Bovine Thymus Poly(Adenosine Diphosphate Ribose) Polymerase

PHYSICAL PROPERTIES AND BINDING TO DNA

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Purified bovine thymus poly(adenosine diphosphate ribose) polymerase is a monomeric protein with a single polypeptide chain having a molecular weight of approximately 130,000, determined by sodium dodecyl sulfate-gel electrophoresis, analytical ultracentrifugation, and gel filtration. A high frictional ratio (1.81) indicated that the molecule has an elongated shape, or a high solvation, or both. The enzyme is a basic protein (pI 9.8), and amino acid analysis showed a relatively high lysine content.

The enzyme activity is dependent on double-stranded DNA and is solely correlated with single- or double-stranded breaks on the DNA. Filter binding assay technique showed that the enzyme-activating efficiency of DNA correlated sufficiently with its enzyme-binding efficiency. Thus, a very high enzyme-activating efficiency of a DNA fraction (active DNA) which was separated from the crude enzyme fraction is mainly due to its high enzyme-binding efficiency. It was also shown that single-stranded DNA and heparin had a strong inhibitory effect on the binding of the enzyme to double-stranded DNA, whereas competitive inhibitors did not affect the binding.

We interpret these results to indicate that the binding of the enzyme to double-stranded DNA is a prerequisite step to its catalytic activity and has a dual function: (a) to position the enzyme on specific binding sites such as single- or double-stranded breaks on the DNA, and (b) to induce an active conformation of the enzyme.

Poly(ADP-ribose) polymerase of eukaryotic cell nuclei catalyzes polymerization of the ADP-ribose moiety of NAD⁺ into a homopolymer of repeating ADP-ribose units, which is known to be bound to various nuclear proteins (Refs. 1–6, for a review, see Ref. 7).

Recently, the enzyme has been purified to near homogeneity from various tissues in several laboratories (8–12). The purified enzyme absolutely requires DNA for its activity, as previously reported (13) and Yamada et al. (14) with partially purified enzyme. In recent reports (8, 15), we described that a DNA fraction (active DNA) which was separated at a purification stage of bovine thymus poly(ADP-ribose) polymerase has a high enzyme-activating ability. Although the precise mechanism of DNA which supports the enzyme activity is still unclear, treatment of isolated nuclei (16) or permeable cells (17) with DNA-endonuclease was reported to elicit a considerable increase in the enzyme activity, indicating that the enzyme is activated by DNA damage. This activation was also implicated in DNA repair (16–20).

In this report, a filter binding assay employed in the study of DNA-protein interaction (21) was used to examine the enzyme binding of active DNA and of various other DNAs including their DNase I-treated products. Physicochemical properties of the purified enzyme will also be presented here.

MATERIALS AND METHODS

Chemicals

[adenine-2-3H]NAD⁺, [adenine-U-¹⁴C]NAD⁺, and [6-¹⁴C]thymidine were purchased from New England Nuclear, Boston, NAD⁺ (grade 5) and thymidine were products of Sigma. Heparin, theophylline, N-ethylmaleimide, and nicotinamide were obtained from Nakarai Chemicals Ltd., Japan. Escherichia coli (16 and 23 S ribosomal RNA was obtained from Boehringer Mannheim, West Germany.

Buffers

Buffer A contained 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM NaCl, 1 mM glutathione, and 0.5 mM dithiothreitol. Buffer B contained 30 mM NaCl, 3 mM sodium citrate, 2 mM Tris-HCl (pH 7.4), and 2 mM EDTA.

DNA Preparations

Calf thymus DNA (type 1, Lot 26C9560) was a product of Sigma. “Active DNA,” a DNA fraction which was separated from the enzyme during the purification and has a very high enzyme-activating efficiency, was prepared as described previously (19). For the preparation of denatured DNA, calf thymus DNA was heated at 95°C for 10 min and rapidly cooled.

