Purification and Characterization of Human Lymphoid Poly(adenosine diphosphate ribose) Polymerase†

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ABSTRACT: Poly(ADP-ribose) polymerase has been purified 12 000-fold from human tonsils with an 83% recovery of enzymatic activity relative to that of the initial homogenate. The specific activity of the purified enzyme is 862 units/mg of protein. The isolated protein has a molecular weight of approximately 116 000 as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The apparent K_m for NAD^+ is estimated to be 185 μM at pH 8.0 and 37 °C. The purified enzyme has an absolute requirement for exogenous DNA for catalytic activity, and the reaction is enhanced by the addition of purified histone H1. The enzyme does not require magnesium or other divalent cations for activity. Enzyme activity is inhibited by *p*-(hydroxymercuri)benzoate and *N*-ethylmaleimide. Thymidine, theophylline, nicotinamide, and 5-methylnicotinamide markedly inhibit enzyme activity whereas ADP-ribose, 3′,5′-cAMP, and sodium fluoride have a minimal effect on enzyme activity. Autoradiograms of labeled products of the reaction catalyzed by the purified enzyme at different concentrations of NAD^+ and at different incubation times show that at low concentrations of NAD^+ and after short incubations, poly(ADP-ribosyl)ation of the enzyme occurs preferentially over that of histone H1; at higher concentrations of NAD^+ or after longer incubations, poly(ADP-ribosyl)ation of histone H1 is increased.

Poly(adenosine diphosphate ribose) polymerase is a chromosomal enzyme which catalyzes the incorporation of the ADP-ribose[1] moiety of NAD^+ into a homopolymer, poly(ADP-ribose) (Hilz & Stone, 1976; Purnell et al., 1980; Hayaishi & Ueda, 1977). This enzyme is able to initiate poly(ADP-ribose) synthesis with the polymer covalently linked to itself (Kawaichi et al., 1981; Jump & Smulson, 1980; Yoshihara et al., 1977; Ogata et al., 1981) or to the chromosomal protein, histone H1 (Kawaichi et al., 1980). Poly(ADP-ribose) polymerase and its products have been reported to function in DNA synthesis (Burzio & Koide, 1970), cellular differentiation (Caplan & Rosenberg, 1975), DNA repair (Smulson et al., 1977; Berger et al., 1979), and DNA transcription (Gartemann et al., 1981).

Poly(ADP-ribose) polymerase has been purified to variable degrees from several tissue sources and mammalian cell lines (Ogata et al., 1981; Yoshihara et al., 1978; Petzold et al., 1981; Okayama et al., 1977; Mandel et al., 1977; Ito et al., 1979; Holtlund et al., 1980). In this paper we report the purification of poly(ADP-ribose) polymerase from normal human tonsils and adenoids. Human tonsils were selected as a tissue source because of the availability of bulk tissue and because lymphoid tissue, particularly animal thymus (Yoshihara et al., 1978; Petzold et al., 1981; Mandel et al., 1977; Ito et al., 1979; Tsopanakis et al., 1978), is a rich source of the enzyme. To our knowledge, this is the first report of purification of the enzyme from normal human lymphoid tissue.

The initial aspects of the purification scheme were similar to others (Yoshihara et al., 1978; Petzold et al., 1981) and employed ammonium sulfate precipitation of protein from a tissue homogenate followed by successive chromatography on DNA–cellulose and hydroxylapatite. In previous purification procedures, the active fraction from the hydroxylapatite column was further purified according to its molecular size on a Sephadex G-200 column (Yoshihara et al., 1978) or a Sepharose 6B column (Petzold et al., 1981). However, these final procedures were usually cumbersome (Yoshihara et al., 1978) or lengthy, requiring 2–3 days (Petzold et al., 1981), and resulted in a significant loss of enzyme activity. Miletich et al. (1980) recently reported the use of an anionic exchange column, Sulfadex S-50, to give wide separation of coagulation glycoproteins of molecular weight 10 000–200 000 with high reproducibility. In the present study, we evaluated the use of this matrix of bead-polymerized dextran (Sephadex G-50) linked to reactive sulfate ester groups to purify efficiently and reproducibly the eluate from the hydroxylapatite column and found that it gave a good separation of poly(ADP-ribose) polymerase from the other proteins. The Sulfadex S-50 purification was rapid (10 h) and gave a high yield of purified enzyme, 55% of the applied material, with a specific activity of 862 units/mg of protein.

Experimental Procedures

Materials

Human tonsils and adenoids, removed at surgery, were obtained from the Department of Pathology, Washington University School of Medicine, and were stored at −20 °C. Sulfadex S-50 was synthesized by the method of Miletich et al. (1980). [^{32}P]NAD^+ (670 Ci/mmol) was donated by New England Nuclear, Boston, MA. A butadiene mercuric acetate adduct was synthesized by Dr. Josef Pitha, National Institute of Aging, Bethesda, MD. [*adenosine*-(U)^{14}C]NAD^+ (563 mCi/mmol) was purchased from New England Nuclear. Calf thymus DNA type I and DNA–cellulose were purchased from Sigma. 5-Methylnicotinamide was obtained from Dr. Kurt Gerzon, Lilly Research Laboratories, Indianapolis, IN. Histone H1 was from Boehringer Mannheim; *Hae*III was from Bethesda Research Laboratories. SV 40 DNA was prepared as previously described (Cohen & Berger, 1981).

