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INHIBITION OF DNA POLYMERASE α, DNA POLYMERASE β, TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE, AND DNA LIGASE II BY POLY(ADP-RIBOSYL)ATION REACTION IN VITRO#

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Incubation of DNA polymerase α,[1] DNA polymerase β, terminal deoxynucleotidyl transferase, or DNA ligase II in a reconstituted poly(ADP-ribosyl)ating enzyme system markedly suppressed the activity of these enzymes. Components required for poly(ADP-ribose) synthesis including poly(ADP-ribose) polymerase, NAD^+, DNA, and Mg^{2+} were all essential for the observed suppression. Purified poly(ADP-ribose) itself, however, was slightly inhibitory to all of these enzymes. Furtheremore, the suppressed activities of DNA polymerase α, DNA polymerase β, and terminal deoxynucleotidyl transferase were largely restored (3 to 4-fold stimulation was observed) by a mild alkaline treatment, a procedure known to hydrolyze alkaline-labile ester linkage between poly(ADP-ribose) and an acceptor protein. All of these results strongly suggest that the four nuclear enzymes were inhibited as a result of poly(ADP-ribosyl)ation of either the enzyme molecule itself or some regulatory proteins of these enzymes.

Poly(ADP-ribose) polymerase, a nuclear enzyme, is activated by binding to a nick or an end of DNA strand (1) and catalyzes the transfer of ADP-ribose portion of NAD^+ to chromatin-bound proteins (2) including the polymerase molecule itself (3). Accumulating evidence (4) suggests that this enzyme is involved in DNA repair in the eukaryotic cell nuclei although the exact mechanism has not yet been clarified. Recent studies elucidated that some nuclear

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[1] Abbreviations used are: DNA pol α-primase; DNA polymerase α with primase activity, DNA pol β; DNA polymerase β, and TDT; terminal deoxynucleotidyl transferase.

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enzymes, Ca^{2+}, Mg^{2+}-dependent endonuclease (5, 6) and DNA topoisomerase I (7) are poly(ADP-ribosyl)ated by the polymerase and that the modification reaction markedly inhibits the activity of the modified enzymes. RNA polymerase I (8) and DNA ligase II (4) are also suggested to be poly(ADP-ribosyl)ated *in vivo*, although the latter enzyme seems to be activated as a result of ADP-ribosylation (9). Recently we have established a reconstituted poly(ADP-ribosyl)ating enzyme system to examine the ADP-ribose accepting ability of various proteins and enzymes *in vitro* (6, 10). In an effort to detect these acceptors in nuclear enzymes, we found that various nuclear enzymes involved in DNA metabolism could be markedly inhibited under appropriate reaction conditions in a reconstituted poly(ADP-ribose) synthesizing enzyme system. The results suggest a possibility that poly(ADP-ribose) polymerase regulates many nuclear enzymes upon a cellular DNA damage.

MATERIALS AND METHODS

Materials A nearly homogeneous preparation of bovine thymus poly(ADP-ribose) polymerase (95% purity) was purified as described previously (11). Bovine thymus DNA polymerase α was partially purified through a successive chromatography on phosphocellulose and DEAE-Sephadex A50 column. Upon DEAE-Sephadex A50 column chromatography, DNA polymerase α activity was separated into two peaks, the one with primase activity (12) and the other without primase: DNA polymerase α fraction with primase activity (DNA pol α-primase[1]) was used through this study unless otherwise indicated. DNA polymerase β (DNA pol β), terminal deoxynucleotidyl transferase (TDT), and DNA ligase II were purified by the methods of Chang (13), Yoshida and Nakamura (14), and Teraoka *et al.* (15), respectively. The purity of DNA pol β, TDT, and DNA ligase II was approximately 10, 90, and 20 %, respectively, judging from SDS-polyacrylamide gel electrophoresis of the prepared enzyme samples.

Calf thymus DNA was obtained from Sigma and NAD^+ from Böhringer Mannheim, West Germany.

