Purification and Properties of Poly(ADP-ribose) Polymerase from Lamb Thymus†

Shirley J. Petzold, Barbara A. Booth, George A. Leimbach, and Nathan A. Berger*

ABSTRACT: Poly(ADP-ribose) polymerase was purified 2900-fold from lamb thymus with a recovery of 5%. Addition of exogenous DNA was essential for activity of the purified enzyme, and the reaction was stimulated by the addition of either a mixture of histones or purified histone H1. The enzyme is inhibited by sulfhydryl binding agents such as phenylmethanesulfonyl fluoride or *N*-ethylmaleimide. It does not require magnesium or other metal ion cofactors for activity. The enzyme migrated as a single polypeptide chain with an apparent molecular weight of 135 000 when gel electrophoresis was performed in the presence of sodium dodecyl sulfate. The apparent molecular weight was 175 000 when determined by gel filtration on Sepharose CL-6B-200. The isoelectric point was pH 9.6, and the pH optimum for activity was 8.6–8.8. The apparent K_m for NAD^+ was 160 μM at 37 °C. The activity of the purified polymerase was unaffected by the presence of ADP-ribose, 3′,5′-cAMP, or NaF. Nicotinamide, 5-methylnicotinamide, theophylline, and thymidine markedly inhibited enzyme activity. Lamb thymus DNA, originally associated with the enzyme, was more effective than commercially obtained calf thymus DNA as an enzyme activator.

Poly(ADP-ribose) polymerase is a chromosomal enzyme which cleaves the N-glycosylic bond between the nicotinamide and adenosine(5′)diphosphoribose moieties of NAD^+ and then joins the ADP-ribose[1] moieties by O-glycosidic linkages to form poly(ADP-ribose) (Hayaishi & Ueda, 1977; Hilz & Stone, 1976; Purnell et al., 1980). The same enzyme is capable of attaching the first residue of the polymer to a macromolecular acceptor such as a chromosomal protein (Kawaichi et al., 1980). The activity of poly(ADP-ribose) polymerase increases when cellular DNA is damaged, and the enzyme may be involved in some aspect of DNA repair as well as other cellular processes which involve DNA strand breaks and/or alterations of chromatin structure (Durkacz et al., 1980; Juarez-Salinas et al., 1980; Sudhakar et al., 1979; Berger et al., 1979). Poly(ADP-ribose) polymerase has been purified to varying degrees from several sources including calf and pig thymus, rat liver, and Ehrlich ascites cells (Yoshihara et al., 1978; Ito et al., 1979; Niedergang et al., 1979; Tsopanakis et al., 1978; Okayama et al., 1977; Kristensen & Holtlund, 1978; Holtlund et al., 1980). The enzymes derived from these different tissues show some common properties and some which are markedly different. For example, the rat liver and calf thymus enzymes appear to be single peptide chains with molecular weights of about 130 000 whereas the pig thymus enzyme has an apparent molecular weight of 63 500 (Tsopanakis et al., 1978). Another difference which has recently become apparent is in the enzyme's requirement for magnesium; while most investigators add magnesium to the enzymatic reaction, Benjamin & Gill (1980) have recently reported that a partially purified preparation of calf thymus enzyme did not require magnesium for full activity. Almost all investigators have found that exogenously added DNA is essential for enzyme activity and that the addition of histone further stimulates enzyme activity. In order to study some of the factors regulating the activity of this enzyme, we have used methods similar to those described by Yoshihara et al. (1978) to purify the enzyme from lamb thymus to apparent homogeneity as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis. In this report, we present the results of the purification of lamb thymus poly(ADP-ribose) polymerase and a comparison of its characteristics to those of enzymes from other tissues.

Materials and Methods

Materials. [^{14}C]NAD^+ ([U-^{14}C]adenosine) (563 mCi/mmol) was purchased from New England Nuclear, Boston, MA. NAD^+, calf thymus DNA, DNA–cellulose, glutathione, dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), *p*-(hydroxymercuri)benzoate (PHMB), ADP-ribose, thymidine, nicotinamide, bovine serum albumin (BSA), catalase, and histone HIIA were purchased from Sigma. Histone H1 was obtained from Boehringer Mannheim. Poly(ethylene glycol) (PEG), molecular weight 6000, was obtained from Research Products Inc., Elk Grove, IL. Hydroxylapatite (HTP grade) and molecular weight standards for electrophoresis were purchased from Bio-Rad, Richmond, CA. Sepharose CL-6B-200 and aldolase were obtained from Pharmacia, Piscataway, NJ. Theophylline and 3′,5′-cAMP were purchased from Calbiochem. 5-Methylnicotinamide was obtained from Lilly, Indianapolis, IN. Whatman GF/C filters were obtained from Fisher, St. Louis, MO, and Aquasol from New England Nuclear.

