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HIGHLY EFFECTIVE DNA IN STIMULATING POLY-(ADP-RIBOSE) POLYMERASE REACTION

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SUMMARY

During the purification of bovine thymus poly(ADP-ribose) polymerase, a fraction was obtained, which is 20 times as effective in activating the enzyme as the bulk of calf thymus DNA. The method for the isolation and purification of this active fraction is described. The results obtained by chemical analysis, UV-spectrophotometry, and ultracentrifugation of the active fraction indicate that the active substance is double stranded DNA. We suggest that the high enzyme-activating ability of the DNA is due to a DNA sequence(s), concentrated on the DNA, with a high enzyme-binding affinity.

Poly(ADP-ribose) polymerase catalyzes the successive transfer of ADP-ribose units from NAD^+ to histones and other nuclear proteins, synthesizing acceptor-bound poly(ADP-ribose) in the cell nuclei (1-5). The enzyme is tightly associated with chromatin and when purified, absolutely requires double-stranded DNA for the activity (6).

During a purification of the enzyme from bovine thymus, a DNA fraction which activates the enzyme 20 times more efficiently than the bulk of calf thymus DNA was obtained from a hydroxylapatite column chromatography (7).

In this paper we will describe the purification method and the results obtained from the kinetic, physical, and chemical study of this DNA fraction.

MATERIALS AND METHODS

Materials [Adenine-2,8-^{3}H]NAD^+ was purchased from New England Nuclear, Boston, USA. NAD^+(grade 5), calf thymus whole histones(type 2) and calf thymus DNA(type 1, Lot 93c9501-95 and 26c9560) were products of Sigma. DNAs of ColE1-Ap^r plasmid RSF2124 (8); closed circular and its EcoR1 digested linear forms were gifts from Mr. Tomoyuki Sako (Yakult Institute for Microbiological Res., Tokyo). Sheared DNA was prepared by passing calf thymus DNA(Lot 26c9560) through a hypodermic needle for several times. Sonicated DNA was prepared from calf thymus DNA(Lot 26c9560) by treating the DNA solution with a Kaijo-Denki Ultrasonifier at the maximum power for 20 min with interrupted cooling and passing through a Chelex 100 column to remove metal ions. DNase 1 treated calf thymus DNA(Lot 26c9560) was prepared by the method of Meyer and Simpson (9).

Purification and assay for poly(ADP-ribose) polymerase The purification and assay for bovine thymus poly(ADP-ribose) polymerase was described previously (7). The enzyme activity was expressed by incorporation of ^{3}H-ADP-ribose (cpm) into acid-insoluble material.

Abbreviations: SDS; sodium dodecyl sulfate, SSC; standard saline-sodium citrate buffer.

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Assay for enzyme-activating activity of DNA The reaction mixture contained 25 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 μM [adenine-2,8-^{3}H]NAD^+ (25 cpm/pmol), 0.1 μg of purified bovine thymus poly(ADP-ribose) polymerase, and varying concentration of DNA tested. The reaction mixture was incubated at 25° C for 10 min. Other procedures were performed as in assay for the enzyme. The enzyme activity is approximately proportional to the concentration of DNA in the reaction mixture in the range between 0 and 20 % saturation of the enzyme activity. Thus the enzyme-activating activity of DNA was expressed as the enzyme activity (cpm) at a fixed concentration of DNA under the assay condition.

SDS-polyacrylamide gel electrophoresis The gel electrophoresis and staining of protein bands were performed according to the method of Hayashi and Ohba (10). DNA was stained with Stainsall after the removal of SDS by electrophoresis in 7 % acetic acid by the method of Dahlberg et al. (11).