Reconstituted poly(ADP-ribosyl)ating enzyme system The reaction mixture contained 5 mM Tris-HCl buffer, pH8.0, 1 mM dithiothreitol, 2 mM NAD^+, 10 μg/0.2 ml calf thymus DNA, 10 mM $MgCl_2$, 5 μg of purified poly(ADP-ribose) polymerase, and an appropriate amount (0.65 to 12 μg) of various nuclear enzymes to be examined in a total volume of 0.2 ml. The reaction was carried out for 40 min at 25°C. The reaction was terminated either by chilling the samples on ice or by the addition of a final concentration of 50 mM nicotinamide as indicated. In some experiments, NAD^+ concentration of the reaction mixture was varied as indicated.

Enzyme assay The activities of DNA pol α and DNA pol β were assayed by the methods described by Matsukage (16). TDT and DNA ligase II activities were assayed by the method of Yoshida *et al.* (14) and Teraoka and Tsukada (17), respectively.

Alkaline-treatment of enzyme Enzyme sample was mixed with a half volume of the alkaline buffer containing 0.2 M glycine-NaOH buffer, pH 10, 50% glycerol, 1 mM EDTA, 200 μg/ml of bovine serum albumin, 50 mM $NaHSO_3$, 1 mM dithiothreitol and 1 mM glutathione. The mixture was incubated at 25°C for the indicated time.

RESULTS

Inhibition of various nuclear enzymes by poly(ADP-ribosyl)ation reaction in vitro. When DNA pol α-primase, DNA pol β, TDT, and DNA ligase II were

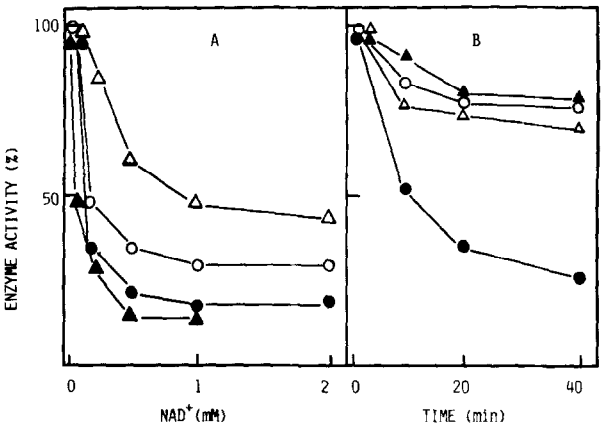

Fig. 1. Inhibition of various nuclear enzymes by poly(ADP-ribosyl)ation reaction in vitro. (A) Partially purified DNA pol α-primase (12 μg ●—●), DNA polymerase β (5 μg: ○—○), highly purified terminal deoxynucleotidyl transferase (0.65 μg; ▲—▲) and DNA ligase II (5 μg; △—△) were incubated in a reconstituted poly(ADP-ribosyl)ating enzyme system as described under "Materials and Methods" except that the concentration of NAD^+ was varied as indicated. After the incubation, the samples were chilled on ice and activity of respective enzyme sample was assayed. The activity of control enzyme sample, which was incubated without NAD^+, was set at 100%. (B) DNA pol α-primase (12 μg) was incubated in a reconstituted poly(ADP-ribosyl)ating reaction mixture (●—●). From a control sample (○—○), NAD^+ was omitted. Other control samples contained either 50 mM nicotinamide (△—△) or purified poly(ADP-ribose) in place of NAD^+ (▲—▲). At the indicated time, an aliquot of each sample was taken out, the reaction was terminated by the addition of nicotinamide at a final concentration of 50 mM, and the activity was assayed.

incubated in a reconstituted poly(ADP-ribosyl)ating enzyme system, the activity of these enzymes markedly decreased with increasing concentrations of NAD^+ in the reaction mixture (Fig. 1A). At 1 mM NAD^+, the activities of DNA pol α-primase, DNA pol β, TDT, and DNA ligase II were 20, 30, 15, and 48 % of the respective control samples incubated without NAD^+: at this concentration of NAD^+, the amount of poly(ADP-ribose) synthesized was 18 to 20 nmoles (the value was determined using [^{3}H]NAD^+ under the reaction conditions described in the legend to Fig. 1A and is expressed as ADP-ribose units).

