PURIFICATION AND PROPERTIES OF CALF THYMUS POLYADENOSINE DIPHOSPHATE RIBOSE POLYMERASE

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1. Introduction

Previous work in our laboratory showed the existence in animal cell nuclei of an enzymatic system which catalyzed the incorporation of the adenosine diphosphate ribose (ADPR) moiety of NAD into an acid-insoluble product, polyadenosine diphosphate ribose (poly ADPR) [1]. We have also demonstrated the mechanism of this reaction, and the structure of the acid-insoluble product to be a polymer of adenosine diphosphate ribose (ADPR) [2,3]. Existence of poly ADPR polymerase and the structure of that polymer were confirmed in other laboratories [4,5]. The function of poly ADPR polymerase is, as yet, unknown, although its involvement in the control of DNA replication [6–8], of cell differentiation [9,10] has been suggested. Evidences suggesting the existence of poly ADPR in vivo were also reported [11]. In spite of many findings of the presence of poly ADPR polymerase, in a variety of tissues and cells, few study of enzyme purification has been published until now. In rat liver nuclei the enzyme was only purified 10-fold [12]. We report here the 540-fold purification of calf thymus poly ADPR polymerase activity and some properties of this enzyme.

Abbreviations used: NAD: nicotinamide adenine dinucleotide, DTT: Dithiothreitol, ADPR: Adenosine diphosphate ribose, poly ADPR: Polymer of adenosine diphosphate ribose.

2. Materials and methods

[^{32}P]NAD with high specific activity was prepared by the method of Colowick and Kaplan [13] modified by Gill [14]. [^{3}H]NAD labelled in the adenine moiety was obtained from NEN (New England Nuclear). Covalently linked nicotinamide-Sepharose was prepared for affinity chromatography in our laboratory. AMP- and NAD-Sepharose were also prepared [15,16]. Sephadex and Sepharose were the products of Pharmacia, NAD, ATP, bovine serum albumin and nicotinamide were obtained from Sigma.

2.1. *Preparation of partially purified poly ADPR polymerase*

Calf thymus was removed immediately after killing and stored in the liquid nitrogen until used. Approx. 20 g of calf thymus was thawed and minced in 0.05 M Tris–HCl buffer, pH 7.9, containing 10 mM 2-mercaptoethanol, 1 mM DTT, 0.1 mM EDTA, and 5% glycerol (w/v). All preparation steps were conducted in the cold. The tissue was disrupted in a mixer for 2 min and suspension was centrifuged for 15 min at 100 000 *g* to sediment crude chromatin fraction. The precipitate was resuspended in the same buffer and saturated $(NH_4)_2SO_4$ was added to give a final concentration of 20% saturation, then subjected to ultrasonic disintegration to decrease the viscosity of suspension. The material precipitated between 60 and 80% saturation of $(NH_4)_2SO_4$ was collected, dissolved in 0.05 M Tris–HCl buffer, pH 7.9, containing 10 mM 2-mercaptoethanol, 1 mM DTT, 0.1 mM EDTA and 25% glycerol (B 25 buffer). B 25

buffer was used throughout purification and also for dialyzing buffer. The solubilized enzyme was purified by successive column chromatography on DEAE-Sephadex A-50, phosphocellulose, and finally by affinity chromatography with nicotinamide-Sepharose. At this step, enzyme was purified to 540-fold and the recovery of enzymic activity was 3%. Partially purified enzyme was stored in the liquid nitrogen.

2.2. *Enzyme assay*

Poly ADPR polymerase activity was measured by the incorporation of radioactivity in acid-insoluble material from labelled NAD. Incubation mixture contained 0.1 M Tris buffer, pH 7.9, 8 mM $MgCl_2$, 0.4 mM DTT, 30 μl inactivated calf thymus chromatin solution [17], 10 nmol of [^{3}H]- or [^{32}P]NAD and enzyme solution in the final vol of 125 μl. After incubation at 37°C for 3 min, the reaction was stopped by the addition of 2 ml of 10% trichloroacetic acid containing 0.02 M sodium pyrophosphate and 0.1 ml of 1% bovine serum albumin. The precipitate was collected and washed by filtration and was counted by liquid scintillation. 1 unit of enzyme activity was defined as 1 nmol ADPR incorporated in acid-insoluble fraction in 3 min at 37°C.

3. Results and discussion

3.1. *Enzyme purification*

It is well known that poly ADPR polymerase is an enzyme bound tightly with chromatin structure of cell nuclei. Among solubilization methods used, only sonic disintegration was successful. Hydrolysis with DNase, or extraction with NaCl or phosphate buffer was unsuccessful because of poor recovery or considerable inactivation of enzymic activity during purification. Table 1 summarizes the procedure used for partial purification of poly ADPR polymerase from the calf thymus. As nicotinamide is a potent inhibitor of poly ADPR synthesis, we have prepared and tested a covalently linked nicotinamide-Sepharose. As expected relatively strong affinity of poly ADPR polymerase activity was observed. On the other hand, NAD- and AMP-Sepharose showed no affinity with enzyme activity. As shown in table 1, poly ADPR polymerase activity was purified about 540-fold with a yield of 3%. At this stage of purification, polyacrylamide gel electrophoresis of enzyme at pH 4.5 showed one predominant coomasie blue-stained band. The partially purified enzyme was stable for several months at −180°C in B 25 buffer. The residual activity of partially purified enzyme stored at 0°C for 1, 4 and 7 days was 95, 70 and 60% respectively.

