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Poly(ADP-ribosylation) of a DNA Topoisomerase*

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A DNA topoisomerase activity, copurifying with poly(ADP-ribose) synthetase from calf thymus, is >95% inhibited if extensive poly(ADP-ribosylation) is allowed to occur. The inhibited DNA topoisomerase, which has drastically different elution properties on hydroxylapatite, can be reactivated by mild alkaline treatment. These results are consistent with a poly(ADP-ribosylation) of the DNA topoisomerase and covalent attachment of the poly(ADP-ribose) moieties to the topoisomerase by alkali-labile bonds.

The phenomenon of poly(ADP-ribosylation) of proteins has been known for many years, and the enzyme catalyzing the reaction, poly(ADP-ribose) synthetase, has been highly purified and extensively studied (1, 2). However, the metabolic role of this phenomenon is still unclear. An increasing body of evidence suggests that extensive poly(ADP-ribosylation) occurs in response to DNA damage (3, 4). It is not established if poly(ADP-ribose) formation is obligatorily required for, facilitates, or is a secondary consequence of the process of DNA repair.

At the biochemical level, it has been established that poly(ADP-ribose) moieties can be covalently attached to the enzyme itself (5–7). In addition to this auto-poly(ADP-ribosylation) phenomenon, however, the enzyme will also modify a number of other acceptor proteins with oligomers of ADP-ribose. These proteins include histone H1 (8–10), histone H6 from trout tissue (8), the sperm-specific protamines (8), SV40 large T antigen (11), and a DNA endonuclease extracted from rat liver chromatin (12). The latter enzyme, which requires both Ca^{2+} and Mg^{2+} for full activity, was inhibited as much as 8-fold as a result of oligo(ADP-ribosylation) (12, 13). There is also evidence that poly(ADP-ribosylation) stimulates DNA ligase II in mammalian cells, although whether the enzyme actually serves as an acceptor for poly(ADP-ribose) is not yet definitively established (14). However, most proteins cannot serve as acceptors for poly(ADP-ribose).

In this communication, we demonstrate that highly purified preparations of poly(ADP-ribose) synthetase have a copurifying DNA topoisomerase activity. We present data consistent with the conclusion that this copurifying DNA topoisomerase serves as an acceptor protein for poly(ADP-ribose). When poly(ADP-ribosylation) occurs, the DNA topoisomerase activity is greatly inhibited. Preliminary data indicate that only a minor fraction of the total DNA topoisomerase activity in crude extracts from calf thymus copurified with the poly(ADP-ribose) synthetase. Since it has been observed that inhibition of poly(ADP-ribosylation) *in vivo* results in uncoiling, and hence slower sedimentation of nucleoids derived from cells that have been exposed to agents which damage DNA (3, 15), the possibility is now raised that the observed effect is in part due to a failure to inhibit a DNA topoisomerase by poly(ADP-ribosylation).

EXPERIMENTAL PROCEDURES

Materials

Calf thymus DNA (D-1501), Sephadex G-150, spermidine (S-2501), proteinase K (P-0390), and crystalline bovine serum albumin (A-7638) were obtained from Sigma. Stock solutions of albumin (10 mg/ml) in 50 mM Tris-Cl, pH 7.2, were heated at 65 °C for 15 min prior to use. New England Nuclear supplied [^{32}P]NAD (23 Ci/mmol) and [^{3}H]NAD (3.4 Ci/mmol). pBR322 plasmid DNA was the generous gift of Allen Laughon and Raymond Gesteland, Howard Hughes Medical Institute, Biology Department, University of Utah.

Assay for DNA Topoisomerase

Samples to be assayed were serially diluted in 35 mM Tris-Cl, pH 8.0, 5 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM spermidine, 100 μg/ml of bovine serum albumin, 72 mM KCl. Aliquots of the dilutions (8 μl) were added to 0.4 μg of pBR322 DNA in the same buffer, and the mixtures (16 μl) incubated at 30 °C for 1 h. The reactions were stopped by the addition of 6 μl of 4.2% sodium dodecyl sulfate, 21% Ficoll 400, 0.2 mg/ml of bromphenol blue, 0.33 mg/ml of proteinase K. Samples were electrophoresed on 1% agarose gels, using 90 mM Tris-borate, pH 8.1, 2.5 mM EDTA as the electrophoresis buffer, at 50 V for 15 h at 10–15 °C. The DNA bands were stained with ethidium bromide and the gels photographed under ultraviolet light using Polaroid 667 Land Pack Film. One unit of DNA topoisomerase relaxed 50% of the plasmid DNA under the conditions of the assay.