Buffers. The enzyme extraction buffer contained 50 mM Tris-HCl, pH 7.4, 0.3 M NaCl, 10 mM EDTA, 1 mM NaN_3,

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[1] Abbreviations: ADP-ribose, adenosine diphosphate ribose; AppppA, diadenosine 5′,5‴,P^1,P^4-tetraphosphate; CT-DNA, calf thymus DNA; HTP, hydroxylapatite; PMSF, phenylmethanesulfonyl fluoride; NaDodSO$_4$, sodium dodecyl sulfate; DTT, dithiothreitol; Cl_3CCOOH, trichloroacetic acid.

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1 mM glutathione, 50 mM $NaHSO_3$, 0.5 mM PMSF (dissolved first in 95% ethanol with final ethanol concentration 0.5%), and 0.5 mM dithiothreitol. Buffer B1 was the same as the extraction buffer except the NaCl concentration was 0.2 M. Buffer B2 was the same as buffer B1 except it 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 dithiothreitol, and 0.5 mM PMSF. Buffer A2 was the same as buffer A1 but also contained 2 M KCl.

Methods

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 each component in the standard reaction mixture was 10 μg calf thymus DNA, 10 μg of histone H1, 0.5 mM [^{14}C]NAD^+ (sp act. 3.8 dpm/pmol), 1.0 mM dithiothreitol, 100 mM Tris-HCl, pH 8.0, and 30 μL of enzyme solution (25 μg of protein/mL of solution) 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 for early points in the time-course experiments, the 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% Cl_3CCOOH. After standing at 0 °C for at least 15 min the samples were filtered through Whatman GF-C filters. The tubes and filters were rinsed 5 times with 20% Cl_3CCOOH and 3 times with cold 95% ethanol. The filters were dried, and their radioactivity was counted in 4 mL of a toluene scintillation fluid containing 4.2 g of 2,5-diphenyloxazole and 52 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per L.

Enzyme Purification Procedure. All procedures were done at 4 °C. A total of 250 g of frozen human tonsil and adenoid tissue 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 cool the suspension. The homogenate was centrifuged at 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 2 volumes of extraction buffer for 1 min and 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 for 1 h, the suspension was centrifuged; the 40%-saturated supernatant was removed and brought to 80% saturation with ammonium sulfate. The suspension was stirred for 90 min and then centrifuged. The 40–80% $(NH_4)_2SO_4$ precipitate was collected and resuspended in 350 mL of buffer B1 by stirring for 15 min.

The resuspended 40–80% $(NH_4)_2SO_4$ pellet (440 mL) was loaded at 100 mL/h on a Sephadex G-25 column (5.5 × 50 cm), and 12-mL fractions were collected. The column was washed with 1 L of buffer B2. Electrical conductivity was measured on all fractions. Fractions preceding the salt peak with the conductivity of buffer B2 were pooled. This Sephadex G-25 pool (500 mL) was loaded at 50 mL/h on a DNA–cellulose column (2.5 × 14 cm) containing approximately 4.1 mg of fixed DNA, and 6-mL fractions were collected. The column was washed with 150 mL of buffer B2, and then the bound enzyme was eluted at 50 mL/h with 400 mL of a linear 0.2–1.5 M NaCl gradient in buffer B2. Fractions containing enzyme activity were pooled (169 mL) and were loaded at 50 mL/h on a hydroxylapatite column (2.5 × 8 cm). The column was washed with 50 mL of buffer A1 and then with 100 mL of buffer A2. The enzyme was eluted at approximately 40 mL/h with a 500-mL linear gradient of 0–50 mM potassium phosphate in buffer A2. A second gradient of 50–300 mM potassium phosphate in buffer A2 was used to elute the human tonsil DNA that had been associated with the enzyme. The hydroxylapatite pool containing enzyme activity (150 mL) was desalted on a Sephadex G-25 column as described above. The active fractions were pooled (371 mL) and loaded at 150 mL/h on a Sulfadex S-50 column (2.5 × 30 cm). The column was washed with 100 mL of buffer B2 and eluted at approximately 100 mL/h with 500 mL of a linear 0.2–to 1.5 M NaCl gradient in buffer B2.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis and Autoradiography. One milliliter of each fraction containing enzyme activity from the Sulfadex column was precipitated with 20% Cl_3CCOOH. The 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 dithiothreitol, 2% $NaDodSO_4$, 20% glycerol, and 0.33% bromophenol blue, pH 7.0, and boiled for 5 min. The 5 or 7.5% $NaDodSO_4$–polyacrylamide gel slabs with 3 or 5% stacking gels were preelectrophoresed at 25 mA in the $NaDodSO_4$–Tris–glycine system of Studier (1973), and then samples were electrophoresed at 25 mA/slab. Gels were stained with 0.25% Coomassie blue in water–methanol–acetic acid, 5:5:1, and then destained with water–methanol–acetic acid, 17:2:1. Fractions from the Sulfadex column were pooled on the basis of the number of protein bands seen on the stained gels. Protein assays were performed on Cl_3CCOOH precipitates according to the method of Lowry et al. (1951). For autoradiography, Kodak XAR-5 X-ray film was exposed to dried gels containing ^{32}P-labeled proteins in a cassette with a Du Pont Cronex Lighting-Plus AC intensifying screen at −70 °C for an appropriate time.