Covalently closed circular ColEI plasmid DNA was prepared from E. coli A745 met thy (ColEl) according to the method of Tomizawa et al. (22), except that the culture was treated with chloramphenicol for 18 h. Thus obtained closed circular ColEI DNA preparation was still contaminated (approximately 10% of the total DNA) with E. coli chromosomal DNA which was approximately 4 S and had a high activation ability on poly(ADP-ribose) polymerase (15). In order to eliminate this DNA, we further purified it by a 5 to 25% sucrose gradient centrifugation in a buffer (0.1 M NaCl, 0.025 M Tris-HCl (pH 7.6), and 0.01 M EDTA) at 200,000 × g for 4 h at 4°C. After centrifugation, samples were fractionated with an ISCO model 640 density gradient fractionator and the peak of closed circular ColEI DNA (23 S) was collected. For preparation of °H-labeled closed circular ColEI DNA, 2.5 μCi/ml of [3H]thymidine was added 60 min after the addition of chloramphenicol. Electrophoretic analysis (described in the next section) of thus purified closed circular ColEI DNA showed a single band when the gels were overloaded with 0.5 μCi of the DNA. The detection limit of the method is 0.04 μCi, suggesting that the DNA was at least 95% pure. E. coli strain A745 was obtained from Dr. Y. Sakakihara (National Institute of Health, Tokyo, Japan).

DNAs used for the enzyme assay and the binding assay were dissolved in Buffer B.

Treatment of Closed Circular ColEl DNA with Enzymes

Closed circular ColEl DNA was converted to full length linear molecules by treatment with EcoRI restriction endonuclease (ColEl DNA contains a single EcoRI site). The reaction mixture (1.1 ml)
Physical and DNA-binding Properties

contained 46 µg of DNA, 80 mM Tris-HCl (pH 7.5), 7 mM MgCl<sub>2</sub>, 7 mM mercaptoethanol, 50 mM NaCl, and 120 units of endonuclease EcoRI (Takara Shuzo Ltd., Japan). The mixture was incubated at 37°C for 60 min and the reaction was terminated by heating at 60°C for 5 min.

In order to control a digestion product of CoIE1 DNA, closing circular CoIE1 DNA was treated with pancreatic DNase I (Worthington, from bovine pancreas, code DPFF). The reaction mixture contained 36 µg/ml of DNA, 10 mM Tris-HCl (pH 8.0), 1.6 mM EDTA, and various amounts of the enzyme. Closed circular H-labeled CoIE1 DNA (33 µg/ml) was also treated with DNase I (10 ng/ml) under the same conditions and used as a standard DNA in the binding assay (Figs. 5 to 7; Table IV). The reaction was carried out at 25°C for 30 min and stopped by adding EDTA to 10 mM. After termination of these reactions, the mixture was phenol-extracted once and dialyzed against Buffer B.

In order to check the extent of digestion of DNAs by endonuclease treatments, 0.4 µg of the endonuclease-digested CoIE1 DNA was run on 1% agarose gel in the presence of 0.5 µg/ml of ethidium bromide using a horizontal slab gel electrophoresis apparatus (23) at 140 V for about 2 h at room temperature. The DNA bands were visualized by the fluorescence from DNA-bound ethidium bromide under ultraviolet irradiation (24) and photographed with a camera equipped with an orange filter (Kenko MC YA3) and Neopan 400 Fuji film. Density of DNA bands on the photographic film was scanned with Joyce, Loebl and Co. microdensitometer MK 3CS. According to the microdensitometric analysis, DNase I (2 ng/ml) of treatment of closed circular CoIE1 DNA produced closed circular and open circular molecules in the ratio of 1:4. The treatment (10 ng/ml of DNase I) produced open circular and full length linear molecules in the ratios of 87:13 and 89:11 for unlabeled and H-labeled CoIE1 DNA, respectively, and the treatment (100 ng/ml of DNase I) produced only fragmented CoIE1 DNA of heterogeneous length.