Assay for poly(ADP-ribose) Synthetase

Reaction conditions are described in the legends to the figures and the tables. At various times, aliquots (1–10 μl) of the reaction mixtures were applied to Whatman GF/C filters, followed by 150 ml of 20% trichloroacetic acid. The filters were dried, and then washed with 10% trichloroacetic acid followed by a wash in 95% ethanol. Radioactivity on the dried filters was determined (7). One unit of synthetase incorporated 1 nmol of NAD into an acid-insoluble form/min.

Poly(ADP-ribose) Synthetase

Two preparations of the synthetase were used in these experiments.

Preparation 1—The synthetase was purified as described previously (7) and then re-chromatographed on hydroxylapatite in the presence of 2 M KCl to remove all traces of DNA (16, 17). Briefly, the enzyme sample in buffer B (50 mM Tris-Cl, pH 8.0, 0.2 M NaCl, 10% glycerol, 2 mM DTT[1]) was applied to a Bio-Gel HTP column (1 × 7 cm) equilibrated with the same buffer. The column was washed with buffer B, followed by 2.5 mM potassium phosphate, pH 7.2, containing 2 M KCl, 10% glycerol, 1 mM DTT. Elution of the enzyme was achieved with a linear gradient (100 ml) of potassium phosphate, pH 7.2, 2.5 to 300 mM, also containing 2 M KCl, 10% glycerol, 1 mM DTT. The entire synthetase peak was pooled (thus including approximately 90% of the DNA topoisomerase activity, see below).

Preparation 2—Poly(ADPR) synthetase was extracted from 1.2 kg of calf thymus and purified on DNA-agarose, as reported (7). The enzyme was next chromatographed on hydroxylapatite in the pres-

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[1] The abbreviation used is: DTT, dithiothreitol, ADPR, ADP-ribose.

ence of 2 M KCl, as described above for preparation 1, except that the column was 2.2 × 8.5 cm, the gradient was 400 ml, and 8-ml fractions were collected. Enzyme assays were carried out for both the synthetase and DNA topoisomerase. Under these chromatographic conditions, the synthetase was eluted just before the topoisomerase (at 55 and 75 mM potassium phosphate, pH 7.2, respectively; details of the purification procedure will be described in a later report) although the two enzyme activities still overlap. Individual fractions were dialyzed against buffer B saturated in ammonium sulfate, and the resulting suspensions centrifuged. The pellets thus obtained were dissolved in buffer B to give a final volume of 0.3 ml.

RESULTS AND DISCUSSION

The purification procedure for poly(ADP-ribose) synthetase, which resulted in nearly homogeneous enzyme preparations, was previously described (7). We have found that all four of the synthetase preparations which have been purified by this method to date contain DNA topoisomerase activity; Fig. 1 shows an assay of this activity.

The presence of DNA topoisomerase activity in highly purified poly(ADP-ribose) synthetase mixtures raises the possibility that the topoisomerase is an intrinsic part of the synthetase protein. However, our results indicate that this is probably not the case. Partial resolution of the synthetase and topoisomerase was obtained on hydroxylapatite, as described under "Experimental Procedures" for enzyme preparation 2.

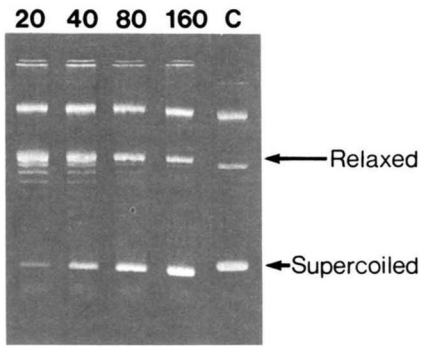

FIG. 1. **Assay of DNA topoisomerase activity in a preparation of poly(ADP-ribose) synthetase.** An aliquot of poly(ADPR) synthetase preparation 1 (see "Experimental Procedures") containing 100 μg/ml of bovine serum albumin was dialyzed against buffer B. The sample was then serially diluted and 8-μl aliquots of the dilutions assayed for DNA topoisomerase. An 60-fold dilution of this enzyme preparation resulted in a 50% relaxation of the plasmid DNA, corresponding to 7,500 units of topoisomerase/ml.