Purification and Preparation of Enzyme-Associated DNA. Although the bulk of the tissue DNA was removed during DNA–cellulose chromatography, some DNA remained associated with the enzyme until hydroxylapatite chromatography. The poly(ADP-ribose) polymerase eluted from the hydroxylapatite column between 0 and 50 mM potassium phosphate. Subsequent elution with a 50–to 300 mM potassium phosphate gradient showed a peak of UV-absorbing material with an OD 260/280 ratio of 1.5 and no detectable protein by protein assay (Lowry et al., 1951). This fraction contained no enzyme activity but was capable of substituting for calf thymus DNA to activate the purified enzyme. It was dialyzed against 0.015 M NaCl, lyophilized, and resuspended in 0.015 M NaCl to give a final concentration of 940 μg of DNA/mL as determined by the diphenylamine reaction (Richards, 1974). The size range of this DNA was determined by electrophoresis in 2% agarose gels at pH 7.5 according to the method of Tegtmeyer & Macasaet (1972) with ethidium bromide staining to detect fluorescence (Cohen & Berger, 1981).

Results

Enzyme Purification. Our purification of poly(ADP-ribose) polymerase from 250 g of pooled human tonsils is presented in Table I. Activity of the enzyme at each purification step was measured by the standard poly(ADP-ribose) polymerase assay in the presence or absence of exogenously added calf thymus DNA.

The whole tissue homogenate and the ammonium sulfate extract showed considerable enzyme activity that was not dependent on exogenously supplied DNA. The use of a

Table I: Summary of the Purification of Human Tonsil Poly(ADP-ribose) Polymerase[a]

fraction	total enzyme act. (units)	total protein (mg)	sp act. (units/mg of protein)	yield (%)	x-fold enzyme purifcn	DNA dependence (%)
homogenate	1843	27000	0.068	100	(1.0)	0
$(NH_4)_2SO_4$ pellet	1395	5100	0.27	76	4.0	0
Sephadex G-25 pool	1750	3800	0.46	95	6.8	0
DNA–cellulose pool	4740	26	182	257	2700	89
HTP pool	3645	7.5	486	198	7100	98
Sulfadex S-50						
pool 1	133	0.35	380	7	5600	100
pool 2	1534	1.78	862	83	12700	100
pool 3	327	0.54	606	18	8900	100

[a] Poly(ADP-ribose) polymerase was purified from human tonsils and the activity was measured in the standard 5-min assay. One unit of enzyme activity is the amount that gives 1 nmol of ADP-ribose incorporated/min from [^{14}C]NAD$^+$ into Cl_3CCOOH-insoluble counts. Percent DNA dependence is calculated as [(activity assayed with added DNA − activity assayed without DNA)/activity assayed with added DNA] × 100. Total enzyme activity at each stage of the purification is based on the maximal activity measured with or without added DNA.

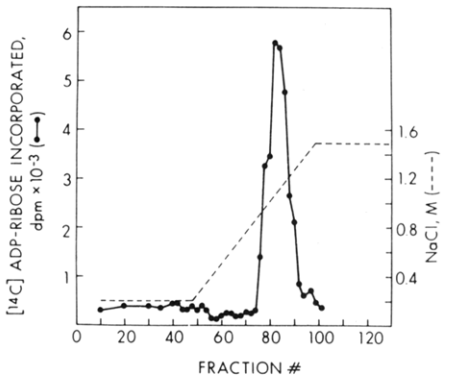

FIGURE 1: Sulfadex S-50 column chromatography. The enzyme pool from the hydroxylapatite chromatography was desalted on Sephadex G-25, then applied to a 2.5 × 30 cm Sulfadex S-50 column in buffer B2, and eluted with a 0.2–1.5 M NaCl gradient in buffer B2. Enzyme activity was measured on each fraction in the complete reaction system by using exogenous DNA as described under Methods.

Sephadex G-25 column to separate the protein fraction from the residual salt in the 40–80% ammonium sulfate fraction resulted in higher activity and an apparent increase in recovery of the poly(ADP-ribose) polymerase. DNA–cellulose chromatography yielded a pool of enzyme that had 2.6-fold more activity than the original homogenate and represented a 2700-fold purification. This marked increase in enzyme activity could be explained by the removal of inhibitors of poly(ADP-ribose) polymerase or enzymes that degrade the polymer. The latter seems likely since 66% of the newly synthesized polymer was degraded during prolonged incubations with enzyme from the Sephadex G-25 pool. As discussed later, there was no polymer-degrading activity in the final enzyme preparation. After DNA–cellulose chromatography, the addition of exogenous DNA to the enzyme assay produced an 89% stimulation of polymerase activity, indicating that the bulk of the native DNA was removed. Application of the active fraction from the DNA–cellulose column to a hydroxylapatite column resulted in a further 2.6-fold purification after the enzyme was eluted from the hydroxylapatite column with a 0–50 mM potassium phosphate gradient. This fraction was totally dependent on the addition of DNA. As shown in other preparations (Yoshihara et al., 1978; Petzold et al., 1981), the hydroxylapatite chromatography dissociated the enzyme from the remaining DNA that was eluted from the hydroxylapatite column with a 50–300 mM potassium phosphate gradient.