Preparation and Assay of Poly(ADP-Ribose) Polymerase

Poly(ADP-ribose) polymerase was purified by the method described in a previous report (8). The purity of the enzyme, examined by SDS-polyacrylamide gel electrophoresis followed by amido black staining, was approximately 97%. The specific activity of the purified enzyme was 160 units/mg of protein. Reaction mixtures for the assay of this enzyme contained 25 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 10 µM [adenine-2,8-'H]NAD<sup>+</sup> (18 cpmpmol), varying amounts of DNA, and 0.2 µg of the purified enzyme in a total volume of 0.2 ml. When H-labeled DNA was used in the assay mixture, 10 µM [adenine-2,8-'H]NAD<sup>+</sup> (18 cpmpmol) was replaced by 10 µM [adenine-2,8-'H]NAD<sup>+</sup> (11 cpmpmol). Histones were not added in all reaction mixtures because the purified enzyme could synthesize poly(ADP-ribose) without histones (8, 25, 26). The mixture was incubated at 25°C for 10 min, and the reaction was terminated by the addition of 2 ml of ice-cold 10% trichloroacetic acid. The acid-insoluble material was collected on a glass fiber filter and the radioactivity was counted by a liquid scintillation spectrometer as described previously (27). One unit of the enzyme activity was defined as being equivalent to 1 nmol of ADP-ribose incorporated into acid-insoluble material/min under the described conditions. The apparent Km for DNA was obtained from the double reciprocal plots of enzyme activity versus DNA concentration as previously described (8).

SDS-Polyacrylamide Gel Electrophoresis

The gel electrophoresis and staining of protein bands with amido black were performed according to the method of Hayashi and Ohba (28). Protein samples were prepared by heating at 100°C for 2 min in 1% SDS, containing 5% β-mercaptoethanol. Electrophoresis was performed on 5.0 and 7.5% polyacrylamide gels containing 0.1% SDS at pH 7.2 in sodium phosphate buffer at a constant current of 8 mA/gel for 5 to 7 h. Marker proteins for molecular weight determination (bovine serum albumin, E. coli RNA polymerase subunits, and soybean trypsin inhibitor) were obtained from Boehringer.

Ultracentrifugal Analysis

All sedimentation velocity and equilibrium experiments were carried out with a Hitachi model 282 analytical ultracentrifuge.

Sedimentation Velocity—The sedimentation coefficients were calculated from the movement of the maximum of the gradient curve with the scanning systems. Before the run, all samples were dialyzed against 0.1 M sodium phosphate buffer (pH 7.4) and were centrifuged in a 12-mm single sector cell for the schlieren system or in a 12-mm double sector cell equipped with quartz windows for the photoelectric scanning apparatus at 280 nm. All measurements were done at 60,000 rpm in a RA-72-TC rotor at 20°C.

Sedimentation Equilibrium—Before the run, all samples were dialyzed against Buffer B containing 0.1 M NaCl and were centrifuged in a 12-mm double sector cell equipped with quartz windows with a 3-mm sample column height. The gradient curve was detected by the photoelectric scanning at 280 nm. The time required to reach equilibrium was reduced by using the overspeed technique of Richards et al. (29). The following sequence of speeds was used: 1) the enzyme was centrifuged for 5 h at 7,900 rpm, 2) the speed was decreased to 4,000 rpm for 15 min, 3) the speed was increased to 5,700 rpm and to equilibrium. The sample was assumed to be at equilibrium when scans taken over the space of several hours showed no more variation than that expected from experimental error. At the end of run, the speed was increased to 40,000 rpm to deplete the meniscus, then measurements were made for base-line correction. All measurements were done in a RA-72-TC rotor at 4°C. The slope of a plot of the logarithm of the optical density at 280 nm as a function of radial distance squared was determined by the method of least squares on a programmable Wang 900 calculator. The partial specific volume of the enzyme was calculated from the amino acid composition (30). Solvent densities were measured by pyknometry.

Gel Filtration and Stokes Radius Approximation

Protein samples of 3 mg/ml were applied in 2.2 ml to a Sephadex G-200 column (3.2 × 40 cm, 320 ml) previously equilibrated with Buffer A, containing 0.1 M sodium phosphate buffer, and the resultant solutions were passed through the filter. The same buffer at 4°C. Fractions of 3.6 ml were collected with a fraction collector. The Sephadex column was calibrated with the following proteins: (a) catalase (Sigma; Stokes radius, 52 Å), (b) yeast alcohol dehydrogenase (Sigma; 46 Å), and (c) bovine serum albumin (Armour; 38 Å). The K<sub>s</sub>, values of above protein standards were used to estimate the Stokes radius of poly(ADP-ribose) polymerase according to the method of Siegel and Monty (31).