Buffers. The enzyme extraction buffer consisted of 50 mM Tris-HCl, pH 7.4, 0.3 M NaCl, 10 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 50 mM $NaHSO_3$, 0.5 mM DTT, and 0.3 mM PMSF (dissolved first in 95% ethanol, final ethanol concentration 0.3%). Buffer B1 was the same as the extraction buffer except the NaCl concentration was 0.2 M and the

† From The Hematology/Oncology Division, The Department of Medicine, Washington University School of Medicine, The Jewish Hospital of St. Louis, St. Louis, Missouri 63110. *Received May 12, 1981.* This work was supported by National Institutes of Health Grants GM26463 and CA23986 and American Cancer Society Grant CH-134. N.A.B. is a Leukemia Society of America Scholar.

[1] Abbreviations used: ADP-ribose, adenosine(5′)diphosphoribose; 3′,5′-cAMP, adenosine cyclic 3′,5′-phosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HTP, hydroxylapatite; NAD, nicotinamide adenine dinucleotide; NEM, *N*-ethylmaleimide; PEG, poly(ethylene glycol); PHMB, *p*-(hydroxymercuri)benzoate; PMSF, phenylmethanesulfonyl fluoride; Me_2POPOP, 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene; PPO, 2,5-diphenyloxazole; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; HPLC, high-performance liquid chromatography.

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EDTA concentration was 1 mM. Buffer B2 was identical with B1 and also contained 10% glycerol. Buffer A1 contained 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 10% glycerol, 0.5 mM DTT, and 0.3 mM PMSF. Buffer A2 was the same as A1 and also contained 2 M KCl.

Poly(ADP-ribose) Polymerase Assay. The reaction components for the standard poly(ADP-ribose) polymerase assay were combined in 12 × 75 mm glass tubes in an ice-water bath. The final concentration of components in the standard reaction mix was 10 μg/100 μL calf thymus DNA, 10 μg/100 μL histone H1, 0.5 mM [^{14}C]NAD^+ (specific activity 3.8 dpm/pmol), 10 mM $MgCl_2$, 1.0 mM DTT, and 100 mM Tris-HCl, pH 8.0, and 30 μL of enzyme solution was added to give a final volume of 100 μL. Reactions were started by placing the tubes in a 37 °C water bath where they were incubated with gentle shaking for 5 min; for the kinetics experiments and the early points in the time courses, tubes were preincubated at 37 °C for 30 s before the enzyme was added. the reactions were stopped by the addition of 4 mL of cold 20% trichloroacetic acid (Cl_3CCOOH). After standing in cold Cl_3COOH for at least 15 min, the samples were filtered through Whatman GF/C filters, and the tubes and filters were rinsed 5 times with 20% Cl_3CCOOH and once with cold 95% ethanol. The filters were washed an additional 3 times with ethanol, dried, and then counted in 8 mL of toluene–PPO–Me_2POPOP scintillation fluid in a Searle Mark III scintillation counter.

Product Analysis. For determination of chain length of poly(ADP-ribose) synthesized in this reaction, the product was precipitated with 20% cold trichloroacetic acid and extracted twice with diethyl ether. The precipitate was solubilized in 6 M guanidine hydrochloride in 0.1 M KH_2PO_4, pH 8.6. The solubilized reaction product was absorbed to a 1-mL dihydroxyboryl-Sepharose column and digested with 270 μg of snake venom phosphodiesterase, and the nucleotides were eluted with 10 mM sodium citrate, pH 4.5, as described by Sims et al. (1980). The eluate was adjusted to 20% trichloroacetic acid and centrifuged to remove residual protein, and the supernatant was extracted 5 times with diethyl ether. The supernatant was analyzed by HPLC using a Varian Model 5000 liquid chromatography system and a micropak AX-10 anion-exchange column, 300 × 4 mm, at a flow rate of 1.5 mL/min at 25 °C. The material was eluted isocratically with 7 mM KH_2PO_4, pH 4.0, in 7 mM KCl for 10 min followed by a 40-min linear gradient between the original buffer and the second buffer which contained 250 mM KH_2PO_4, pH 5, with 500 mM KCl, followed by an isocratic elution at the high salt concentration for 16 min. Three-milliliter fractions were collected throughout the elution, and aliquots were diluted into 10 mL of Aquasol for scintillation counting. All radioactivity was found at the elution times of 5′-AMP or phosphoribosyl-AMP.

Other Assays. Protein assays were performed on Cl_3CCOOH precipitates according to the method of Lowry et al. (1951). Liquid phase isoelectric focusing was performed for 15 h in an LKB ampholine column 8101 (Vesterberg, 1975) with 0.5 mM DTT and 1 mM EDTA incorporated into the gradient. Electrophoresis of DNA was performed in 2% agarose gels at pH 7.5 according to the method of Tegetmeyer & Macasset (1972). DNA was assayed by a diphenylamine method as described by Richards (1974).

Enzyme Purification Procedure. Lamb thymus was obtained within 2–3 h of slaughter and brought to the laboratory in crushed ice. The thymus was trimmed free of fat and connective tissue, rinsed twice in cold isotonic saline, and frozen at −70 °C until use. All subsequent procedures were done at 4 °C. A 500-g sample of frozen thymus in 3 volumes of extraction buffer was homogenized for 4 min in a 1-gal Waring blender. The homogenization was interrupted at 1-min intervals to allow the suspension to cool. The homogenate was centrifuged at 9000 rpm (13000*g*) for 15 min in a Sorvall GSA rotor. All subsequent centrifugations were the same unless otherwise indicated.