The enzymatically active fraction from the hydroxylapatite column was applied to a Sulfadex S-50 column and eluted with a gradient of 0.2–1.5 M sodium chloride. Figure 1 shows that the enzyme eluted in a narrow range. Each fraction of the Sulfadex eluate showing polymerase activity was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. As shown in Figure 2, fractions at the leading and tailing edges showed several protein bands and were combined as pool 1 and pool 3, respectively. Pool 2 consisted of fractions migrating as a single protein band, and this pool contained 42% of the enzyme activity applied to the column. As shown in Table I, pool 2 contains an 83% recovery of enzyme activity as compared to the initial homogenate, but when compared to the activity

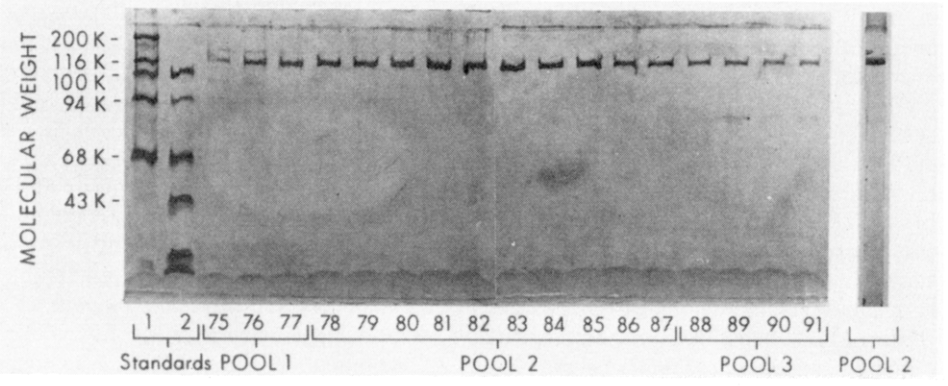

FIGURE 2: $NaDodSO_4$–polyacrylamide gel of active fractions from Sulfadex S-50 column stained with Coomassie Blue. 1 mL of each fraction was Cl_3CCOOH precipitated and analyzed on a 7.5% $NaDodSO_4$–polyacrylamide gel. Pools 1 and 3 were composed of fractions that stained for the major protein band at a molecular weight of 116 000 and for minor protein bands at lesser molecular weights. Pool 2 was composed of fractions that stained only for the major protein band. The column at the extreme right represents 62 μg of protein from pool 2 applied to a cylindrical $NaDodSO_4$–polyacrylamide gel that shows Coomassie Blue staining at molecular weights of 116 000, 72 000, and 60 000. Molecular weight standards were as follows: myosin 200 000; β-galactosidase 116 000; phosphorylase *b* 94 000; bovine serum albumin 68 000; ovalbumin 43 000.

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

reaction conditions	dpm of [^{14}C]ADP-ribose incorporated	% of total
complete	13 657	100
minus calf thymus DNA	56	0.4
minus histone H1	808	5.9
minus dithiothreitol	13 386	98

[a] The complete reaction system for poly(ADP-ribose)polymerase contained 8.4 μg of calf thymus DNA, 10 μg histone H1, 0.5 mM [^{14}C]NAD$^+$, 1.0 mM dithiothreitol, 100 mM Tris-HCl, pH 8.0, and 30 μL of enzyme solution (25 μg of protein/mL of solution) in a final volume of 100 μL. Incubations were for 5 min at 37 °C.

found in the DNA cellulose fraction, the recovery was 32%. This represents a 12 000-fold purification of the enzyme from the homogenate. Figure 2 also shows the cylindrical Na-DodSO$_4$–polyacrylamide gel electrophoresis of 62 μg of protein from pool 2 stained with Coomassie blue. This demonstrates a major band of protein at a molecular weight of 116 000 and two minor bands at molecular weights of approximately 72 000 and 60 000. The purity of the enzyme at this stage was estimated to be 98% from the density of each band as measured with a gel scanning apparatus on a Gilford 250 spectrophotometer. Each minor band was less than 1% of the density of the major band. Further studies to characterize the purified poly(ADP-ribose) polymerase were performed on pool 2 from the Sulfadex S-50 column.

Stability of Poly(ADP-ribose) Polymerase. The purified enzyme in buffer B2 and 1.0 M NaCl remained stable at −70 °C for at least 7 months. Before being assayed, a sample of enzyme was dialyzed at 4 °C for 6 h against two exchanges of 200-fold excess of buffer B2. When stored at −70 °C, the activity of the dialyzed enzyme remained stable for 72 h.

Progress of the Reaction and Determination of Apparent K_m for NAD$^+$. As shown in Table II, the purified enzyme required DNA and histone H1 for full activity. There was insignificant loss of enzyme activity when DTT was omitted from the final reaction system. Omission of DNA resulted in complete loss of enzyme activity. In the absence of histone H1, product formation was only 6.6% of that in the complete reaction system.