Amino Acid Analysis

Amino acid analysis was performed by the laboratory of Dr. K. Narita (Institute for Protein Research, Osaka University, Osaka, Japan) using a Hitachi KLA-5 amino acid analyzer. The poly(ADP-ribose) polymerase samples were extensively dialyzed against 0.1 M KCl and then deionized water and hydrolyzed under reduced pressure in 6 n HCl for 24, 48, and 72 h at 110°C. Cysteine and half-cystine were determined as cysteic acid after performic acid oxidation according to the method of Schram et al. (32). Tryptophan was determined spectrophotometrically according to the method of Edelhoch (33).

Isoelectric Focusing

Isoelectric focusing of the enzyme in 10 to 30% glycerol gradient was performed with 1 KB/14 Amphioline electofocusing equipment (beta-Mercaptoethanol 0.1%) was added to the solution to stabilize the enzyme activity. The concentration of carrier Amphioline (pH range, 7 to 10) was 1.8%. The enzyme (0.9 mg) was applied and focusing was performed at 4°C for 3 days. The recovery of the enzyme activity after the run was approximately 30%.

Binding Assay

Binding of poly(ADP-ribose) polymerase to DNA was measured by a glass fiber filter assay which was employed in the study of adenovirus DNA-terminating protein complex (21). Just prior to use, filters (Whatman GF/F, 21 mm in diameter) were rinsed with 1 ml of binding buffer (25 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, and 0.1 M NaCl). For all binding experiments, varying amounts of purified poly(ADP-ribose) polymerase, as indicated in each experiment, and 0.4 µg (5,000 cpm) of H-labeled CoIE1 plasmid DNA were mixed in a final volume of 0.2 ml of the binding buffer. The reaction was started by the addition of the enzyme and incubated at 25°C for 10 min, then 1.0 ml of the chilled binding buffer was added. The diluted sample was filtered at a flow rate of 30 to 40 ml/min. The reaction tube was rinsed twice with 1 ml of the binding buffer, and the resultant solutions were passed through the filter. The collected filter was dried and counted in a toluene-based scintillation fluid. In the absence of the enzyme, less than 3% of labeled CoIE1 DNA was retained by the filter.
Physical and DNA-binding Properties

**Competitive Binding Assay**

When increasing amounts of unlabeled DNA are added to a constant amount of labeled CoEl DNA and then the enzyme is added, the unlabeled DNA competes with the labeled DNA for the enzyme, resulting in a decrease in labeled DNA-enzyme complex. This experiment was done in the presence of 0.4 μg (0.995 pmol) of DNase I-treated (10 ng/ml) CoEl [3H]DNA, 0.6 μg (4.5 pmol) of the enzyme, and varying amounts of unlabeled DNA, the concentration of which is indicated in Fig. 7. Other conditions of this binding assay are the same as described above.

**Other Methods**

RNA was estimated spectrophotometrically (34). Protein and DNA were estimated by the method of Lowry et al. (35) and Burton (36), respectively. DNA was also estimated by measuring its absorbance at 260 nm.

**RESULTS**

**Physical Properties of Poly(ADP-Ribose) Polymerase**

**Molecular Weight Determinations**—The molecular weight of the purified enzyme was determined by sedimentation equilibrium using four different concentrations of the enzyme (A260 = 0.18, 0.26, 0.28, and 0.3). In each case, the plot of log A260 versus the radial distance squared was linear, indicating a homogeneous enzyme solution. A result of the determination with the most concentrated sample is shown in Fig. 1. From the slope of the plot, the molecular weight of the enzyme was calculated to be 131,500 ± 4,500 (mean ± S.D.).

The minimum subunit molecular weight of the enzyme was determined by SDS-polyacrylamide gel electrophoresis. The calibration curve with proteins of known molecular weights showed no apparent molecular weight dependence on the gel concentration and a molecular weight value of 130,000 was obtained. This value is in good agreement with that obtained from sedimentation equilibrium data, indicating the absence of oligomer under the chosen buffer condition. Although the salt composition of the buffer was different from the enzyme salt composition, the enzyme was obtained by the above methods.