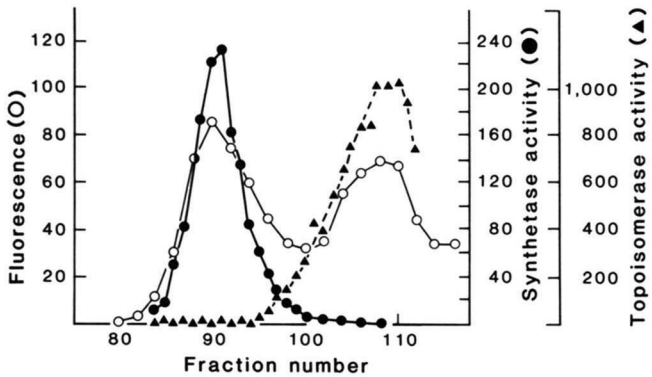

FIG. 2. **Separation of poly(ADP-ribose) synthetase and DNA topoisomerase on Sephadex G-150.** The fractions from the hydroxylapatite column containing the peak of DNA topoisomerase activity (see poly(ADPR) synthetase preparation 2 under "Experimental Procedures") were applied to a Sephadex G-150 column (2.6 × 162 cm) equilibrated against buffer B. Fractions of 3.8 ml were collected. The units for fluorescence are arbitrary. The units of enzyme activity are total units/fraction.

If the fractions containing the peak of the DNA topoisomerase activity are pooled separately from the fractions containing the bulk of the poly(ADP-ribose) synthetase activity, and the topoisomerase fractions run on Sephadex G-150 (Fig. 2), an almost total resolution of synthetase and DNA topoisomerase was obtained. Preliminary measurements indicate that the DNA topoisomerase activity that copurifies with poly(ADP-ribose) synthetase is a very small fraction of the total DNA topoisomerase activity in extracts of calf thymus. The relationship between the copurifying topoisomerase with the other DNA topoisomerases is currently being investigated.

In the course of our studies, we discovered that the copurified topoisomerase activity was severely inhibited by poly(ADP-ribosylation). In the experiment described in Table I, a poly(ADP-ribose) synthetase preparation was preincubated under conditions in which poly(ADP-ribose) was formed, and subsequently assayed for DNA topoisomerase. It was found that the topoisomerase was severely inhibited in the complete preincubation reaction but was not inhibited when NAD or DNA were omitted from the reaction mixture. Since NAD is the substrate and DNA is necessary for the stimulation of poly(ADP-ribose) synthetase, these results suggest that poly(ADP-ribosylation) inhibits the DNA topoisomerase activity. The severe inhibition of topoisomerase activity could be due to poly(ADP-ribose) itself being inhibitory or to

TABLE I

Conditions for the inhibition of DNA topoisomerase by poly(ADP-ribosylation)

Preincubation mixtures (60 μl) contained 50 mM Tris-Cl, pH 8.0, 1 mM DTT, 10 mM $MgCl_2$, 50 μg/ml of calf thymus DNA, 0.2 mM NAD, [^{32}P]NAD (final specific activity for NAD = 23,000 cpm/nmol) and poly(ADP-ribose) synthetase preparation 2. After 20 min at 23 °C, aliquots of the reaction mixture were analyzed for poly(ADP-ribose) synthesis and DNA topoisomerase activity.

Preincubation reaction	NAD incorporated during preincubation	DNA topoisomerase
	nmol/ml	*units/ml*
Complete	28	≤30
−DNA	0.025	625
−NAD		750

TABLE II

Automodified poly(ADP-ribose) synthetase does not inhibit DNA topoisomerase

Preincubation mixtures (50 μl) contained 40 mM Tris-Cl, pH 8.0, 1 mM DTT, 10 mM $MgCl_2$, 50 μg/ml of calf thymus DNA, 0.2 mM NAD, [^{32}P]NAD (final NAD specific activity = 97,000 cpm/nmol), and aliquots (8 μl) of synthetase and/or topoisomerase as indicated. After 20 min at 23 °C, 40-μl aliquots were added to 5.5 μl of 0.1 M nicotinamide containing 6.4-ml aliquots of synthetase, topoisomerase, or buffer. Assays for DNA topoisomerase were then performed.

The enzyme preparations were obtained from fractions of poly(ADPR) synthetase preparation 1 (see "Experimental Procedures") which had been chromatographed on Sephadex G-150. The peaks of synthetase and topoisomerase were separately pooled and concentrated by the ammonium sulfate dialysis procedure described under "Experimental Procedures" for synthetase preparation 2. The concentrated preparations were then dialyzed against buffer B.