Figure 3 shows the time course obtained for poly(ADP-ribose) synthesis when the purified enzyme was incubated with either 9 or 500 μM NAD$^+$. At the low concentration, there was an initial rapid rate of polymer synthesis that leveled off after the first 5 min. At 500 μM NAD$^+$, there was a rapid rate of polymer synthesis for the first 10 min and then a much slower rate of synthesis for the subsequent 50 min. This change in rate may reflect the increase in apparent K_m of the enzyme due to auto(ADP-ribosyl)ation as recently suggested by Kawaichi et al. (1981). In a more detailed analysis, we found 2 min to be within the linear portion of enzymatic activity and therefore used 2-min incubation periods for the kinetic determinations. The apparent K_m for NAD$^+$ of human tonsil poly(ADP-ribose) polymerase as estimated by the method of Lineweaver & Burk (1934) was 185 μM at 37 °C.

Effects of Calf Thymus DNA and Enzyme-Associated Human Tonsil DNA on Poly(ADP-ribose) Polymerase Activity. Human tonsil DNA was separated from the enzyme by hydroxylapatite chromatography. Figure 4 shows an ethidium bromide stained 2% agarose gel electrophoresis of calf thymus DNA, and this hydroxylapatite dissociated human DNA at concentrations of 840 and 940 μg/ml, respectively. SV 40 DNA, form I and form II, and *Hae*III cut SV 40 DNA

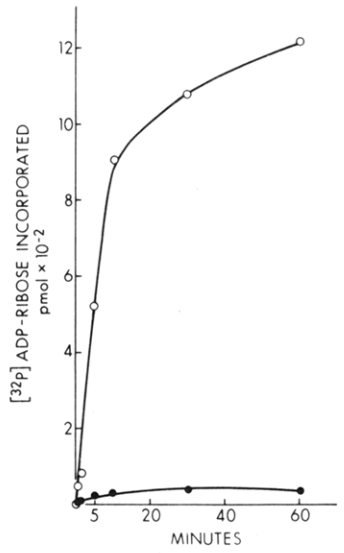

FIGURE 3: Time course of the poly(ADP-ribose) polymerase reaction. The reaction system for poly(ADP-ribose) polymerase was the same as in Table II except for the use of 9 μM [^{32}P]NAD$^+$, sp act. 2920 cpm/pmol (closed circles), or 500 μM [^{32}P]NAD$^+$, sp act. 44 cpm/pmol (open circles). Picomoles of [^{32}P]ADP-ribose incorporated into poly(ADP-ribose) are shown on the ordinate.

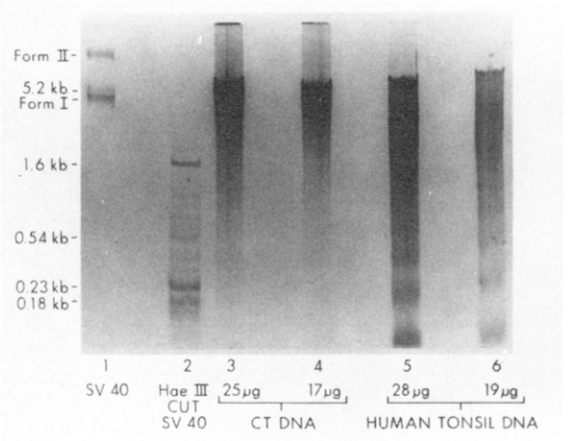

FIGURE 4: Photograph of ethidium bromide stained 2% agarose gel electrophoresis. Lane 1 is SV 40 DNA; lane 2, an *Hae*III digest of SV 40 DNA; lanes 3 and 4, Sigma calf thymus DNA type I; lanes 5 and 6, human tonsil DNA obtained from the 50–300 mM potassium phosphate gradient of the hydroxylapatite column.

were chromatographed simultaneously to size the DNA fragments. The hydroxylapatite-dissociated DNA was distributed over a range of smaller molecular weights than the calf thymus DNA, suggesting the presence of smaller fragments with more available free ends.

Figure 5 shows the activity of the purified poly(ADP-ribose) polymerase in reaction systems with 10 μg of histone H1 and with variable amounts of calf thymus DNA or hydroxylapatite-dissociated human DNA. As has been previously demonstrated for the small DNA fragments associated with calf thymus polymerase (Benjamin & Gill, 1980), the smaller size range of the hydroxylapatite-dissociated human DNA may contribute to its ability to stimulate greater enzyme activity.