The supernatant (crude extract 1) was removed, and the pellet was rehomogenized in 1 volume of extraction buffer for 1 min and then recentrifuged. The second supernatant (crude extract 2) was combined with crude extract 1, and solid ammonium sulfate was added to the suspension to give 40% saturation. After being stirred 1 h, the suspension was centrifuged, and the 40% saturated supernatant was removed, brought to 80% saturation with solid ammonium sulfate, and stirred for an additional hour. The 40–80% $(NH_4)_2SO_4$ precipitate was collected by centrifugation and resuspended to 1 L with B1 by stirring for 15 min. This solution was brought to 5% PEG by the addition of an appropriate volume of a 50% w/v solution of PEG and then stirred for 30 min. The PEG suspension was centrifuged, and the supernatant was adjusted with 50% PEG to a final concentration of 14% PEG. The 14% PEG solution was stirred 30 min and centrifuged; the pellet was resuspended with the aid of a 15-mL Dounce homogenizer in approximately 150 mL of B2.

DNA–Cellulose Chromatography. The resuspended 5–14% PEG pellet (300–600 mL) was loaded at 30–50 mL/h on a DNA–cellulose column (2.5 × 14 cm), and 10-mL fractions were collected. The column was washed with 150 mL of B_2, and then the bound enzyme was eluted at 50 mL/h with 400 mL of a 0.2–1.5 M NaCl gradient in B2. Fractions containing enzyme activity were pooled and frozen at −70 °C.

Hydroxylapatite Chromatography. The DNA–cellulose enzyme pool was loaded onto a hydroxylapatite column (2.5 × 8 cm) at approximately 40 mL/h. The column was washed with 50 mL of A1 and then 100 mL of A2. The enzyme was eluted at approximately 35 mL/h with a 500-mL linear gradient of 0–50 mM potassium phosphate in buffer A2. A second gradient from 50 to 300 mM potassium phosphate in A2 was used to elute the lamb thymus DNA that had been associated with the enzyme. Enzymatically active fractions from the first gradient were pooled and concentrated to approximately 10 mL in a 50-mL Amicon concentrator using a PM-10 membrane (Amicon, Lexington, MA).

Sepharose Gel Filtration. The concentrate from the hydroxylapatite column was loaded onto two Sepharose CL-6B-200 columns arranged in series (2.5 × 85 cm each) and eluted with B2 at a flow rate of approximately 9 mL/h. Fractions of 5 mL were collected and assayed for enzyme activity, with peak fractions taking about 60 h to elute from the column.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. One milliliter of each active fraction from the Sepharose column was precipitated with 20% Cl_3CCOOH, precipitates were collected by centrifugation at 12000*g* for 10 min, and the pellets were washed once with cold 95% ethanol. Precipitates were resuspended in 100 μL of 10 mM potassium phosphate, 0.1 M DTT, 2% $NaDodSO_4$, 20% glycerol, and 0.33% bromophenol blue, pH 7.0, and boiled for 5 min. $NaDodSO_4$ (7.5%)–polyacrylamide gel slabs with a 5% stacking gel were preelectrophoresed at 32 mA in the $NaDodSO_4$–Tris–glycine system of Studier (1973), and then samples were run at 25 mA. Gels were stained with 0.25% Coomassie Blue in 5:5:1 water–methanol–acetic acid and then destained with 17:2:1

Table I: Purification of Lamb Thymus Poly(ADP-ribose) Polymerase[a]

fraction	total enzyme act. (units)	total protein (mg)	sp act. (units/mg of protein)	yield (%)	enzyme purification	DNA dependence[b] (%)
crude extract	25000	28200	0.89	100	(1.0)	8
40–80% $(NH_4)_2SO_4$ pellet	24200	8470	2.9	97	3	0
5–14% PEG pellet	25300	1330	19	100	21	0
DNA–cellulose pool	5070	76.5	66	20	74	32
HTP pool	4520	5.04	897	18	1009	100
Sepharose CL-6B-200						
pool 1	1140	0.45	2540	4.6	2860	100
pool 2	871	0.56	1550	3.5	1747	100
pool 3	361	0.44	819	1.4	921	100

[a] Poly(ADP-ribose) polymerase was purified from lamb thymus, and activity was measured in the standard 5-min assay described under Materials and Methods. One unit of enzyme activity is the amount which gives 1 nmol of ADP-ribose incorporated per min from [^{14}C]NAD$^+$ into Cl_3CCOOH-insoluble counts. [b] DNA dependence (%) calculated as [(activity assayed with added DNA − activity assayed without added DNA) × 100]/(activity assayed with added DNA).

water–methanol–acetic acid. Fractions from the Sepharose column were pooled on the basis of the number of protein bands seen on the stained gels.