RESULTS

Preparation of active DNA Poly(ADP-ribose) polymerase was extracted from bovine thymus gland and purified to homogeneity by selective precipitation with ammonium sulfate and column chromatographies on DNA-cellulose, hydroxylapatite, and Sephadex G-200, successively. When two steps linear gradient elution (0-50, and 50-300 mM potassium phosphate buffer, pH 7.4) was applied at the step of hydroxylapatite column chromatography, the enzyme eluted at 30 mM phosphate buffer and, on further elution, a fraction with an absorbance at 280 nm emerged at 150 mM phosphate buffer (7). The latter did not show the enzyme activity by itself alone, but had a very high enzyme-activating efficiency when added to the reaction mixture in place of DNA and histones. Since this fraction had an UV-absorption maximum at 260 nm and the ratio of E_{260}/E_{280} was 1.3-1.5, we tried to purify the active substance according to a method for purifying nucleic acids as follows. This fraction was collected, combined, and dialyzed against 2 liters of 1/5 x SSC overnight. After the addition of two volumes of absolute ethanol, the sample was kept at -20° C overnight. The resultant sediment was collected by centrifugation, dissolved in 4 ml of 1/5 x SSC and dialyzed against the same buffer. The sample was extracted with an equal volume of a phenol mixture (70 ml of m-cresol, 55 ml of water, 0.5 g of 8-hydroxylquinoline, and 500 g of phenol) according to the method for isolating nucleic acids by Parish (12). The aqueous phase was dialyzed against 2 liters of 1/5 x SSC overnight and mixed with two volumes absolute ethanol. After keeping the sample at -20° C overnight, the precipitates were collected by centrifugation, dissolved in a small volume of 1/5 x SSC and kept at -20° C. This method yielded 1-2 mg of DNA from 800 g of bovine thymus. The specific activity of the DNA to support the enzyme reaction did not change during the procedure described above.

Since the DNA fraction has a much higher efficiency than the bulk of calf thymus DNA in stimulating the enzyme reaction, we designate the DNA "active DNA" in the present report.

Physical and chemical analysis of active DNA Buoyant density of active DNA was examined by CsCl density equilibrium centrifugation. As shown in Fig. 1, the material gave a single symmetrical peak of absorbance at 260 nm at a density of 1.704. The enzyme-activating activity was found to be distributed parallel to the optically observed peak.

The UV-absorption spectrum showed the characteristic profile of DNA with an absorption maximum at 260 nm and an E_{260}/E_{280} ratio of 1.6 (data not shown).

The analysis of base composition showed that the content of adenine is approximately equal to that of thymine, and guanine is equal to cytosine, giving an approximate purine/pyrimidine ratio of 1.0 (table 1). The G:C content was 43 %. Spots other than these four bases were undetected on the analysis by paperchromatography.

The Burton's diphenylamine reaction was strongly positive and the ratio of deoxyribose to bases (estimated from the absorbance at 260 nm) was approximately equal to that of calf thymus DNA. The purified material contained negligible amount of protein judging from the protein staining after SDS-polyacrylamide gel electrophoresis.

Kinetic properties of active DNA The saturation of the enzyme activity was examined by increasing the concentration of DNA in the reaction mixture. In spite of relatively small differences in buoyant density and base composition, active DNA showed a much higher enzyme-activating efficiency than the bulk of calf thymus DNA (Fig. 2). When the enzyme-activating efficiency was compared by determining the concentration of various DNAs required for 10 % saturation of the enzyme activity (the enzyme activity saturated with active DNA was set at 100 %), the DNA concentrations were 1 ng and 20-100 ng/0.2 ml for active DNA and other DNAs, respectively (Fig. 2). The fact that high activity of the DNA is abolished by heating to 100° C and rapidly cooling the DNA indicates that the double-stranded structure of the DNA is a prerequisite for the maintenance of its high enzyme-activating activity.

The fact that active DNA could be isolated at the relatively late stage of purification of the enzyme indicates that the DNA is preserved in the crude enzyme preparation by binding to the enzyme even during DNA-eliminating process such as DNA-cellulose column chromatography. When the molecular size was examined by sucrose gradient centrifugation, active DNA gave a relatively broad peak of 3-9 S centered at approximately 6 S.

In order to see whether mere random scission of calf thymus DNA do yeild active DNA, calf thymus DNA was fragmented by DNase 1 treatment, sonication, and shearing, giving the size of 8 S, 8 S, and 10 S, respectively. As seen in Fig. 2, the enzyme-activating activity of calf thymus DNA was not affected greatly by these treatments. The result indicates that the enzyme-activating