**S Value Determination**—Results from sedimentation velocity experiments are shown in Fig. 2. A symmetrical single peak was always observed, indicating a homogenous enzyme solution. The sedimentation coefficient value was dependent on the enzyme concentration and the $s_{20w}$ value obtained by extrapolation was 4.94 S.

**Stokes Radius Determination**—The Stokes radius of the enzyme was obtained by comparing the elution position of the enzyme on a column of Sephadex G-200 with those of proteins of known Stokes radii and was estimated to be 59 Å.

From the Stokes radius and $s_{20w}$ obtained by the above determinations, the molecular weight of the enzyme was calculated to be 127,000 using the following equation (31):

$$M_c = \frac{6\pi n\eta s}{(1 - c\varphi)}$$

where $M_c$ = molecular weight, $s$ = Stokes radius, $s$ = sedimentation coefficient, $\eta$ = partial specific volume, $n$ = viscosity of medium, $\rho$ = density of medium, and $N$ = Avogadro’s number.

From the $s_{20w}$ and molecular weight determined by equilibrium centrifugation, the frictional ratio ($f/f_{in}$) of the enzyme was calculated to be 1.81 using the following equation (37):

$$f/f_{in} = \frac{(4/3)^{1/2} - 1 - c\varphi}{6\pi n(\pi N)^{1/2}} M_c^{1/2} \left(1 - \frac{c\varphi}{N}ight)$$

**Amino Acid Analysis and pl**—The amino acid composition of the enzyme is shown in Table I. In addition to these amino acids, a small unidentified peak was found just before marker glucosamine on the short column used for the separation of basic amino acids. The lysine content was the highest in this protein and the isoelectric focusing data showed a single peak of the enzyme activity at pH 9.8, indicating the basic nature of the enzyme.

Physical properties of the enzyme described above are summarized in Table II.

**Enzyme Activation and Binding Properties of DNA**

As previously described (13), the enzyme is tightly bound to chromatin in cells and, when isolated and purified, it definitely requires DNA not as a substrate (8, 26) but as an enzyme activator with an unknown mechanism. Recently, the activity of a partially purified enzyme is reported to be enhanced in response to DNA strand breaks (19). The activity of DNA polymerase using an analytical ultracentrifuge. $s_{20w}$ of the enzyme is plotted as a function of the enzyme concentration. The buffer and procedure are described under "Materials and Methods." The enzyme had a starting $A_{260}$ of 0.3.

**Fig. 1 (left). Sedimentation equilibrium of poly(ADP-ribose) polymerase.** Optical density of a logarithmic scale is plotted as a function of the square of radial distance in centimeters. The buffer and procedure are described under "Materials and Methods." The enzyme had a starting $A_{260}$ of 0.3.

**Fig. 2 (right). Sedimentation constants of poly(ADP-ribose) polymerase using an analytical ultracentrifuge.** $s_{20w}$ of the enzyme is plotted as a function of the enzyme concentration. The experimental procedure is described under "Materials and Methods."○, UV scanner experiment; ●, schlieren experiment.

**Table I**

| Amino acid composition of poly(ADP-ribose) polymerase | mol/100 mol of all amino acids |
|------------------------------------------------------|-------------------------------|
| Lysine                                               | 11.87                         |
| Histidine                                            | 1.96                          |
| Arginine                                             | 2.76                          |
| Tryptophan                                           | 1.02                          |
| Aspartic acid, asparagine                            | 9.29                          |
| Throneine                                            | 4.79                          |
| Serine                                               | 7.58                          |
| Glutamic acid, glutamine                             | 11.11                         |
| Proline                                              | 4.83                          |
| Glycine                                              | 7.46                          |
| Alanine                                              | 6.38                          |
| Half-cystine                                         | 2.25                          |
| Valine                                               | 6.78                          |
| Methionine                                           | 2.31                          |
| Isoleucine                                           | 4.71                          |
| Leucine                                              | 8.67                          |
| Tyrosine                                             | 3.00                          |
| Phenylalanine                                        | 3.23                          |

"Average of 24-, 48-, and 72-h hydrolysis times, except as noted below.

* Determined separately as described under "Materials and Methods."

* Extrapolated to zero hydrolysis time.