Results

Enzyme Purification. Table I is a summary of our purification of poly(ADP-ribose) polymerase starting from 500 g of pooled lamb thymus glands. Column fractions obtained during the enzyme purification were analyzed by using the standard poly(ADP-ribose) polymerase assay with exogenously added calf thymus DNA to provide maximum enzyme activation. However, during the initial stages of purification, the addition of exogenous DNA did not significantly increase enzyme activity, suggesting that the extracts contained sufficient DNA to provide maximal activation of the polymerase. For example, a 30-μL aliquot of the crude extract incorporated 5546 dpm of [^{14}C]NAD$^+$ into poly(ADP-ribose) in the presence of exogenously added DNA and 5098 dpm when no DNA was added. Thus addition of DNA to the reaction stimulated enzyme activity by only 8%, which is indicated in Table I as 8% dependence on exogenously added DNA. Differential ammonium sulfate precipitation gave a 3-fold purification of polymerase activity; this fraction was not stimulated by addition of exogenous DNA, suggesting that the preparation still contained sufficient DNA to completely activate all of the poly(ADP-ribose) polymerase present.

In an attempt to separate the polymerase from residual DNA, we introduced a differential precipitation with poly(ethylene glycol) (PEG) (Alberts & Herrick, 1971). While this step resulted in a 7-fold purification of enzyme activity, the activity of the polymerase was still not stimulated by exogenous DNA, suggesting that some residual DNA was very tightly associated with the enzyme. However, since the differential PEG precipitation resulted in a significant purification of enzyme activity, it was included in subsequent isolation procedures. Chromatography of the solubilized 5–14% PEG pool on DNA cellulose and elution with a gradient of 0.2–1.5 M NaCl yielded a pool of enzyme activity which was purified another 3-fold. The addition of exogenous DNA to assays of the DNA–cellulose pool resulted in a partial stimulation of polymerase activity. Chromatography of the DNA–cellulose pool on hydroxylapatite and elution with a 0–50 mM potassium phosphate gradient resulted in a 1000-fold purification of enzyme activity relative to the crude extract. At this point in the purification, enzyme activity was totally dependent on the addition of exogenous DNA, suggesting that hydroxylapatite chromatography dissociated the enzyme from the remaining DNA. Upon subsequent elution of the hydroxylapatite column with a 50–300 mM potassium phosphate

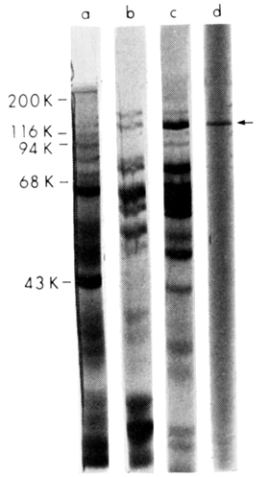

FIGURE 1: $NaDodSO_4$–polyacrylamide gels of fractions from the purification of lamb thymus poly(ADP-ribose) polymerase. Gels (7.5%) were electrophoresed as described under Materials and Methods. The molecular weight standards indicated on the left in kilodaltons were determined on simultaneously run gels by using the following markers: myosin, M_r 200 000; β-galactosidase, M_r 116 500; phosphorylase *b*, M_r 94 000; BSA, M_r 68 000; ovalbumin, M_r 43 000. (Lane a) Crude extract; (lane b) DNA–cellulose pool; (lane c) hydroxylapatite pool; (lane d) Sepharose pool 1. The arrow indicates the position of lamb thymus poly(ADP-ribose) polymerase.

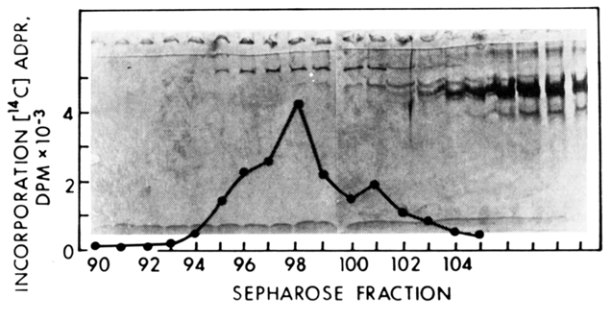

FIGURE 2: $NaDodSO_4$–polyacrylamide slab gels of active fractions from the Sepharose column. A plot of the enzyme activity in each fraction is superimposed on the gel. Enzyme activity was measured as [^{14}C]ADP-ribose incorporated into Cl_3CCOOH-insoluble material as described under Materials and Methods. Fractions were pooled as described in the text.

gradient, a small peak of DNA was eluted with an OD 260/280 ratio of 1.5. This fraction was free of protein as determined by Lowry protein assay.