Table 1
Purification of calf thymus poly ADPR polymerase activity

Step	Total protein (mg)	Total activity (units)	Specific activity (units per mg protein)	Over-all yield (per cent)
Homogenate	2497	1254	0.50	100
Crude chromatin	1716	1754	1.02	140
Ammonium sulfate	486	436	0.89	35
DEAE-Sephadex A50	33.7	256	10.74	20
Phosphocellulose	0.49	100	203	8
Nicotinamide-Seph.	0.16	43.3	269	3.4

Enzyme was obtained from 20 g calf thymus. Activities were measured and unit s defined as described in Materials and methods. Proteins were determined by the method of Lowry et al. [21] after precipitation with 20% trichloroacetic acid.

3.2. *Properties of the partially purified poly ADPR polymerase*

Requirements of the poly ADPR synthesis reaction: partially purified poly ADPR polymerase required chromatin, DTT and $MgCl_2$ for its action. Without chromatin there was no incorporation of ADPR into acid-insoluble material. The amount of incorporation of ADPR without DTT and without $MgCl_2$ were about 5 and 46% respectively, of that of the complete system.

Effects of divalent cations: fig.1 shows the effect of divalent cations on the poly ADPR synthesis. A marked increase of enzymic activity was obtained with $MnCl_2$ at the concentration of 4 mM while a less dramatic stimulation was observed in the presence of $MgCl_2$ and $CaCl_2$. An inhibition of 30% was obtained in the presence of KCl at the concentration of 0.05 M. It is interesting to note that animal RNA polymerase is also activated with 2–4 mM $MnCl_2$ and slightly stimulated with higher concentration of $MgCl_2$.

pH optimum: measured between pH 7.6 and 9.2 of the Tris–HCl buffer, the partially purified poly

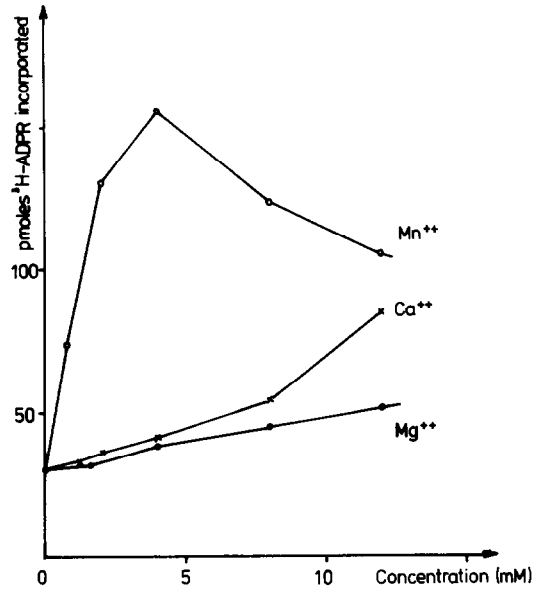

Fig.1. The influence of metal ion concentration on the rate of polymerase activity. The standard chromatin-primed assay was used, except that the metal ion concentration was varied, as shown.

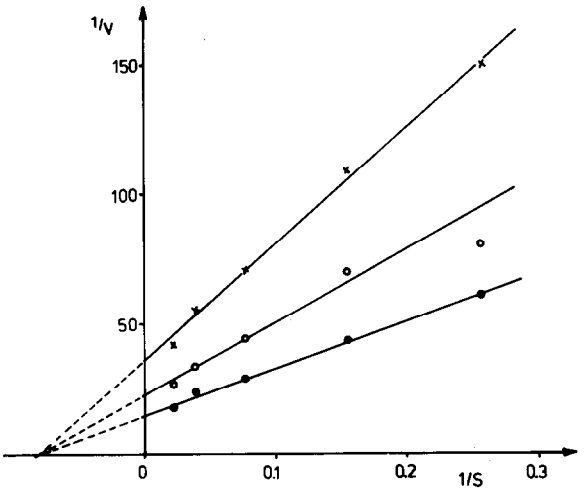

Fig.2. Lineweaver–Burk plot for calf thymus poly ADPR polymerase. The standard assay was used with varying amounts of [^{3}H]NAD: (•——•) NAD alone, K_M = 0.10 mM; (○——○) + 0.25 mM nicotinamide; (X——X) + 0.25 mM thymidine.

ADPR polymerase showed the maximum activity at pH 8.8, although the enzymic activity did not show remarkable changes under the conditions described for enzyme assay. At most a half of activity was obtained with phosphate buffer at 50 mM probably because of the inhibitory effect of the ionic strength and approximately the same enzymic activity was obtained between pH 7.4 and 8.1.

Optimal temperature: the highest enzyme activity was obtained between 21.5 and 30°C. The enzymic activities at 40 and 50°C were 59 and 20%, respectively, of that at 25°C.

Kinetic data: the enzyme was incubated with different concentrations of NAD. An apparent K_M of 100 μM was obtained from double reciprocal plot [18] of substrate concentration and enzymatic activity. A typical non competitive inhibition curve was obtained in the presence of nicotinamide and thymidine at the concentration of 0.25 mM. It is interesting to note that competitive inhibition was obtained with mouse fibroblast nuclear [19] and Ehrlich ascites nuclear [20] poly ADPR polymerase.

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