* Represented by 72-h hydrolysis time.
of our purified bovine thymus enzyme was also increased by DNase I or DNase II treatment of calf thymus DNA (38). To determine further the interaction between the enzyme and DNA, we analyzed the formation of the enzyme-DNA complex by its retention on glass fiber filters and correlated the complex formation with poly(ADP-ribose) synthesis. In order to avoid structural ambiguity in high molecular DNA of animal origin and inhibitory activity of single-stranded DNA (8), covalently closed circular molecules of CoIE1 plasmid DNA and its controlled DNase I-digested products were used as a standard DNA in the present experiment.

Enzyme Activating Ability of CoIE1 DNA—As shown in Fig. 3, closed circular CoIE1 DNA which has no single-stranded breaks (nicks) shows essentially no enzyme-activating ability. When this DNA is digested mildly with increasing amounts of DNase I, the enzyme-activating ability of the DNA increases (Fig. 3) and the initial part of the response is linear to the number of nicks on the DNA (Fig. 4).

Binding Characteristics—In the binding assay used in this experiment, the composition of the buffer was the same as that of the enzyme assay except for the absence of NAD+ and the inclusion of 0.1 M NaCl, as described later. Addition of NAD+ reduced the binding efficiency to some extent, probably through the formation of the product. The maximum formation of the complex was attained within 1 min of incubation at 25°C, and the complex was detected even when the reaction mixture was incubated at 0°C.

As shown in Fig. 5, whereas free [H]-labeled CoIE1 DNA is not retained on glass fiber filter, the DNA-enzyme complex is. When increasing amounts of the enzyme are added to a solution of CoIE1 [3H]DNA, an increasing proportion of the DNA is retained on the filter. The efficiency of retention of the complex with nicked CoIE1 DNA (DNase I-treated CoIE1 DNA) is higher than that with closed circular CoIE1 DNA, but the higher efficiency decreased to the level which was attained by the complex with closed circular CoIE1 DNA when the enzyme was heat-inactivated (65°C, 15 min).

Effect of NaCl—The enzyme is bound to chromatin in vitro and can be easily separated from chromatin by a buffer containing 0.3 M NaCl. In the enzyme purification step, the enzyme bound to DNA-cellulose was dissociated by a buffer containing 0.5 to 1.0 M NaCl (8). If the binding of the enzyme to nicked DNA is a prerequisite for the enzyme to be catalytically active, the addition of NaCl to the nicked DNA-enzyme complex may eventually lead to a loss of the enzyme activity accompanying the dissociation of the complex. Fig. 6 shows that both the activity and the binding efficiency of the enzyme decrease approximately in a parallel manner when the concentration of NaCl is increased. It is also noted that the binding of the enzyme to closed circular CoIE1 DNA is catalytically active and is thus nonspecific. The specificity of the binding is clear at 0.1 M NaCl, where the binding efficiency of the enzyme to closed circular CoIE1 DNA is much less than that to nicked CoIE1 DNA. Thus, in routine binding assay, 0.1 M NaCl was always added to the binding mixture. These results support the notion that the specific binding of the enzyme to nicked DNA is a prerequisite for the enzyme-activating process.

Competitive Binding Assay—If our binding assay could detect the formation of a catalytically active enzyme-DNA complex, the enzyme-activating efficiencies of various double-stranded DNAs should be well correlated with their enzyme-binding efficiencies since various double-stranded DNAs, including their DNase I-digested products, activate the enzyme with varying efficiencies.

The formation of complexes between the enzyme and var-
various DNAs was tested by a competitive binding binding assay. As shown in Fig. 7, when increasing amounts of unlabeled DNAs are added to 0.4 μg of DNase I-digested (10 ng/ml) ColEI [H]DNA and then 0.6 μg of the enzyme is added, the unlabeled DNA competes with the labeled DNA for the enzyme, resulting in a decrease in the labeled DNA-enzyme complex.

The results obtained also indicate that all unlabeled DNAs are not equally effective competitors. Relative amounts of various unlabeled DNAs necessary to reduce the filter-bound counts to 50% of control counts obtained in the absence of the unlabeled DNA were used to estimate the efficiencies of the binding of the enzyme to the DNAs (Table III). To a fairly good approximation, these amounts reflect the efficiency of DNA in the formation of a catalytically active enzyme-DNA complex because the enzyme-binding efficiencies of various double-stranded DNAs are reasonably correlated with their enzyme-activating efficiencies. Although active DNA shows high enzyme-binding and enzyme-activating efficiencies, similar high efficiencies are achieved by extensive treatments of ColEI DNA and calf thymus DNA with DNase I (100 ng/ml).