As shown in Figure 1, an $NaDodSO_4$–polyacrylamide gel of the enzyme pool from the hydroxylapatite chromatography showed multiple bands. This material was further purified

Table II: Requirements for Poly(ADP-ribose) Polymerase Reaction[a]

reaction mixture	[^{14}C]ADP-ribose incorporated (dpm)	control (%)
complete	7884	100
minus DNA	51	0.6
minus H1	872	11
minus H1 + HIIA*	6358	81
minus DTT	8995	114
minus DTT + PHMB (1 mM)	0	0
minus DTT + NEM (1 mM)	3963	50
minus $MgCl_2$	10153	129

[a] The complete reaction system for poly(ADP-ribose) polymerase contained 10 μg of calf thymus DNA, 10 μg of histone H1, 0.5 mM [^{14}C]NAD, 4 mM $MgCl_2$, 1.0 mM DTT, 100 mM Tris-HCl, pH 8.0, and 30 μL of enzyme solution in a final volume of 100 μL. Incubations were for 5 min at 37 °C. Components were omitted or added as indicated in the table. [b] HIIA is a histone mixture from Sigma (St. Louis, MO).

on Sepharose 6B; each fraction of the Sepharose eluate showing polymerase activity was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The results of a typical analysis are shown in Figure 2. The enzymatic activity recovered from the Sepharose column represented approximately 10% of the starting material. However, as seen in Figure 2, only the leading edge of the enzyme peak appeared to contain a single protein band; these fractions were combined, designated as pool 1, and used for all subsequent studies in this paper. Fractions containing the enzyme and two additional bands were combined as pool 2. The remainder of the active fractions containing multiple protein bands were combined as pool 3. For example, in a preparation such as that shown in Figure 2, fractions 95–97 would be combined in pool 1, fractions 98–100 in pool 2, and fractions 101–103 in pool 3. As shown in Table I, the polymerase activity in pool 1 represented a 4.6% recovery and a 2860-fold purification relative to the crude extract. Figure 1 shows that the enzyme migrated as a single polypeptide chain with a molecular weight of 135 000 when $NaDodSO_4$–polyacrylamide gel electrophoresis was performed in the presence of DTT. The molecular weight of the purified enzyme from several different preparations was 175 000 when estimated by Sepharose gel filtration. The isoelectric point of the purified enzyme was 9.6. Analysis of the fractions from the isoelectric focusing column by $NaDodSO_4$–polyacrylamide gel electrophoresis showed the enzyme activity corresponded with the presence of a single polypeptide chain of molecular weight 135 000.

For confirmation that this enzyme preparation could actually synthesize poly(ADP-ribose), the purified enzyme was incubated in the standard reaction system containing 1 mM [^{14}C]NAD for 30 min at 37 °C. The product was processed for HPLC analysis as described under Materials and Methods. This procedure yields phosphoribosyl-AMP as the unique degradation product of internal residues of poly(ADP-ribose) and 5′-AMP as the degradation product of the terminal residues of poly(ADP-ribose) (Sims et al., 1980). All of the radioactivity in the hydrolysate of our reaction product chromatographed with these compounds in a ratio of 14 phosphoribosyl-AMP residues to 1 5′-AMP residue. Thus the product of this reaction was clearly poly(ADP-ribose) with an average chain length of 15 residues.

As indicated in Table II, poly(ADP-ribose) polymerase activity was totally dependent on added DNA. Histone H1 was not absolutely required, but its presence accounted for a 10-fold stimulation in enzyme activity. Higher concentrations of H1 depressed enzyme activity. Replacement of H1 with

Table III: Effect of Glycohydrolase and Phosphodiesterase Inhibitors on the Activity of Poly(ADP-ribose) Polymerase[a]

sample	[^{14}C]ADP-ribose incorporated (dpm) 5 min	60 min
control	5677	7950
ADP-ribose	5337	6220
cAMP	5991	6384
NaF	5922	6448

[a] The complete reaction system for poly(ADP-ribose) polymerase was the same as indicated in Table II. The difference in enzyme activity for control reactions reported in Tables II and III was due to the use of different enzyme preparations with different specific activities. ADP-ribose, cAMP, or NaF was added to give final concentrations of 1 mM just prior to the addition of enzyme. The reactions were run at 37 °C for either 5 or 60 min.

Table IV: Effect of Various Compounds on Poly(ADP-ribose) Polymerase Activity[a]

sample	[^{14}C]ADP-ribose incorporated (dpm)	inhibition (%)
control	5113	
nicotinamide (1 mM)	693	86
5-methylnicotinamide (1 mM)	1036	80
theophylline (2 mM)	578	89
thymidine (2 mM)	609	88

[a] The effects of various potential inhibitors of poly(ADP-ribose) polymerase activity were tested by using the standard assay system described under Materials and Methods. The final concentrations of inhibitors in the reaction were as indicated.

HIIA, a mixture of histones, also stimulated enzyme activity. The enzyme remained active when DTT was omitted from the final assay mixture; however, addition of 1 mM PHMB totally inhibited and 1 mM NEM caused 50% inhibition of enzyme activity.

When magnesium was omitted from the reaction mixture, there was no decrease in enzyme activity. The requirement for a metal ion cofactor was further investigated by testing the effects of 4 mM manganese, 4 mM calcium, 4 mM zinc, 10 mM EDTA, and 10 mM EGTA on enzyme activity. Neither the presence nor absence of magnesium, manganese, or calcium affected enzyme activity. When the chelating agents EDTA or EGTA were added to the reaction mixture, there was also no significant effect on enzyme activity. The enzyme therefore appears to have no requirement for exogenously added metals. $ZnCl_2$ (4 mM) inhibited enzyme activity by 94%, probably due to the ability of zinc to bind to an essential sulfhydryl group.

