Activation of DNA ligase by poly(ADP-ribose) in chromatin
(nicotinamide-adenine dinucleotide/poly(ADP-ribose) synthetase/histone/DNA repair)

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ABSTRACT To elucidate the molecular mechanism by which poly(ADP-ribose) participates in DNA excision repair, we examined the effect of poly(ADP-ribose) on DNA ligase activity in DNA/histone and reconstituted chromatin systems. The ligase activity was markedly inhibited by histones; the inhibition varied depending on histone subfraction and DNA/histone ratio. Poly(ADP-ribose), either exogenous or synthesized in situ by poly(ADP-ribose) synthetase, reversed this inhibition by histone almost completely. This effect was specific for poly(ADP-ribose); polyanions such as mRNA, tRNA, rRNA, and synthetic poly(A) were less effective or ineffective. The ligase activity with reconstituted chromatin as the substrate was about half of that with free DNA whereas the activities with these two substrates were almost the same in the presence of poly(ADP-ribose) synthesized in situ. The polymers synthesized under these conditions were exclusively bound to the synthetase. Together with our previous finding that the enzyme is the main acceptor of the polymer in DNA-damaged cells, these results suggest that poly(ADP-ribose) in the synthetase-bound form counteracts inhibition by histones and activates DNA ligase to rejoin DNA strands in polynucleosomal structures.

Poly(ADP-ribose) is a macromolecule synthesized from NAD+ in eukaryotic cells by a nuclear enzyme, poly(ADP-ribose) synthetase (1). The synthesis proceeds by covalent attachment of ADP-ribose with protein acceptors such as histones, nonhistone chromosomal proteins, and the synthetase itself followed by chain elongation by a terminal addition mechanism (2). The biological function of this macromolecule is not yet fully understood, but several lines of evidence suggest its possible involvement in DNA excision repair; DNA strand breaks produced by various agents markedly stimulate poly(ADP-ribose) synthesis (3-5), and a number of inhibitors of poly(ADP-ribose) synthetase retard the rejoining of damaged DNA strands (6, 7). In an attempt to clarify the role of poly(ADP-ribose) in DNA rejoining, we identified poly(ADP-ribose) synthetase as the main acceptor of the polymer in DNA-damaged cells (8). In the present study, we examined the effect of poly(ADP-ribose) on DNA ligase activity in DNA/histone and reconstituted chromatin systems. The results presented here show that the ligase activity is strongly inhibited by histones, but the inhibition is reversed by poly(ADP-ribose) formed on the synthetase itself in response to DNA breakage.

MATERIALS AND METHODS

Chemicals and Enzymes. [γ-32P]ATP, [adenine-14C]NAD+, and [adenylate-32P]NAD+ were obtained from Amersham International. 3-Aminobenzamide was purchased from Tokyo Kasei. Escherichia coli alkaline phosphomonoesterase and Crotalus adamanteus venom phosphodiesterase were products of Worthington; the latter was further purified by the method of Oka et al. (9). Calf thymus DNA was obtained from Sigma, yeast tRNA, E. coli SS and 16S/23S rRNAs were from Boehringer, and poly(A) (φ20 = 6) was from Miles. Globin mRNA (9S) was prepared from rabbit reticuloocytes by the method of Aviv and Leder (10). Whole (unfractionated) histone and histone subfractions (H1, H2A, H2B, H3, and H4) were prepared from calf thymus according to Johns (11). A mixture of high-mobility group chromosomal proteins was from the same source after Sanders (12). ADP-ribosyl histone H1 was synthesized with isolated rat liver nuclei by the method of Okayama et al. (13). Poly(ADP-ribose) with average chain lengths of 10, 17, and 25 was prepared according to Sugimura et al. (14). [5-32P]Phosphoryl DNA with single-strand breaks was prepared from calf thymus DNA according to Weiss et al. (15). Poly(ADP-ribose) synthetase was purified to apparent homogeneity from calf thymus by the method of Ito et al. (16). DNA ligase was purified approximately 400-fold from calf thymus by 0.1 M NaCl extraction, streptomycin treatment, (NH4)2SO4 fractionation, and sequential chromatography on DEAE-Sephacel, phosphocellulose, hydroxypatite, and Bio-Gel P-300. The final preparation had a specific activity of 8 units/mg of protein, and no detectable activity of DNase, phosphomonoesterase, phosphodiesterase, poly(ADP-ribose) synthetase, or poly(ADP-ribose) glycohydrolase. Details of the purification and properties of the enzyme will be reported elsewhere.

