Polycytidylate Polymerase From Ehrlich Ascites Cells[1]

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Summary: The synthesis of a homopolymer of cytidylate is specifically catalyzed by an enzyme isolated from Ehrlich ascites cells. The enzyme requires the presence of CTP, Mg^{2+} and ATP, although ATP is not incorporated into product. The enzyme, as isolated, contains a polynucleotide primer.

A DNA-dependent ATP polymerase from Escherichia coli (1) and enzymes isolated from calf thymus nuclei which are specific for the synthesis of polyadenylate (2) and polycytidylate (3) have been studied. The isolation of homopolymers of adenylate from the nuclei of Ehrlich ascites cells has been reported (4). This report describes the properties of an enzyme from Ehrlich ascites cells which catalyzes the formation of polycytidylate from CTP.

Cytidylate polymerase was purified from Ehrlich ascites cells. The Ehrlich ascites cells were taken from the mice 7 days after transplantation. The cells were removed from the fluid by centrifugation, and the packed cells washed with 0.0153 M NaCl (containing 1×10^{-3} M dithioerythritol) two or three times and centrifuged at low speed. This treatment removed the red blood cells. The packed cells were suspended in 2 volumes of 1×10^{-3} M dithioerythritol and homogenized for 1.5 min at full speed in a Sorvall Omni-Mixer. The homogenate was centrifuged at 27,000 x g for 1 hour at 2-5°. The supernatant fluid was used as the crude extract, and could be quick-frozen in

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an acetone-dry ice bath and stored at -18°. The frozen crude extract was thawed, centrifuged and dialyzed against 0.02 M Tris-HCl, pH 7.0 for 1 hour. The pH of the dialyzed fraction was adjusted to pH 5.2 with 1 M acetic acid and quickly centrifuged. The precipitate was dissolved in 0.05 M Tris-HCl, pH 8.0 (one-fourth the original volume of the crude extract) and the pH adjusted to 7.0-7.5 if necessary. The pH 5.2-insoluble fraction, after dissolving in buffer, could also be stored at -18° after quick-freezing. The pH 5.2-insoluble fraction was then made up to 45% saturation (5) with ammonium sulfate, stirred and centrifuged. The precipitate was discarded. The supernatant fluid was brought to 60% saturation with ammonium sulfate, stirred and centrifuged. The precipitate was dissolved in a volume of 0.02 M Tris-HCl pH 7.0 (containing 10^{-3} M dithioerythritol) equal to the volume of the pH 5.2-insoluble fraction used. This ammonium sulfate fraction was dialyzed against 0.02 M Tris-HCl, pH 7.0 containing 1×10^{-3} M dithioerythritol and was quick-frozen for storage at -18°.

The primer was isolated from the 45-60% ammonium sulfate fraction by heat treatment of the enzyme fraction, followed by ethanol precipitation of the primer material (2). The ratio of the absorbance at 260 mμ to the absorbance at 280 mμ was 1.5 for the two preparations of primer used in these experiments. This corresponds to approximately 48 μg of nucleic acid/ml (6). Where more concentrated solutions of primer were needed, aliquots were lyophilized to dryness and taken up in smaller volumes of water. The lyophilized primer material was still biologically active.

The enzyme activity was routinely assayed by the filter paper technique of Bollum (7). The incubation mixture containing ATP (2.5×10^{-4}), magnesium acetate (1.4×10^{-2} M), Tris-HCl buffer (pH 8.3, 5×10^{-2} M) and the 45-60% ammonium sulfate fraction (150-300 μg) was preincubated at 37° for 3 min. The reaction was initiated by the addition of the substrate, ^{14}C-CTP (0.05 μC). The total reaction volume was 0.20 ml.

For the determination of chain lengths of the polymer the standard

reaction mixture was incubated for 1 hour and the entire volume streaked on Whatman 3 MM paper. The chromatogram was developed in a solvent of 95% ethanol - H_2O (70:30, by vol.). The chromatogram was dried, and redeveloped in a solvent of 1 M ammonium acetate-95% ethanol (70:30, by vol.) (8). After drying the chromatogram the origin was cut out, added to a tube containing 1.0 N KOH, cytidine (0.36 μmoles) and 2',(3')-CMP (0.33 μmoles), and incubated for 24 hours at 37°. The KOH hydrolysate was neutralized with $HClO_4$, the $KClO_4$ removed by centrifugation, and the supernatant fluid lyophilized. The lyophilized sample was mixed with a small volume of H_2O and spotted on Whatman 3 MM. This chromatogram was developed in a solvent containing 1 M ammonium acetate-95% ethanol (30:75 by vol.) The cytidine and 2', (3')-CMP areas were detected under UV light, cut out, and eluted with 0.01 N HCl and aliquots taken for ^{14}C-counting and for absorbance measurements at 280 mμ.

The ammonium sulfate fraction, for maximal incorporation of a cytidylic acid into the acid-insoluble polymer, required CTP, ATP, and magnesium ions. At this stage of purity CTP served as a better substrate than CDP or CMP. The optimal pH was approximately pH 8.3 in Tris-HCl buffer.