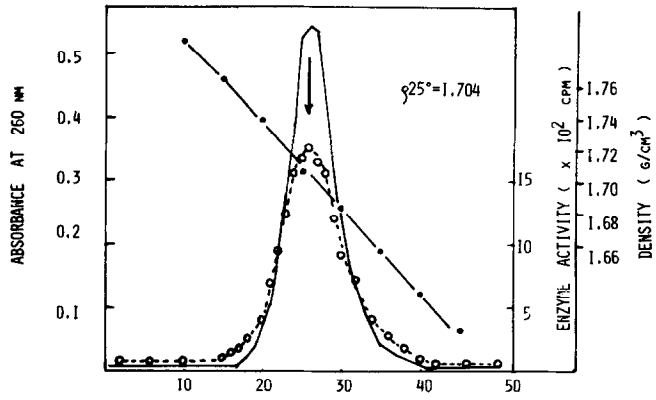

Fig. 1. CsCl density gradient equilibrium centrifugation of active DNA
Active DNA (345 μg) was centrifuged at 30,000 rpm for 89 hours at 10° C in 4.2 ml of CsCl solution ($\rho^{25°}$=1.683) in a SW 40 roter using a Hitachi model 65P ultracentrifuge. After the run, a hole was pierced at the bottom of the tube and six-drop fractions were collected sequentially. Density of each fraction was measured by refractometry (●—●). The sample was diluted with 1.5 ml of 1/5 x SSC and the sample was assayed for absorbance at 260 nm (———) and for enzyme-activating activity (O---O) as described in Methods. For an assay of enzyme-activating activity, 0.2 μl of each diluted sample and 0.2 μg of the purified enzyme were used. The arrow in the figure indicates the point of $\rho^{25°}$=1.704.

Table 1. Base composition of active DNA

base	adenine	thymine	guanine	cytosine
mol %	29.4	27.8	21.8	21.0

Base composition was analyzed according to the method described by Bendich (16). An amount of 140 μg of active DNA was hydrolyzed in a sealed glass tube with formic acid and bases were separated by two-dimensional paper chromatography with solvent systems; (1) iso-propanol: 6 N HCl (68:33.5), and (2) conc. HCl: water: methanol (20:10:70).

ability of DNA does not depend on its size, intrastrand nicks nor number of the terminal. This conclusion is strengthened by the fact that a closed circular plasmid DNA (RSF 2124) supports the enzyme activity as equally as its linear whole-length counterpart (an EcoRI product of RSF 2124), as shown in Fig. 2 and table 2.

As previously described (7), the enzyme reaction supported with DNA contaminated with a relatively small portion of denatured DNA is stimulated by the addition of whole histones at a histone/DNA ratio of 1-2 by weight, probably by its masking of the inhibitory effect of single stranded DNA. As shown in table 2, this histone-effect was very slight in the active DNA-supported enzyme reaction. The result indicates that active DNA has no appreciably

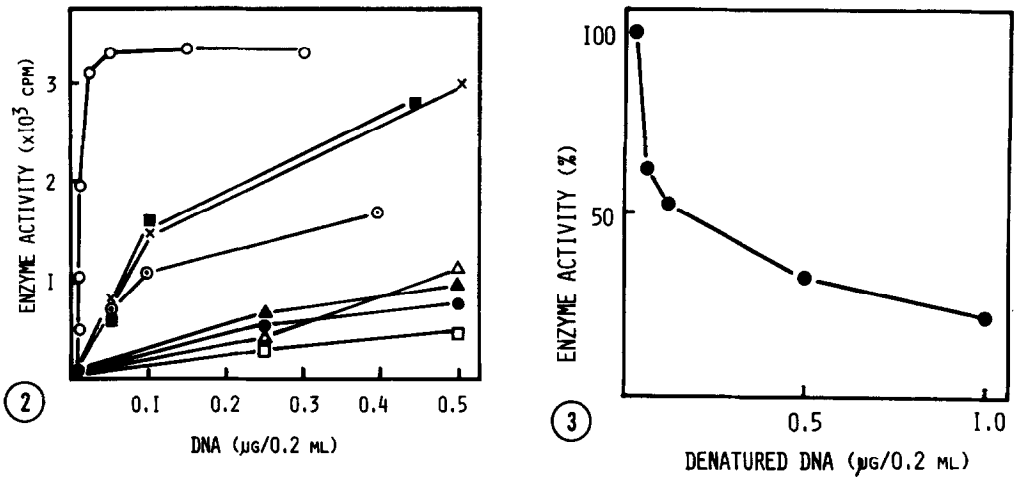

Fig. 2. Activation of enzyme with various DNA Assay for enzyme-activating activity of DNA was performed as described in Methods. The indicated concentration of active DNA (○—○), calf thymus DNA (Lot 93c9501-95; ⊙—⊙, Lot 26c9560; ●—●), sonicated DNA (△—△), sheared DNA(▲—▲), DNase I treated DNA (□—□) and RSF 2124 DNA (covalently closed circular; ×—×, and linear; ■—■) were used. The preparation of these DNAs and their S_{w}^{20} values are given in Methods and in text, respectively.

