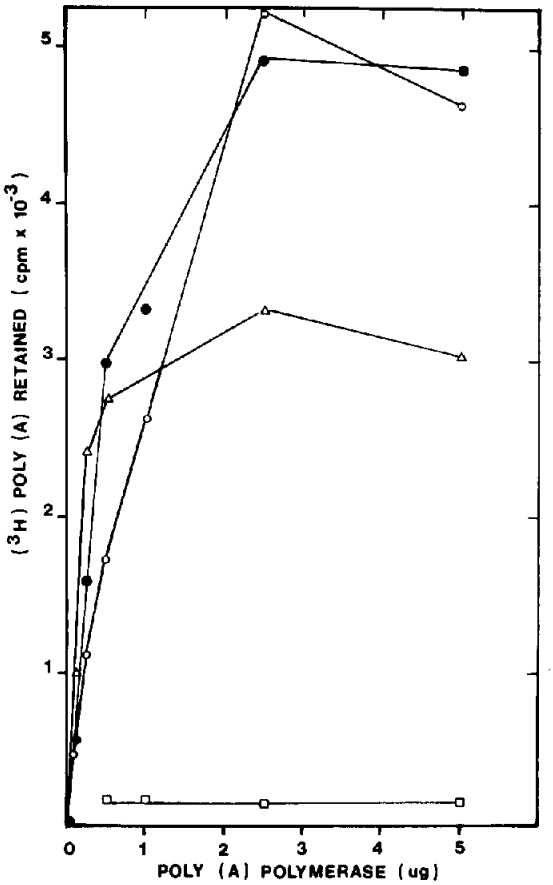

Fig.1. Retention of [^{3}H] poly(A) on nitrocellulose membrane filter in the presence of increasing amounts of poly(A) polymerase at different potassium chloride concentrations. Ten μl [^{3}H] poly(A) were mixed with the indicated amounts of poly(A) polymerase in 1.0 ml of binding buffer. The reaction mixture was washed and assayed as described in Materials and methods. Binding buffer + 0.001 M KCl (○—○), binding buffer + 0.01 M KCl (●—●), binding buffer + 0.1 M KCl (△—△), and binding buffer + 0.5 M KCl (□—□).

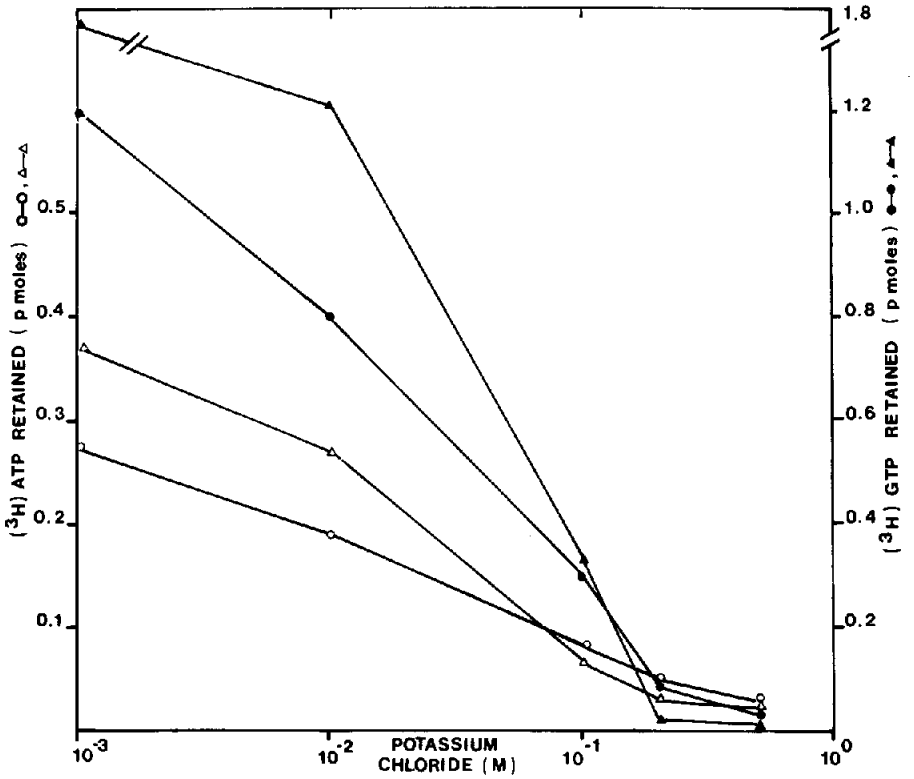

Fig.2. Dissociation of enzyme–substrate and enzyme–substrate–primer complexes at increasing KCl concentration. Ten μl [^{3}H] ATP containing 271 pmol ATP (spec. act. 11 150 cpm/pmole) or 10 μl [^{3}H] GTP containing 416 pmol of GTP (spec. act. 3 250 cpm/pmole) were mixed with 1 μg poly(A) polymerase in the presence or absence of 50 μg poly(A) in 1.0 ml binding buffer. The complexes thus formed were treated with different concentrations of KCl in binding buffer, and washed with the same salt–buffer solution. The amount of radioactive ATP or GTP retained on the filter was determined. [^{3}H] ATP + enzyme (○—○), [^{3}H] ATP + enzyme + poly(A) (△—△), [^{3}H] GTP + enzyme (•—•), [^{3}H] GTP + enzyme + poly(A) (▲—▲).

bound was about four times higher than [^{3}H] ATP. The dissociation of enzyme–substrate or enzyme–substrate–primer complexes with KCl further indicates that the chemical bonds involved in complex formation are ionic in nature.