Assays. DNA ligase activity was assayed by a modification of the method of Weiss et al. (17) as follows. The reaction mixture (200 μl) consisted of 75 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM dithiothreitol, 0.4 mM ATP, 1.5 μg of [5-32P]phosphoryl DNA (5,000-10,000 cpm/pmol), and about 1 milliunit of enzyme. The relatively low concentration of DNA, compared with the Km value (=75 μg/ml; variable with content of strand ends), was used to avoid the formation of insoluble material on mixing with histone. After incubation for 30 min at 37°C, 25 μg of calf thymus DNA and 5% (wt/vol) Cl3CCOOH were added. The mixture was centrifuged at 8,000 × g for 10 min. The precipitate was dissolved in 200 μl of 0.1 M NaOH, and the solution was allowed to stand for 10 min at 65°C. Then, a 185-μl aliquot was removed and neutralized with 98 μl of 0.3 M Tris-HCl (pH 7.4). This mixture was incubated with alkaline phosphomonoesterase (10 μg) for 30 min at 65°C and then treated with 4 ml of ice-cold 5% Cl3CCOOH. The acid-insoluble material was collected on a Millipore filter and radioactivity was determined with a liquid scintillation spectrometer. One unit of DNA ligase was defined as the amount converting 1 nmol of 32P to a phosphomonoesterase-resistant form under these conditions. Protein was assayed by the method of Lowry et al. (18) using bovine serum albumin as the standard. The average chain length of poly(ADP-ribose) was determined by the phosphodiesterase digestion method (19).

Reconstitution of Chromatin. Chromatin was reconstituted from [5-32P]phosphoryl DNA and purified core histones (H2A, H2B, H3, and H4) by the method of Gould et al. (20). As judged
by discrete DNA fragments (160 ± 20 and 400 ± 50 base pairs long) produced by micrococcal nuclease digestion (data not shown), the reconstituted chromatin had a typical polynucleosomal structure.

RESULTS

Inhibition of DNA Ligase Activity by Histones. The activity of partially purified DNA ligase from calf thymus was markedly inhibited by histones, as reported by Zimmerman and Levin (21) for the rat liver enzyme. The inhibition was dose dependent and varied between histone H1 and whole (unfractionated) histone (Fig. 1); at the same concentration (7.5 μg/ml) as substrate DNA, these histones inhibited the ligase activity by 80% and 40%, respectively. Almost complete inhibition was observed with histone H1 at ≥10 μg/ml. When both DNA and histone H1 were increased to 150 μg/ml (keeping the unit ratio), the extent of inhibition remained 80%, indicating that the inhibition by histone was dependent on the relative concentration to DNA. A mixture of core histones (H1-depleted whole histone) was as effective as whole histone (data not shown). Analysis with oligo(ADP-ribosylated histone H1 (Fig. 1) indicated that modification by short ADP-ribosyl chains did not affect the inhibitory effect of this histone. The inhibitory effect appeared to be highly specific for histones; other basic proteins, such as high-mobility group chromosomal proteins, cytochrome c, and egg white lysozyme, did not significantly inhibit the ligase activity under similar conditions (data not shown).