Effect of Other Agents on Enzyme Activity. If the purified enzyme were contaminated by glycohydrolases or phosphodiesterases, then reactions run in the presence of inhibitors of these enzymes should increase the amount of polymer that accumulates during the assay period. Table III shows that when poly(ADP-ribose) polymerase activity was measured in the presence of 1 mM ADP-ribose or 1 mM 3′,5′-cAMP, which inhibits poly(ADP-ribose) glycohydrolase (Tanaka et al., 1976; Burzio et al., 1976), or in the presence of 1 mM NaF, which inhibits phosphodiesterases (Miwa et al., 1975), there were no significant changes in the amount of Cl_3CCOOH-insoluble counts incorporated during either a 5-min or a 60-min incubation period. Raising the concentration of these compounds to 5 mM did not change the amount of poly(ADP-ribose) synthesized. These results indicate that the purified polymerase is not comtaminated by active glycohydrolases or phosphodiesterases. These results contrast with those of

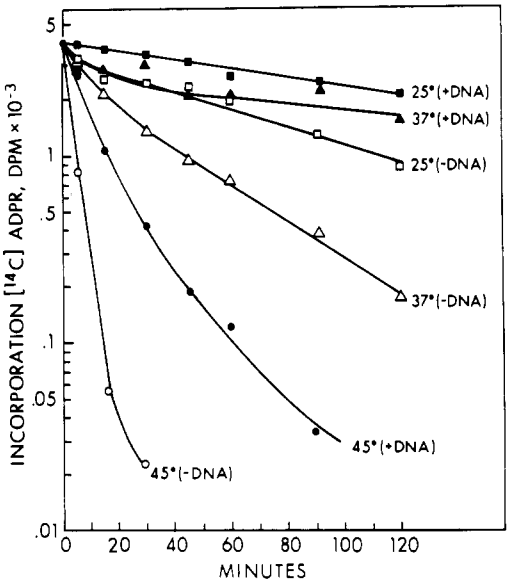

FIGURE 3: Effect of temperature on stability of poly(ADP-ribose) polymerase. Purified poly(ADP-ribose) polymerase was preincubated at 25, 37, or 45 °C in the presence or absence of DNA for the length of time indicated on the abscissa. At the time indicated on the abscissa, samples of enzyme preincubated alone or enzyme preincubated with DNA were removed from the preincubation temperature, and activity was assayed at 37 °C in a 5-min reaction by using the standard assay conditions described under Materials and Methods. Preincubation at 25 °C with DNA (■) or without DNA (□), at 37 °C with DNA (▲) or without DNA (△), and at 45 °C with DNA (●) and without DNA (○).

Niedergang et al. (1979), who found that 3′,5′-cAMP and ADP-ribose inhibited calf thymus poly(ADP-ribose) polymerase.

Table IV shows the effects of a number of agents that are known to inhibit poly(ADP-ribose) polymerase derived from other sources. All of these compounds, nicotinamide, 5-methylnicotinamide, theophylline, and thymidine, markedly inhibited the lamb thymus enzyme.

Effect of pH and Temperature. When poly(ADP-ribose) polymerase was activated with highly polymerized, double-stranded calf thymus DNA, the pH optimum for enzyme activity was between 8.6 and 8.8. Enzyme activity decreased above pH 8.8 and then increased again at pH 10. Since several investigators have shown that poly(ADP-ribose) polymerase activity increases in the presence of DNA strand breaks (Benjamin & Gill, 1980; Miller, 1975; Berger & Sikorski, 1981; Cohen & Berger, 1981; Ohgushi et al., 1980), it is possible that the increase in activity occurring at pH 10 was due to induction of strand breaks at alkali labile sites in the calf thymus DNA. When DNA was completely denatured by boiling and rapid quenching prior to its use in the assay, the enzyme activity was very low at all pHs. These latter results indicate that single-stranded DNA is a poor activator of poly(ADP-ribose) polymerase and that the increase in activity that occurred with double-stranded DNA at pH 10 was probably not due to any strand unwinding. These results are similar to those of Benjamin & Gill (1980) who showed that heat-denatured DNA and single-stranded homopolymers were not effective activators of poly(ADP-ribose) polymerase.