Fig. 3. Effect of denatured DNA The activity of the enzyme (0.1 μg) was assayed in the presence of 1.0 μg of active DNA and the indicated concentration of denatured calf thymus DNA, as described in Methods. With the concentration of active DNA used, the enzyme activity reaches a saturated level where the activity is set at 100 %. Denatured calf thymus DNA was prepared from Lot 26c9560 DNA by heating the DNA solution in 1/10 x SSC at 100° C for 10 min and quickly cooling to 0° C.

Table 2. Effect of histones

DNA used		enzyme activity (cpm) concentration of histones (μg/0.2 ml) 0	2	20
calf thymus DNA	(2 μg)	1,468	4,008	660
active DNA	(1 μg)	4,312	4,419	4,171
RSF 2124 (ccc)	(1 μg)	4,853	4,507	1,072
RSF 2124 (linear)	(1 μg)	4,540	4,562	---

The enzyme reaction was performed as described in Methods with and without calf thymus whole histones and in the presence of various DNAs. The concentrations of DNAs used are sufficient to saturate the activity of the enzyme used (0.1 μg per assay).
ccc; covalently closed circular DNA.

denatured portion in the molecule as is the case in closed circular plasmid DNA (RSF 2124) and its EcoR1 digested linear form.

When the histone/DNA ratio was increased to more than 10, histones strongly inhibited the reaction. This inhibitory effect of histones was however very slight in the enzyme reaction supported with active DNA (table 2).

The enzyme activity supported with active DNA was also inhibited by the addition of relatively small amount of denatured DNA (Fig. 3). The enzyme activity was inhibited by 50 % by the addition of 10 % of denatured DNA. This inhibition could be restored by adding histones in the reaction mixture (data not shown).

DISCUSSION

The present study has shown that a DNA fraction obtained from a purification stage of bovine thymus poly(ADP-ribose) polymerase highly stimulates this enzyme reaction. When the enzyme-activating efficiency of active DNA was compared to that of several different batches of calf thymus DNA from Sigma, the former gave a value 20-50 times more effective than the latter. Although the precise basis for the high enzyme-activating efficiency has not been clarified yet, it is shown that the high efficiency is due to neither its small molecular size, the terminal structure, the internal nicks in the DNA strand, nor contamination of some protein in the preparation. In spite of relatively small differences in the base composition and buoyant density between active DNA and the bulk of calf thymus DNA, the reaction supported by the former was remarkably different from the latter in the response to histones. In our previous study (13), with synthetic deoxyhomopolynucleotides, the enzyme from rat liver showed preference for some base sequences, though not perfectly specific. Therefore, the high enzyme-activating efficiency of active DNA may be interpreted as that this DNA is rich in a unique sequence(s) where the enzyme can bind with high efficiency and be activated. In this regards we can estimate the number of the enzyme-binding site on an active DNA molecule. Since the curve in Fig. 2 is essentially a titration of the enzyme present with DNA as previously described (7), the DNA/enzyme ratio required for activation of the enzyme is obtained. Based on the value, the number of the enzyme molecule which is expected to be bound to an active DNA molecule of 2×10^5 dalton (300 base pairs) is estimated to be 15 on average (this value is a maximum estimate since the calculation was made on an assumption that all enzyme molecules in our enzyme preparation were active). Since the numbers estimated for two lots of calf thymus DNA, 93c9501-95 and 26c9560, were 0.7 and 0.3 per 300 base pairs, respectively, the enzyme-activating site on active DNA may be more than 20 times denser than that on the bulk of calf thymus DNA.

Our recent binding studies with the enzyme and DNA using membrane filter also revealed that active DNA has a much higher enzyme-binding capacity than the bulk of calf thymus DNA (14).

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