Similar inhibition could be observed also with the use of homogeneously purified DNA pol β from bovine thymus and mouse ascites hepatoma cells (18), where an approximately 80 and 50 % inhibition was observed respectively at 2 mM NAD^+. The time course of the inhibition of DNA pol α-primase is shown in Fig. 1B. The rapid inhibition of this enzyme could be blocked either by omitting NAD^+ from the reaction mixture or by adding 50 mM nicotinamide, an inhibitor of poly(ADP-ribose) polymerase (2), to the mixture.

The requirements for the observed inhibition of DNA pol α-primase by poly(ADP-ribosyl)ation are summarized in Table I. All the components required for poly(ADP-ribosyl)ation reaction (11) including poly(ADP-ribose) polymerase, NAD^+, DNA, and Mg^{2+} were essential for the inhibition of DNA pol α-primase.

Table I. Requirements for inhibition of DNA polymerase α

components added or omitted	inhibition (%)	components added or omitted	inhibition (%)
1. complete	77	5. - $MgCl_2$	0
2. - NAD^+	0	6. + nicotinamide(25 mM)	19
3. - ADPR pol.	3	7. + 3AB(2.5 mM)	25
4. - DNA	0	8. - NAD^+, + poly(ADPR)	0

Incubation of DNA pol α-primase (9 μg) in the reaction mixture for poly(ADP-ribosyl)ation and the assay of the enzyme were carried out as described under "Materials and Methods". From some control samples, either NAD^+, poly(ADP-ribose) polymerase, DNA, or $MgCl_2$ (experiment No. 2,3,4,5, respectively) was omitted. Other control samples contained inhibitors of poly(ADP-ribose) polymerase, nicotinamide (No.6) or 3-aminobenzamide (No.7), or 27 μg/0.2 ml of poly(ADP-ribose) in place of NAD^+ (No.8). The enzyme activity of a control sample incubated without NAD^+ (No.2) was set at 100% and percent inhibition was calculated based on this value.

The inhibition of DNA pol β, TDT, and DNA ligase II also showed quite similar requirements (data not shown), indicating that poly(ADP-ribosyl)ation reaction did cause the observed inhibition of these enzymes.

Purified poly(ADP-ribose) (24 nmol/0.2 ml, added to the ADP-ribosylating reaction mixture in place of NAD^+) had little effect on the activity of DNA pol α-primase (Fig. 1B) or the other enzymes (data not shown), suggesting that a modification with poly(ADP-ribose) of either the enzyme molecule itself or some enzyme-regulating protein may be the basis for the observed inhibition.

Reactivation of inhibited enzyme by a mild alkaline-treatment. It is well known that the linkage formed between poly(ADP-ribose) and a protein is a glycosidic ester linkage between ADP-ribose and a carboxyl residue of protein (19), which is extremely labile at an alkaline pH (20). Thus, a mild alkaline treatment of poly(ADP-ribosyl)ated Ca^{2+}, Mg^{2+}-dependent endonuclease and DNA topoisomerase I, resulted in reversal of the inhibition by hydrolyzing the linkage (5 , 7). As shown in Fig. 2, the suppressed DNA pol α primase activity resulting from the poly(ADP-ribosyl)ation reaction was largely restored during the mild alkaline-treatment at pH 10, while the activity of a control sample remained almost unchanged. DNA pol β and TDT, which were inhibited by poly(ADP-ribosyl)ation, also showed quite similar responses to the alkaline-treatment (data not shown), although an attempt to reactivate the inhibited DNA ligase II was unsuccessful because of the extreme lability of this enzyme in the alkaline buffer.

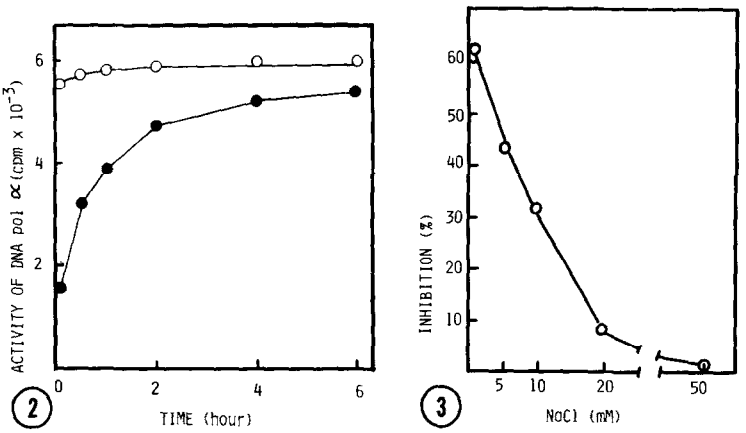

Fig. 2. Restoration of activity of DNA pol α-primase inhibited by poly(ADP-ribosyl)ation.