Reversal of Histone Effect by Poly(ADP-Ribose). When free poly(ADP-ribose) (average chain length, 25) was added to the DNA ligase/histone H1 reaction mixture, a dose-dependent activation of the ligase was observed (Fig. 2). At 7.5 μg/ml (a concentration identical to those of DNA and histone H1), the polymer stimulated the ligase activity >4-fold and restored >60% of the original activity. The activity was almost completely recovered by 10 times more poly(ADP-ribose). There was little activation by poly(ADP-ribose) in the absence of histone. The activating effect was specific for long-chain poly(ADP-ribose); mono(ADP-ribose) had no effect, and the effect increased with increasing average chain length of poly(ADP-ribose) (Table 1, experiment 1). Some other polyanions also reversed the histone inhibition but to lesser extents than poly(ADP-ribose) (experiment 2); globin mRNA was about 80% and 16S/23S rRNA was about 50% as effective. Smaller polynucleotides [5S rRNA, tRNA, and poly(A)] were almost inert. Poly(ADP-ribose) synthesized in situ—i.e., in the mixture of DNA with single-strand breaks and histone H1—also effectively activated DNA ligase (Fig. 3). With increasing amounts of poly(ADP-ribose) synthetase, increasing amounts of poly(ADP-ribose) were synthesized and, almost in parallel to this increment, DNA ligase activity increased up to >50% of the original level. Little activation of DNA ligase was observed when poly(ADP-ribose) synthesis was inhibited by the specific inhibitor 3-aminobenzamide (22), suggesting that the activation was due not to the synthetase protein but to its product, poly(ADP-ribose). 3-Aminobenzamide had no effect on DNA ligase activity by itself (data not shown). Analysis of products using [32P]NAD in parallel experiments indicated that poly(ADP-ribose) molecules synthesized under these conditions had long chain lengths and were bound exclusively to the synthetase (data not shown). Based on these observations, the effect of poly(ADP-ribose) synthesized in situ was estimated to be comparable with that of free long-chain polymer.

Table 1. Effect of poly(ADP-ribose) of various chain lengths and of various polyanions on histone H1 inhibition of DNA ligase activity

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Polyanion</th>
<th>DNA ligase activity, % original</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>ADP-ribose</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>Poly(ADP-ribose)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chain length, 10</td>
<td>32.7</td>
</tr>
<tr>
<td></td>
<td>Chain length, 17</td>
<td>38.2</td>
</tr>
<tr>
<td></td>
<td>Chain length, 25</td>
<td>50.7</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>Poly(ADP-ribose) (chain length, 25)</td>
<td>51.9</td>
</tr>
<tr>
<td></td>
<td>Globin mRNA</td>
<td>46.0</td>
</tr>
<tr>
<td></td>
<td>16S/23S rRNA</td>
<td>33.5</td>
</tr>
<tr>
<td></td>
<td>5S rRNA</td>
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</tr>
<tr>
<td></td>
<td>Poly(A)</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>tRNA</td>
<td>15.6</td>
</tr>
</tbody>
</table>

DNA ligase activity was assayed under standard conditions except that histone H1 (7.5 μg/ml) and various polyanions (5 μg/ml) were added as indicated.

Fig. 1. Inhibition of DNA ligase activity by histones. DNA ligase activity was assayed under standard conditions except for the addition of histone H1 (●), histone H1 modified by oligo(ADP-ribose) (average chain length, 1.5) (◇), or whole histone (▲).

Fig. 2. Effect of poly(ADP-ribose) on DNA ligase activity with or without histone H1. DNA ligase activity was assayed under standard conditions except for the addition of various amounts of poly(ADP-ribose) (average chain length, 25) in the absence (○) or presence (●) of histone H1 (7.5 μg/ml).

Fig. 3. Effect of poly(ADP-ribose) of various chain lengths and of various polyanions on histone H1 inhibition of DNA ligase activity.
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Fig. 3. Relationship between amount of poly(ADP-ribose) synthetase and poly(ADP-ribose) synthesis (A) and activity of DNA ligase (B) in the presence of histone H1. Identical sets of reaction mixtures (50 μl each) containing 100 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 1 mM di-thiothreitol, 0.4 mM [adenine-32P]NAD+ (10,000 cpm/pmol), 1.5 μg of [5'-32P]phosphoryl DNA, 1.5 μg of histone H1, and the indicated amounts of poly(ADP-ribose) synthetase were incubated with (●) or without (○) 4 mM 3-aminobenzamide for 1 hr at 37°C. The reaction was terminated by the addition of 3-aminobenzamide to a final concentration of 4 mM. Then, one set of reaction mixtures was treated with 10-μg portions of alkaline phosphomonoesterase, and these mixtures were incubated for 30 min at 65°C (this phosphomonoesterase treatment helped the subsequent 3H determination by reducing 32P). Then, 3 ml of ice-cold 20% Cl3CCOOH was added, and 3H incorporation into acid-insoluble materials was quantified (A). The other set of the reaction mixture was treated with about 1 milliunit of DNA ligase and examined for DNA ligase activity (B).