The activation of the enzyme can also be introduced by the addition of full length linear ColEI DNA produced by EcoRI restriction endonuclease (Table III, Line 6). This observation supports the fact observed first by Benjamin and Gill (19) that some of the DNA ends activate the enzyme. Our previous kinetic studies (8) suggested that the strong inhibitory effect of denatured DNA on the enzyme activity may be due to its high affinity with the enzyme, resulting in easy formation of an abortive enzyme-denatured DNA complex. This prediction is confirmed directly in the competitive binding assay (Table III) in which heat-denatured DNA is shown to be the strongest competitor among various DNAs used in this experiment.

Effect of Various Inhibitors—Table IV shows that heparin has a strong inhibitory effect on both the binding of the enzyme to nicked ColEI DNA and the enzyme activity. Similar inhibitory effects are seen in the presence of an excess amount of ribosomal RNA or of closed circular ColEI DNA. The inhibitory effects of closed circular ColEI DNA are another indication of an abortive binding of the enzyme to this DNA.

Thymidine and nicotinamide inhibit the enzyme reaction competitively with NAD⁺ (39, 40). N-Ethylmaleimide (8) and theophylline (41, 42) also inhibit the enzyme activity. As shown in Table IV, at the enzyme-inhibitory concentrations, these inhibitors show little effect on the binding efficiency.

**TABLE III**

| DNA Type          | Relative enzyme-binding efficiency* | Relative enzyme-activating efficiency |
|-------------------|-------------------------------------|--------------------------------------|
| ColEI DNA         |                                     |                                      |
| Untreated (closed circular) | 0.13                                | 0.004                                |
| DNase I-treated   |                                     |                                      |
| (2 ng/ml of DNase I) | 0.46                                | 0.34                                 |
| (10 ng/ml of DNase I) | 1.0                                 | 1.0                                  |
| (100 ng/ml of DNase I) | 4.3                                 | 4.2                                  |
| EcoRI-treated     |                                     |                                      |
|                   | 0.46                                | 0.19                                 |
| Calf thymus DNA   |                                     |                                      |
| Untreated         | 1.6                                 | 0.16                                 |
| DNase I-treated   |                                     |                                      |
| (100 ng/ml of DNase I) | 2.8                                 | 2.2                                  |
| Heat-denatured    | 12.8                                | 0.004                                |
| Active DNA        | 4.3                                 | 6.7                                  |

* The efficiency was calculated from the data in Fig. 7 and is represented as the reciprocal value of the relative amount of unlabeled DNA to reduce the filter-bound counts to 50% of control counts obtained in the absence of the unlabeled DNA.

**FIG. 6.** Effect of NaCl on the efficiency of binding of poly(ADP-ribose) polymerase to [H]-labeled ColEI DNA and of the enzyme activity. The binding was determined as described under “Materials and Methods” except that NaCl was added in the binding buffer as indicated and the amount of the enzyme was 0.4 μg (3 pmol). The assay of the enzyme was described as described under “Materials and Methods,” except that NaCl was added in the assay mixture as indicated and 0.1 μg of the [H]-labeled ColEI DNA was used. ○, the efficiency of the binding with DNase I-treated (10 ng/ml) ColEI [H]DNA; □, that with closed circular ColEI [H]DNA. The binding efficiency of the enzyme to the DNase I-treated ColEI [H]DNA was set at 100%. ▲, enzyme activity by DNase I-treated ColEI [H]DNA; ■, enzyme activity by closed circular ColEI [H]DNA. The enzyme-activating efficiency by the DNase I-treated ColEI [H]DNA in the absence of NaCl was set at 100%.