As shown in Figure 3, preincubation of the enzyme at increasing temperatures between 25 and 45 °C resulted in progressively more rapid rates of enzyme inactivation. The rate of enzyme inactivation at any of the temperatures could be slowed down by preincubating the enzyme in the presence of DNA. At 45 °C, poly(ADP-ribose) polymerase incubated in the absence of DNA was inactivated by 50% in 2 min, and

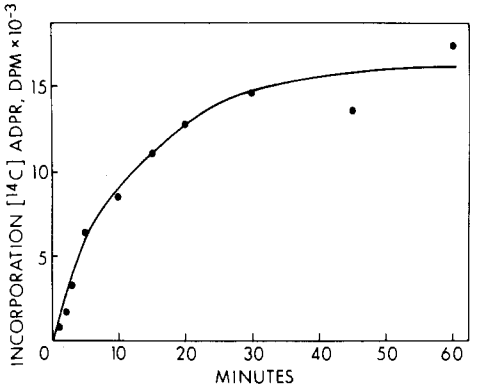

FIGURE 4: Progress of the poly(ADP-ribose) polymerase reaction at 37 °C and 4 mM NAD^+. Reactions were run with standard concentrations of calf thymus DNA and histone H1 as indicated under Materials and Methods. Enzyme activity was measured as [^{14}C]-ADP-ribose incorporated into Cl_3CCOOH-insoluble material.

in the presence of DNA, it was inactivated by 50% in 11 min. At 37 °C, the enzyme preincubated without DNA was inactivated by 50% in 18 min, whereas when preincubated with DNA, it was 50% inactivated in 62 min. Similarly at 25 °C, there was 50% loss of enzyme activity in 48 min in the absence of DNA and in 128 min in the presence of DNA. Thus DNA has a protective effect on enzyme stability.

Progress of the Reaction and Apparent K_m for NAD. At 37 °C in the presence of 4 mM NAD^+, poly(ADP-ribose) synthesis progressed linearly for 5 min following which there was a continuous accumulation of Cl_3CCOOH-precipitable material for 1 h (Figure 4). When the reaction was run at 37 °C in the presence of 500 μM NAD^+, the reaction progressed linearly for 3 min and usually leveled off after 5–10 min of incubation. In some of our earlier preparations when the reaction was run with 500 μm NAD^+, there was a subsequent decrease in the amount of Cl_3CCOOH-precipitable radioactive material when the incubation was continued from 5 to 60 min. Although these enzyme preparations showed a single protein band on $NaDodSO_4$–polyacrylamide gel electrophoresis, these effects could have been due to the presence of trace contaminants of phosphodiesterase, glycohydrolase, protease, or ADP-ribosyl-protein hydrolase (Okayama et al., 1978). The first three possibilities seem unlikely since addition of agents such as NaF, 3′,5′-cAMP, ADP-ribose and PMSF, which inhibit such enzymes, had no effect on the reaction. We have not pursued this degradation reaction any further since as shown by the 60-min value for the control sample in Table III, in most of our subsequent enzyme preparations incubated with 500 μM NAD^+, the amount of label incorporated during the first 5–10 min of the reaction was not degraded during prolonged incubation.

Assays to determine the apparent K_m for NAD^+ were run for 2 min at 37 °C over a wide range of NAD^+ concentrations (5 μM–4 mM). The apparent K_m for NAD^+ was 160 μM.

Effect of Lamb Thymus DNA. The polymerase remained associated with some DNA until it was separted by hydroxylapatite chromatography. As shown in Figure 5, the lamb thymus DNA, originally associated with the enzyme, was more effective than commercially available calf thymus DNA in its ability to support polymerase activity. In the standard 0.1-mL reaction system, calf thymus DNA produced maximal stimulation in the range of 400 μg/mL. At higher concentrations, it inhibited enzyme activity, while at concentrations below 10 μg/mL, it did not stimulate enzyme activity at all. In contrast, addition of lamb thymus DNA at concentrations below 10 μg/mL gave marked stimulation of polymerase activity, with

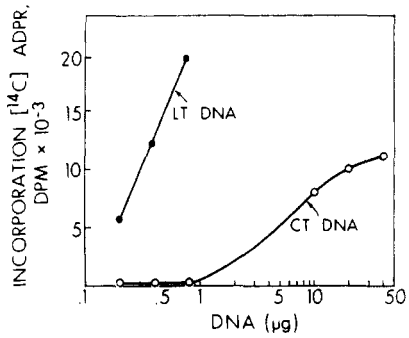

FIGURE 5: Comparison of the effects of lamb thymus DNA and calf thymus DNA on the activity of poly(ADP-ribose) polymerase. Poly(ADP-ribose) polymerase activity was assayed at 37 °C for 15 min at 500 μM NAD^+ and 100 μg/mL histone H1. Variable amounts of either lamb thymus DNA isolated from the hydroxylapatite column or commercial, highly polymerized calf thymus DNA (Sigma) were used in the 0.1-mL reaction system. Amount of DNA on abscissa is given in μg/0.1-mL reaction system. Lamb thymus (LT) DNA (●). Calf thymus (CT) DNA (○).

8 μg/mL of lamb thymus DNA stimulating almost twice as much poly(ADP-ribose) synthesis as occurred with 400 μg/mL of calf thymus DNA. Neutral agarose gel electrophoresis was perfomed to compare the sizes of the calf and lamb thymus DNAs by using *Hae*III restriction fragments of SV40 DNA as molecular weight markers. The lamb thymus DNA originally associated with the enzyme showed a heterogeneous distribution of DNA fragments which in general were much smaller than those present in the commercial highly polymerized calf thymus DNA. Most of the calf thymus DNA was greater than 5000 base pairs in length whereas most of the lamb thymus DNA was distributed in a broad band between 200 and 1600 base pairs in length. Since poly(ADP-ribose) polymerase has been shown to be stimulated by DNA strand breaks, it is possible that the increased ability of the lamb thymus DNA to stimulate poly(ADP-ribose) polymerase activity is due to the small size of this DNA which provides many more strand ends to stimulate the purified enzyme.