Even though ATP stimulated the polymerization of CTP by the Ehrlich ascites cell enzyme, ^{14}C-ATP was not incorporated into the acid-insoluble product by this enzyme fraction either in the presence or absence of CTP. ^{3}H-GTP or ^{14}C-UTP were, likewise, not incorporated into the acid-insoluble product.

The requirement for magnesium ions could be partially met by manganese ions. The optimum concentration for Mg^{2+} was 1.4×10^{-2} M while the optimum concentration for Mn^{2+} was much lower (3.2×10^{-4} M). However, at the optimum concentrations of both metal ions, Mn^{2+} gave only 80% of the activity seen with Mg^{2+}. Concentrations of Mn^{2+} greater than 3.2×10^{-4} M caused a sharp loss in enzyme activity.

Addition of isolated primer (at concentrations which were saturating)

Table I

Effect of Primer on CTP Polymerization

The enzyme fraction was incubated at 25° with or without the addition of T_1 RNase or pancreatic RNase for 1 hour. Aliquots were then added to the reaction mixture containing ^{14}C-CTP, Mg^{2+}, ATP and buffer. Primer was added where indicated at t=0.

Preincubation Addition	Addition at t = 0	μμmoles CMP Incorporated/15 min.
none	none	25
none	primer	33
none	T_1 RNase	19
T_1 RNase	none	1
T_1 RNase	primer	16
none	none	26
none	pancreatic RNase	21
pancreatic RNase	none	1
pancreatic RNase	primer	11
none	yeast RNA (10 μg)	28
T_1 RNase	yeast RNA (10 μg)	2
none	poly C (5 μg)	28
T_1 RNase	poly C (5 μg)	1

to the native enzyme stimulated poly C formation 2.7-fold. Pre-incubation of the enzyme fraction with low levels of pancreatic RNase or T_1 RNase resulted in the complete loss of enzymatic activity. However, activity could be partially restored by the addition of heat-treated enzyme fraction or the isolated primer. The addition of primer at t=0, to the T_1 RNase-treated samples resulted in a marked stimulation of poly C formation (Table I). Attempts to inactivate the added RNase with bentonite also resulted in the loss of polymerase activity. The primer requirement could not be met by either poly C or yeast RNA. The inactivation of the enzyme by treatment with either T_1 RNase or pancreatic RNase indicated that the primer was a heteropolymer.

The average chain length of the polymer formed seemed to depend on the pH and the buffer used. The results of these experiments are shown in Table

Table II

Average Chain Lengths of Polycytidylate

The reaction mixtures were the same as indicated in Table I, except that the buffer, and pH were changed as indicated.

buffer	pH	average chain length[a]
Tris-HCl	6.6	9, 13, 9, 10
	8.3	52, 55, 63, 45
Phosphate	6.6	18, 31, 28
	8.3	99, 85, 79

[a]Each value represents the average chain length of the poly C obtained in different experiments.

II. It can be seen that the average chain lengths of the polymers were larger in phosphate buffer than in Tris-HCl buffer, and also larger at pH 8.3 than at pH 6.6. While a detailed study of the effect of magnesium ions on the polymer length was not undertaken, it was observed that at concentrations of magnesium ions lower than the optimum, the average chain length of the polymer was decreased.

Actinomycin D at a concentration as high as 116 μg/ml had absolutely no inhibitory effect on the CTP polymerization reaction.

The role of these enzymes from thymus nuclei (3) and Ehrlich ascites cells which catalyze the synthesis of homopolymers of cytidylate and homopolymers of adenylate (2) and the function of polyadenylate in Ehrlich ascites cell nuclei (4) is not known. It has been suggested by Edmonds and Abrams that these homopolymers could function as storage forms for nucleoside triphosphates or perhaps as part of a repressive control mechanism in protein synthesis (9). If the function of these homopolymers were as storage forms, the polymers, in effect, would lower the intracellular concentration of the nucleotide pools which in turn could eliminate many of the allosteric effects

of the nucleotide pools. This would serve an important physiological role.

However, evidence is available which suggests that nucleotide homopolymers can have a pronounced effect on certain proteins. Firshein et al, (10) have shown that polycytidylate markedly stimulates the enzymatic activity (presumably through enzyme synthesis) of deoxycytidylate and deoxguanylate kinases when added to resting cultures of pneumococci. In addition, Braun and Nakano (11) have reported that polyadenylate-polyuridylate and polycytidylate-methylated albumin complexes can enhance antibody formation in mouse spleen cells. It is possible, therefore, that these nucleotide homopolymers play an important role in control mechanisms in protein synthesis. In the cases of the pneumococci and the spleen cells, the effect of the homopolymers would be an inductive or derepressive effect rather than a repressive effect as suggested by Edmonds and Abrams (9). It could be that this polymer of cytidylate is related to interferon synthesis, since it has been repeatedly demonstrated that synthetic poly I-C stimulates interferon synthesis (12).

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