Activation of DNA Ligase by Poly(ADP-ribose) in Reconstituted Chromatin. The effect of poly(ADP-ribose) on DNA ligase under conditions closer to physiological was examined using chromatin reconstituted from substrate DNA and core histones. With this form of DNA, the activity of DNA ligase was about a half of that with free DNA. A similar effect of chromatin formation on DNA ligase activity has been reported by Zimmerman and Levin (21). In this reconstituted chromatin system, as in the DNA/histone H1 system described above, more poly(ADP-ribose) was synthesized by larger amounts of poly(ADP-ribose) synthetase and, almost in parallel, the DNA ligase activity increased (Fig. 4). Again, poly(ADP-ribose) synthetase per se had little effect on the ligase activity in the absence of poly(ADP-ribose) synthesis. Parallel experiments showed that almost all molecules of poly(ADP-ribose) formed in the chromatin system were also bound to the synthetase (data not shown).

DISCUSSION

Poly(ADP-ribose) synthetase is activated by DNA breakage in vitro (23, 24) as well as in vivo (3-5). The biological significance of this phenomenon is inferred from the finding that poly(ADP-ribose) is indispensable for certain types of DNA excision repair in eukaryotes, probably at the stage of strand ligation (6, 7). In the present study, we investigated the effect of poly(ADP-ribose) on DNA ligase activity in a DNA/histone mixture and also in reconstituted chromatin. Our study confirmed and extended the previous report (21) that histones are very inhibitory to DNA ligase and that the ligase activity in chromatin is only about a half of that with free DNA. It seems reasonable to assume that the DNA ligase activity is dormant under physiological conditions. When DNA is damaged, poly(ADP-ribose) is synthesized in situ in response to DNA breakage and activates the ligase. The effect of poly(ADP-ribose) is size dependent, and all other polyanions so far tested are less effective or totally ineffective. Since poly(ADP-ribose) synthesis increases after DNA damage (3-5) while that of RNA decreases markedly (25), these results support the view that long-chain poly(ADP-ribose) is the most potent and physiological activator of DNA ligase in DNA-damaged cells. Long-chain poly(ADP-ribose) has been found in association with poly(ADP-ribose) synthetase from various sources, including DNA-damaged cells (8), while monomers and short oligomers have been found mostly with histones (26, 27). Because essentially all poly(ADP-ribose) molecules synthesized in DNA/histone or reconstituted chromatin systems were bound to the synthetase, the enzyme-bound polymer appears to be the actual form of the activator.

All these findings support our current working hypothesis (28) that poly(ADP-ribose) synthetase produces long-chain poly(ADP-ribose) on itself in response to DNA breakage and that this polymer counteracts inhibition of DNA ligase by histones, thus facilitating the joining of broken DNA strands in chromatin. An important feature of this hypothesis is that poly(ADP-ribose) synthetase links DNA damage to DNA ligase through its affinity for DNA strand ends and the activator function of its bound product, poly(ADP-ribose). Poly(ADP-ribose) increases the ligase action, possibly by two mechanisms—first, by locally neutralizing the positive charge of histones and loosening DNA–histone interactions, and, second, by locating the ligase at strand breaks. The latter mechanism is suggested by our recent finding that DNA ligase has high affinity for poly(ADP-ribose) (28, 29). These mechanisms appear to be more important to DNA repair than DNA replication, because nascent DNA fragments in replication, but not in repair, are reported not associated with histones until ligation to bulk DNA (30).

Recently, Creissen and Shall (31) reported that an isozyme of DNA ligase, termed DNA ligase II (32), might be ADP-ribosylated and activated in DNA-damaged cells. However, we have not so far detected any activity suggestive of DNA ligase II during our purification from calf thymus nor found any ADP-ribosylation of our DNA ligase preparation (29). In this context, Teraoka et al. (33, 34) have presented evidence suggesting that DNA ligase II might be a proteolytic fragment of a single species of DNA ligase.
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