DNA pol α-primase (12 μg) was incubated in the mixture for poly(ADP-ribosyl)ation as described under "Materials and Methods". The sample (●—●) and a control sample incubated as above without NAD^+ (○—○) were treated with an alkaline pH as described under "Materials and Methods". At the indicated time, an aliquot of 30 μl was taken out and DNA polymerase α activity was assayed.

Fig. 3. Effect of NaCl on the inhibition of DNA pol α-primase by poly(ADP-ribosyl)ation.

DNA poly α-primase (12 μg) was incubated in a poly(ADP-ribosyl)ating system as described under "Materials and Methods" except that the mixture contained the indicated concentration of NaCl. The activity of control enzyme sample, which was incubated without both NAD^+ and NaCl, was set at 100% and percent inhibition of each sample was calculated based on this value.

Effect of ionic strength of poly(ADP-ribosyl)ating reaction mixture on the inhibition of enzymes. As shown in Fig. 3, a relatively low concentration of NaCl in poly(ADP-ribosyl)ating reaction mixture markedly decreased the extent of inhibition of DNA pol α-primase. At 10 mM NaCl, the inhibition was reduced to approximately one half of that wihtout NaCl, and, at 50 mM, the inhibition was almost completely abolished (Fig. 3). Similar effect of NaCl was also observed with TDT and DNA pol β : the extent of inhibition of these enzymes was reduced to one half at 25 mM. Increasing concentrations of 25 to 50 mM of Tris-HCl buffer, pH 8.0 in the poly(ADP-ribosyl)ating reaction mixture also markedly decreased the extent of the observed inhibition (data not shown).

DISCUSSION

The present study clearly indicates that many nuclear enzymes, which are involved in DNA metabolism, can be inhibited as a result of poly(ADP-ribosyl)-ation reaction in vitro. Ineffectiveness of purified poly(ADP-ribose) in the inhibition (Fig. 1B) and restoration of the inhibited enzyme activity by a mild alkaline treatment (Fig. 2) suggest that a covalent modification with poly(ADP-ribose) of either the enzyme molecule or some enzyme - regulating proteins is the basis for the observed inhibition. A relatively low ionic strength seems

to be essential for either the initiation or the elongation process of the modification reaction, since a low concentration of NaCl (10 to 20 mM) markedly suppressed the observed inhibition (Fig. 3). In this respect, our recent study on poly(ADP-ribosyl)ation of micrococcal nuclease (MNase) *in vitro* indicated that, whereas the elongation of poly(ADP-ribose) chain bound to the enzyme is quite limited at 25 mM Tris-HCl buffer (pH 8.0), extensive elongation occurred at 5 mM[2/].

In contrast to the observation that DNA ligase II was activated by poly(ADP-ribosyl)ation of either the enzyme molecule itself or a closely associated protein *in vivo* (4), our results indicated that the enzyme is strongly inhibited as a result of poly(ADP-ribosyl)ation reaction *in vitro*. At present, we can not reconcile the two observations.

In the present study, we examined six nuclear enzymes (DNA pol α with and without primase activity, DNA pol β, TDT, DNA ligase I and II), which are all involved in DNA metabolism and thus, possessing DNA binding affinity. With one exception (DNA ligase I[3/]), all these enzymes were markedly inhibited by poly(ADP-ribosyl)ation reaction *in vitro*. Although the occurrence of these events *in vivo* remains to be proved, the present results suggest that one biological function of poly(ADP-ribose) polymerase may be to cause the halt of chromatin metabolism at the damaged site on DNA to protect cells from abnormal metabolism in chromatin upon cellular DNA damage.

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