**FIG. 7.** Competitive binding assay: competition of various DNAs with DNase I-treated (10 ng/ml) ColEI [H]DNA for poly(ADP-ribose) polymerase. The amount of unlabeled DNA as indicated was mixed with 0.4 μg (0.095 pmol) of DNase I-treated (10 ng/ml) ColEI [H]DNA and 0.6 μg (4.5 pmol) of the enzyme was added. The procedure is described under “Materials and Methods.” ——X, closed circular ColEI DNA; ——O, DNase I-treated (2 ng/ml) ColEI DNA; ——□, DNase I-treated (10 ng/ml) ColEI DNA; ——△, EcoRI-treated ColEI DNA; ▲—▲, calf thymus DNA; ●—●, DNase I-treated (100 ng/ml) calf thymus DNA; ●—○, heat-denatured calf thymus DNA; ○—○, active DNA.
**TABLE IV**

| Additions          | Enzyme activity<sup>a</sup> | Binding efficiency<sup>b</sup> |
|--------------------|-----------------------------|-------------------------------|
| None               | 100                         | 100                           |
| Heparin            | 0.005 μg                    | 75                            |
|                    | 0.05 μg                     | 0                             |
| Ribosomal RNA      | 1 μg                        | 93                            |
|                    | 10 μg                       | 81                            |
| ColEl DNA (closed circular) | 0.5 μg       | 88                            |
|                    | 4 μg                        | 73                            |
| Thymidine          | 10 μM                       | 30                            |
|                    | 1 mM                        | 1                             |
| Nicotinamide       | 10 μM                       | 68                            |
|                    | 1 mM                        | 2                             |
| Theophylline       | 10 μM                       | 77                            |
|                    | 1 mM                        | 8                             |
| N-Ethylmaleimide   | 10 μM                       | 79                            |
|                    | 1 mM                        | 6                             |

<sup>a</sup> The enzyme activity was assayed as described under "Materials and Methods" except that various inhibitors were added to the assay mixture as indicated and 0.1 μg of unlabeled DNase I-treated (10 ng/ml) ColEl DNA was used.

<sup>b</sup> The binding efficiency was determined by the same method as the competitive binding assay described under "Materials and Methods" except that various inhibitors were added instead of unlabeled DNA.

We previously reported (44) a higher enzyme-activating efficiency for poly(d(A))-poly(d(T)) than for poly(d(G))-poly(d(C)) as an indication of sequence preference in the enzyme activation. However, this notion seems to be incorrect because the number of nicks or ends per unit weight of the poly(d(G))-poly(d(C)) (about 13 S) is much lower than that of the poly(d(A))-poly(d(T)) (about 5 S), mainly owing to different preparative procedures. Therefore, the difference in enzyme-activating efficiencies of these DNAs is largely due to the difference in the number of nicks or ends. High enzyme-activating and enzyme-binding ability of active DNA may also be attributable to its small $e_{\text{vis}}$ of 6 S and a high content of nicks.  

**DISCUSSION**

As shown in Table II, poly(ADP-ribose) polymerase from bovine thymus has a molecular weight of approximately 130,000 and is composed of a single polypeptide. Precise structure of activated enzyme bound to DNA is, however, not clear. A high fractional ratio of 1.81 of the enzyme calculated from the molecular weight and the $e_{\text{vis}}$ suggests a marked degree of hydration of the protein, or pronounced nonspherical molecular asymmetry, or both. Our previously reported (8) overestimation of the molecular weight (150,000 to 160,000) determined by gel chromatography according to the method of Andrews (43) may be due to the hydrodynamic property of the molecule.

Recently, poly(ADP-ribose) polymerase has been purified from various tissues by several investigators. The molecular weights of these purified enzymes, determined by SDS-polyacrylamide gel electrophoresis, are 120,000 for the calf thymus enzyme by Mandel et al. (9), 130,000 for the enzyme of Ehrlich ascites tumor cells by Ikeda, Nara Medical University, for their valuable comments and support concerning the analytical ultracentrifugal analysis; Dr. H. Toda and Ms. Y. Yagi, Institute for Protein Research, Osaka University, for carrying out the amino acid analysis; and Dr. Takai, Kobe University, for helping us to perform isoelectric focusing. We are also indebted to Mr. T. Sako, Yakult Institute for Microbiological Re-

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<sup>a</sup> K. Yoshihara, unpublished results.

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**Physical and DNA-binding Properties**

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