3.2. *Inhibition of enzyme activity by potassium chloride.*

Poly(A) polymerase reaction is inhibited as increasing KCl concentration (fig.3). The amount of KCl required for 50% enzyme inhibition (ID_{50}) was 100 mM. This inhibition can be explained by the dissociation of enzyme–substrate and enzyme–primer complexes by KCl (figs.1,2).

4. Discussion

Analogous to the RNA polymerase reaction mechanism [17,18], different steps involved in the poly(A) polymerase reaction can be written as follows:

(1) E + ATP $\rightleftharpoons$ E–ATP (substrate binding)

(2) E + P $\rightleftharpoons$ E–P (primer binding)

(3) E + ATP + P $\rightleftharpoons$ $E<^{ATP}_{P}$ (primer–substrate binding)

(4) $E<^{ATP}_{P}$ $\longrightarrow$ E–P–AMP + PPi (initiation)

(5) E–P–AMP + nATP $\longrightarrow$ E–P–$(A)_{n+1}$ + nPPi (elongation)

(6) E–P–$(A)_{n+1}$ $\longrightarrow$ E + P–$(A)_{n+1}$ (termination).

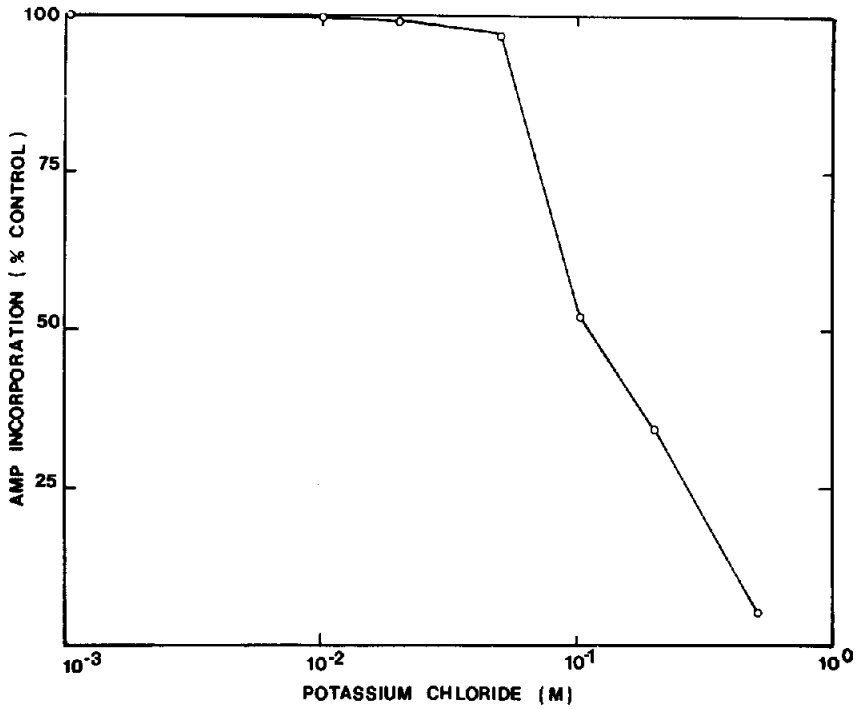

Fig.3. Inhibition of poly(A) polymerase activity with increasing concentrations of potassium chloride. Poly(A) polymerase activity was determined as described in Materials and methods. Two μg of tRNA-Sepharose purified enzyme were used.

Results presented here demonstrate that steps 1–3 of the above scheme indeed occur. For example, poly (A) polymerase binds to both ATP and GTP, but not UTP and CTP. Hence the enzyme has specificity of binding to purine nucleotides. This difference in binding explains the fact that poly(A) polymerase cannot synthesize poly(U) or poly(C). The enzyme does not utilize GTP in the polymerization reaction although GTP binds to the enzyme. Therefore, it can be speculated that the block in poly(G) synthesis is at a step subsequent to binding of the substrate. Poly(A) polymerase binds to the primer and shows a typical saturation curve suggesting the enzyme binding to the primer at a specific site. Poly(C) also binds to the enzyme, but is a weak primer [16]. Poly(U) is a good primer [16] but binds poorly to the enzyme. The reasons for these observations cannot be explained by our results. More substrate binds to the enzyme–primer complex than to the enzyme alone. The reason for this phenomenon is also not clear. Enzyme–primer, enzyme–substrate or enzyme–substrate–primer complexes can be dissociated at high potassium chloride concentrations. These data explain the inhibitory effect of KCl on poly(A) polymerase reaction.

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