Effect of Histone H1 on Enzyme Activity. Figure 5 shows that peak enzyme activity occurs at H1:DNA ratios between 1.0 and 2.0. For determination of the optimal histone to DNA ratio for enzyme activity, various concentrations of histone H1 were incubated with poly(ADP-ribose) polymerase while the concentration of calf thymus DNA was held constant at 8.4

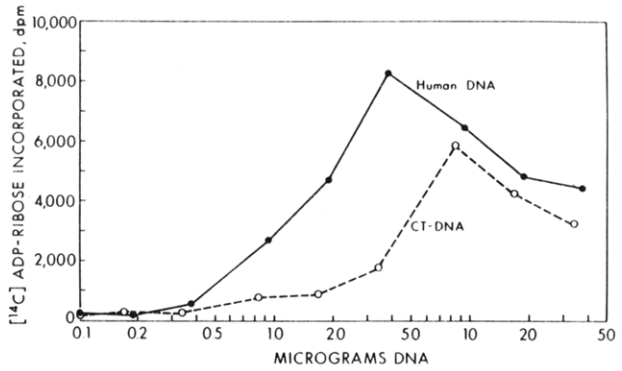

FIGURE 5: Effect of DNA concentration on the purified poly(ADP-ribose) polymerase reaction. The reaction system for poly(ADP-ribose) polymerase was the same as in Table II with varying concentrations of calf thymus DNA or hydroxylapatite-dissociated human tonsil DNA as indicated on the graph.

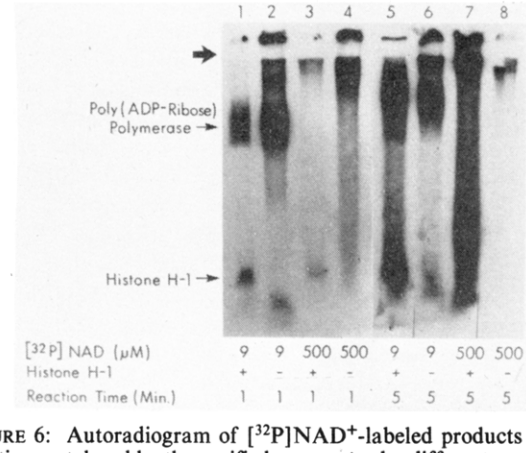

FIGURE 6: Autoradiogram of [^{32}P]NAD$^+$-labeled products of the reactions catalyzed by the purified enzyme under different conditions as specified under each lane. The specific activities of [^{32}P]NAD$^+$ for 9 and 500 μM are 2200 and 37 cpm/pmol, respectively. Lanes 1–4 had a film exposure time of 20 h and lanes 5–8, 72 h. The broad arrow indicates the interface between the 3% stacking gel and the 5% separating gel. The localization of poly(ADP-ribose) polymerase and histone H1 was determined by staining simultaneously chromatographed lanes of each protein with Coomassie Blue.

μg of DNA/100 μl of reaction. These studies showed that the enzyme was most active when the H1:DNA ratio was between 1.5 and 2.0. Enzymatic activity dropped rapidly at lower and greater ratios.

An autoradiogram of the polyacrylamide gel separation of the labeled products formed during incubations with [^{32}P]-NAD$^+$ and with purified enzyme under different conditions is shown in Figure 6. Lane 1 shows poly(ADP-ribosyl)ation of the enzyme after a 1-min incubation with 9 μM NAD$^+$ and 10 μg of histone H1; lane 2 is the same reaction without histone H1. Both of these lanes show the presence of a poly(ADP-ribosyl)ated band at the molecular weight of 116 000 corresponding to the molecular weight of the enzyme. Lanes 3 and 4 are also 1-min incubations plus and minus histone H1, respectively, but with 500 μM NAD$^+$. These reactions also show poly(ADP-ribosyl)ated bands at molecular weights near the top of the 5% NaDodSO$_4$–polyacrylamide gel. In addition, lanes 1 and 3 show the presence of a poly(ADP-ribosyl)ated band comigrating with histone H1. Lanes 5–8 show the same reaction systems after 5-min incubations. In contrast to lanes 1 and 2, the poly(ADP-ribosyl)ated polymerase migrates near the top of the 5% NaDodSO$_4$–polyacrylamide gel at both concentrations of NAD$^+$. The decrease in mobility of the poly(ADP-ribosyl)ated enzyme after incubation with higher NAD$^+$ concentrations as in lanes 3 and 4 or for longer time

Table III: Effect of Divalent Cations on Poly(ADP-ribose) Polymerase Activity[a]

addition	dpm of [^{14}C]ADP-ribose incorporated	% of control
control	14 331	100
$CaCl_2$	13 901	97
$HgCl_2$	314	2.2
$MgCl_2$	13 284	93
$MnCl_2$	4 665	33
$ZnCl_2$	389	2.7

[a] The reaction system for poly(ADP-ribose) polymerase contained the various divalent cations at 4 mM concentrations. Purified enzyme was dialyzed against buffer B2 containing no EDTA and no dithiothreitol. Dithiothreitol was excluded from the reaction mixture.

periods as in lanes 5 and 6 is presumably due to lengthening of the covalently linked poly(ADP-ribose) chain, thus increasing the molecular weight of the modified enzyme protein. Similarly, the increased amount of radioactive material that occurs between histone H1 and the enzymes in lanes 5 and 7 may be due to the synthesis of long chains of poly(ADP-ribose) attached to histone H1. Alternatively, it is possible that it reflects free poly(ADP-ribose) chains that were cleaved from their protein linkages during the conditions (pH 6.8–8.8) of the gel electrophoresis (Wong et al., 1977).