Discussion

Poly(ADP-ribose) polymerase has been purified to varying degrees from a variety of sources (Yoshihara et al., 1978; Ito et al., 1979; Niedergang et al., 1979; Tsopanakis et al., 1978; Okayama et al., 1977; Holtlund et al., 1980), and although there are similarities in the reported properties of many of the preparations, there are also some major differences. In the present studies, we found that lamb thymus poly(ADP-ribose) polymerase has a molecular weight estimated at 175 000 by gel filtration and 135 000 by $NaDodSO_4$–polyacrylamide gel electrophoresis. A similar discrepancy between molecular weights obtained by gel filtration (M_r 150 000–160 000) and other methods (M_r 130 000) was also found for calf thymus polymerase. Ohgushi et al. (1980) indicated that this may be due to molecular asymmetry of the enzyme since they obtained a frictional ratio of 1.81 for the calf thymus polymerase. Ito et al. (1979) obtained a somewhat lower frictional ratio of 1.39 for the enzyme from calf thymus. Both of these groups estimated the molecular weight of the calf thymus enzyme to be about 130 000, which is similar to what we obtained for the lamb thymus enzyme using $NaDodSO_4$–polyacrylamide gel electrophoresis. These estimates are significantly higher than the molecular weight of 63 500 obtained by Tsopanakis et al. (1978) for the enzyme from pig thymus. Since the polymerase isolated by Mandel's group (Niedergang et al., 1979) was bound to DNA, the possibility exists that some of the high molecular weight forms of the enzyme are due to its residual association with DNA. However, in our studies, the activity of the high molecular weight enzyme purified from lamb thymus was totally dependent on the addition of exogenous DNA, indicating that any associated DNA was removed during the purification.

The activity of lamb thymus poly(ADP-ribose) polymerase is greatly stimulated by the addition of histone H1 presumably because it acts as an acceptor for attachment of ADP-ribose moieties. Our preliminary autoradiographic results indicate that the enzyme is capable of both self-poly(ADP-ribosyl)ation and poly(ADP-ribosyl)ation of histone H1.

The enzyme from lamb thymus was similar to the calf thymus poly(ADP-ribose) polymerase in that it was inhibited by PHMB and NEM (Yoshihara et al., 1978; Ito et al., 1979; Niedergang et al., 1979). It is interesting to note that while PHMB totally inhibited enzyme activity, NEM only suppressed activity by 50%, suggesting that an essential sulfhydryl group is more accessible to organomercurials than to NEM. Similar patterns of inhibition have been found for other enzymes (Yamaguchi & Fujisawa, 1978; Johnson et al., 1980).

DNA can be considered to be an essential enzyme activator since the activity of the purified enzyme is totally dependent on the addition of DNA. In addition to its ability to activate poly(ADP-ribose) polymerase, DNA also protected the enzyme and slowed its rate of temperature-dependent inactivation. Lamb thymus DNA was considerably more effective in activating the enzyme than calf thymus DNA. Several investigators (Benjamin & Gill, 1980; Miller, 1975; Berger & Sikorski, 1981; Cohen & Berger, 1981; Ohgushi et al., 1980) have shown that the ability of DNA to stimulate the activity of poly(ADP-ribose) polymerase is dependent on the number of strand breaks or free ends present in the DNA. Since the lamb thymus DNA was smaller than the calf thymus DNA, it should have had more free ends available for enzyme activation.

Lamb poly(ADP-ribose) polymerase does not have any metal ion requirement; neither magnesium, manganese, nor calcium stimulated enzyme activity, and chelating agents did not significantly inhibit activity. Although several other investigators have reported that the enzyme from calf thymus (Yoshihara et al., 1978; Ito et al., 1979; Niedergang et al., 1979) and other sources is magnesium dependent (Okayama et al., 1977; Holtlund et al., 1980), Benjamin & Gill (1980) recently reported that the activity of a partially purified preparation from calf thymus was not dependent on magnesium.

Our results indicate that lamb thymus poly(ADP-ribose) polymerase has an apparent K_m for NAD^+ of 160 μM. This is somewhat higher than the apparent K_m of 50–60 μM published by other investigators for calf thymus polymerase (Yoshihara et al., 1978; Ito et al., 1979). However, recent publications by Ogata et al. (1980) and Butt & Smulson (1980) suggest that the K_m for NAD may be greater than those earlier reported values. With an apparent K_m for NAD^+ of 160 μM, the lamb thymus enzyme would be active at physiologic NAD^+ levels (Kaplan, 1960; Bassham et al., 1959; Silber et al., 1962). We are currently investigating the possibility that under physiological conditions, other components which activate the enzyme may further lower its K_m for NAD^+; for example, the association of the enzyme inside the cell with specific types of damaged DNA may lead to a lower K_m for NAD^+.

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