Divalent Cation Requirements. For evaluation of the possibility of a tightly bound metal ion, samples of purified enzyme were dialyzed for 72 h against buffer B2 containing 10 mM EDTA or against the same buffer containing 2.5 mM *o*-phenanthroline instead of EDTA. Extensive dialysis against the chelating agents EDTA or *o*-phenanthroline did not result in significant loss of enzyme activity; in fact, enzyme activity was enhanced by 30% following EDTA dialysis. This study suggests that no tightly bound cations are required for enzyme activity. In addition, the increase in enzyme activity after dialysis against 10 mM EDTA suggests that the enzyme may be partially inhibited by traces of divalent cations in the water used.

For evaluation of the effect of added divalent cations, purified enzyme was dialyzed against buffer B2 containing 10 mM EDTA and 0.5 mM dithiothreitol or against buffer B2 without EDTA and dithiothreitol. Table III shows that zinc chloride and mercuric chloride markedly inhibited poly(ADP-ribose) polymerase activity. Manganese chloride inhibited enzyme activity by 33%, whereas calcium chloride and magnesium chloride had minimal effects on enzyme activity. Similar effects were observed at divalent cation concentrations of 1 mM, and there was no significant difference between these data and those obtained with enzyme dialyzed against buffer B2 with EDTA and dithiothreitol.

Effect of Substrate Analogues, Nucleotides, and Potential Inhibitors on Poly(ADP-ribose) Synthesis. Studies of isolated nuclei (Preiss et al., 1971), permeabilized cells (Berger et al., 1978), and purified calf thymus (Niedergang et al., 1979) poly(ADP-ribose) polymerase showed inhibition of poly(ADP-ribose) synthesis by agents that were capable of competing with the nicotiamide portion of NAD$^+$. As shown in Table IV, both 5-methylnicotinamide and nicotinamide inhibited the poly(ADP-ribosyl)ation reaction. ADP-ribose, which represents the other product when nicotinamide is cleaved from NAD$^+$, showed no inhibition of poly(ADP-ribose) synthesis.

In contrast to the results of Niedergang et al. (1979), cyclic AMP and ATP had insignificant effects on poly(ADP-ribose) synthesis even at concentrations of 5 mM. Theobromine and

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

addition	% of control
control	100
5-methylnicotinamide	39
nicotinamide	10
ADP-ribose	118
3′,5′-cAMP	113
ATP	94
theobromine	15
theophylline	14
diadenosine 5′,5‴,P^1,P^4-tetraphosphate	78
thymidine	13
pyridoxal	88
pyridoxal 5′-phosphate	110
sodium fluoride	92

[a] The complete reaction system for poly(ADP-ribose) polymerase was the same as indicated in Table II. The various compounds at a final concentration of 1 mM were added to the indicated reactions prior to the addition of enzyme. In the control reaction, the amount of incorporation from [^{14}C]NAD$^+$ was 4656 dpm/5 min.

Table V: Effect of Sulfhydryl Reagents on Poly(ADP-ribose) Polymerase Activity[a]

addition	% of control
none (control)	100
dithiothreitol, 1 mM	101
iodoacetamide, 1 mM	78
iodoacetamide, 5 mM	51
N-ethylmaleimide, 1 mM	77
N-ethylmaleimide, 5 mM	71
p-(hydroxymercuri)benzoate, 1 mM	1.2
butadiene mercuric acetate adduct, 1 mM	1.4

[a] The complete reaction system for poly(ADP-ribose) polymerase was the same as indicated in Table II with the omission of 1.0 mM dithiothreitol. The inhibitors were added to the indicated reactions prior to the addition of enzyme. In the control reaction, the amount of incorporation from [^{14}C]NAD$^+$ was 7592 dpm/5 min.

theophylline, analogues of the purine portion of NAD$^+$, were effective inhibitors of the enzyme. Diadenosine 5′,5‴,P^1,P^4-tetraphosphate was shown by Yoshihara & Tanaka (1981) to function as an apparent inhibitor of poly(ADP-ribose) polymerase due to formation of products that were not Cl_3CCOOH precipitable. Our results also show slight inhibition of poly(ADP-ribose) synthesis by AppppA. We have not determined whether this is due to (ADP-ribosyl)ation of AppppA.

Studies by Preiss et al. (1971) and Berger et al. (1978) showed inhibition of the poly(ADP-ribose) polymerase reaction by thymidine in permeabilized cells. This study shows that thymidine inhibited the activity of the purified human poly(ADP-ribose) polymerase. Pyridoxal compounds have also been shown to inhibit the poly(ADP-ribose) polymerase reaction in permeabilized cells (Berger et al., 1978); however, they had negligible effects on the purified enzyme. Thus, inhibition of poly(ADP-ribose) synthesis by pyridoxal compounds in permeabilized cells is probably due to a mechanism other than direct inhibition of the poly(ADP-ribose) polymerase. Sodium fluoride had no effect on the enzyme activity. This indicates that the enzyme preparation is not contaminated by any phosphodiesterase activity that may interfere with poly(ADP-ribose) formation.

As shown in Table V, iodoacetamide and *N*-ethylmaleimide inhibited the poly(ADP-ribose) polymerase reaction by 25–50%. Like mercuric chloride and zinc chloride, the organomercurials, *p*-(hydroxymercuri)benzoate and the butadiene mercuric acetate adduct, inhibited the formation of poly(ADP-ribose) by more than 98%. These findings suggest that essential sulfhydryl groups that are readily accessible to the latter compounds are critical for the activity of the purified human enzyme.

Discussion

Poly(ADP-ribose) polymerase from normal human lymphoid tissue has been purified to near homogeneity by using a novel column chromatographic procedure (Miletich et al., 1980) as the final purification step. With the aid of a sulfated bead-polymerized dextran, Sulfadex S-50, we have isolated poly(ADP-ribose) polymerase with a specific activity of 862 units/mg of protein and a recovery of 83% relative to the initial crude material; however, this apparent recovery value is elevated because of the marked increase in enzyme activity following DNA–cellulose chromatography. When enzyme activity of the purified polymerase is compared to the activity of the DNA–cellulose pool, recovery is 32%. $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of the purified enzyme shows a major protein band at a molecular weight of 116 000 and two minor protein bands at molecular weights of 72 000 and 60 000. While most purified poly(ADP-ribose) polymerases have molecular weights between 110 000 and 130 000 (Jump & Smulson, 1980; Yoshihara et al., 1978; Petzold et al., 1981; Mandel et al., 1977; Ito et al., 1979), some laboratories (Okayama et al., 1977; Tsopanakis et al., 1978) have reported molecular weights of 55 000–63 500 for the purified enzyme or for persistent contaminating proteins. As suggested by Holtlund et al. (1981), these smaller proteins may represent proteolytic fragments of the larger polymerase or heterogeneity among different animal species.

The Sulfadex S-50 purification scheme produces a high yield of homogeneous poly(ADP-ribose) polymerase in a short time period. Lengthy dialysis and concentration steps and prolonged exposure to low ionic strength buffers are eliminated; thus, the opportunities for inactivation of the enzyme and proteolysis are minimized.

The purified human enzyme has properties similar to the purified enzymes from rat liver nuclei (Yamada & Sugimura, 1973) and calf or lamb thymus (Yoshihara et al., 1978; Petzold et al., 1981). Enzyme activity is totally dependent on the presence of DNA and is stimulated 16-fold by the addition of histone H1. The hydroxylapatite-dissociated human DNA is more effective in activating the enzyme than is the calf thymus DNA, probably because of the greater number of strand breaks or free ends in this isolated DNA compared to that in the commercially prepared calf thymus DNA. This effect of strand breaks has been demonstrated in several laboratories (Cohen & Berger, 1981; Benjamin & Gill, 1980; Ohgushi et al., 1980).

Autoradiograms of the products of the reactions catalyzed by the purified human poly(ADP-ribose) polymerase indicate that the enzyme is capable of both auto(ADP-ribosyl)ation and poly(ADP-ribosyl)ation of histone H1. The modification of the enzyme occurs as the preferential reaction at low NAD$^+$ concentrations and at early reaction times, and the product appears to be the poly(ADP-ribosyl)ated polymerase. At higher substrate concentrations and with prolonged incubation, there is an increase in poly(ADP-ribosyl)ation of the enzyme, resulting in a greater molecular weight such that the poly(ADP-ribosyl)ated product fails to enter the gel. This accumulation occurs in the presence or the absence of histone H1. At the higher NAD$^+$ concentrations and with longer incubations, histone H1 also becomes poly(ADP-ribosyl)ated to a

greater extent. These findings support the evidence from Ogata et al. (1981) that the polymerase is a primary acceptor of poly(ADP-ribose) and that under appropriate conditions, histone H1 also serves as an acceptor.

Some laboratories (Yoshihara et al., 1978; Niedergang et al., 1979) have reported a magnesium ion cofactor requirement for purified calf thymus poly(ADP-ribose) polymerase, whereas others have reported no requirement for this cofactor by enzyme extracted from lamb (Petzold et al., 1981) or calf (Benjamin & Gill, 1980) thymus. After extensive dialysis against 10 mM EDTA or 2.5 mM *o*-phenanthroline, the purified human enzyme showed no requirement for any divalent cation cofactor. These studies also indicate the absence of any tightly bound divalent cation as is present with DNA polymerase (Slater et al., 1971).

The purified human poly(ADP-ribose) polymerase has physical and chemical properties similar to those recently reported for the polymerases isolated from Ehrlich ascites tumor cells, pig thymus, and HeLa S3 cells by Holtlund et al. (1981). Characteristics common to the polymerases from each of these tissue sources are a molecular weight of 112 000–116 000, an absolute requirement for DNA in the reaction mixture, inhibition of the reaction by nicotinamide, and little effect on enzyme activity by ADP-ribose. Holtlund et al. (1981) indicated that the enzyme extracted from HeLa cells only showed 44% activity in the absence of magnesium ion; however, these studies were performed before the enzyme was separated from its associated DNA by hydroxylapatite chromatography. Using a purified human enzyme preparation, our studies indicate no requirement for divalent cations. This and other comparisons suggest that many similarities exist among the poly(ADP-ribose) polymerases isolated from different sources and that many of the apparent differences may be explained by variations in the purification procedure and the extent of purity of the final product.

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