Preincubation reaction	NAD incorporated during preincubation	Added with nicotinamide quench	DNA topoisomerase
	nmol/ml		*units/ml*
Complete	40		≤20
−Topoisomerase (synthetase only)	32	Topoisomerase	750
−Synthetase (topoisomerase only)	5	Synthetase	250

modification of the topoisomerase by poly(ADP-ribosylation). The experimental results are consistent with topoisomerase modification. When the synthetase is preincubated with NAD, which results in the production of a high concentration of poly(ADP-ribose) in the reaction mixture, inhibition of the DNA topoisomerase was not observed when the two enzymes were subsequently mixed (Table II). However, when the topoisomerase and the synthetase were incubated together, a strong inhibition of the DNA topoisomerase activity was observed. When the DNA topoisomerase was preincubated alone, a slight inhibition of topoisomerase activity was observed since the topoisomerase preparation was not completely resolved from the poly(ADP-ribose) synthetase, and a small amount of synthetase activity was detected therein. These experiments make it clear that the synthetase and topoisomerase have to be incubated together in the presence of NAD to get full inhibition, and that the presence of auto-poly(ADP-ribosylated) synthetase alone is not detectably inhibitory to the topoisomerase, consistent with modification of the topoisomerase.

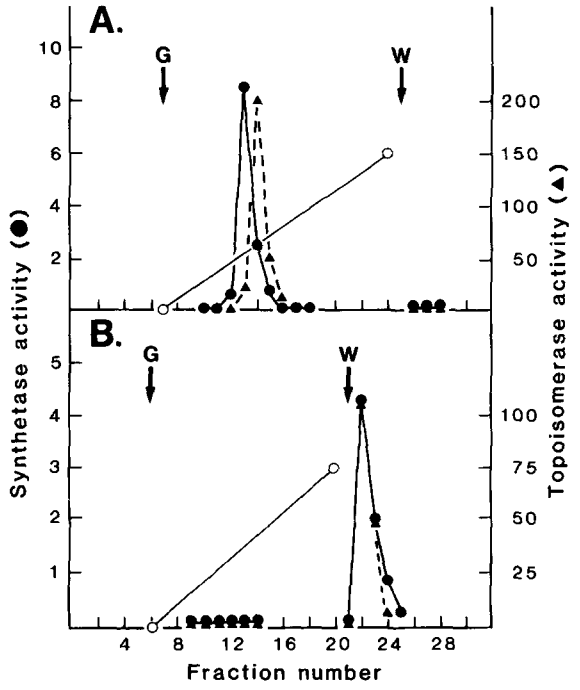

FIG. 3. **Change in the chromatographic behavior of DNA topoisomerase as a result of poly(ADP-ribosylation).** The reaction mixture (0.7 ml) contained 10 mM Tris-Cl, pH 8.0, 1 mM DTT, 10 mM $MgCl_2$, 100 μg/ml of calf thymus DNA, 2 mM NAD, [^{3}H]NAD (final NAD specific activity, 1,900 cpm/nmol) and enzyme preparation 1. In a control sample, NAD was omitted from the mixture. After a 20-min incubation at 23 °C, the buffer was exchanged by the basket centrifugation technique (18) with 30 mM Tris-Cl, pH 7.0, 0.2 M NaCl, 10% glycerol, 2 mM DTT. The sample was applied to a hydroxylapatite column (0.5 × 8 cm) equilibrated with the same buffer. The column was developed with a linear gradient (20 ml) of potassium phosphate, pH 7.2, 2.5 to 300 mM, containing 2 M KCl, 10% glycerol, and 2 mM DTT, followed by a stepwise elution using 1.5 M potassium phosphate, pH 7.2, 10% glycerol, 2 mM DTT. Column fractions (1.2 ml) were made 0.1 mg/ml in bovine serum albumin, and dialyzed individually against a buffer (30 mM Tris-Cl, pH 7.0, 0.2 M NaCl, 10% glycerol, 2 mM DTT) saturated in ammonium sulfate. The resulting suspensions were centrifuged, and the pellets dissolved in the same Tris buffer mentioned above (final volume = 30 μl/fraction). Prior to assay for poly(ADPR) synthetase and DNA topoisomerase, the samples were diluted 20-fold with 25 mM $NaHCO_3$, pH 10, 0.2 M NaCl, 10% glycerol, 2 mM DTT, and stood 1 h at 23 °C. *G* = start of gradient (2.5 to 300 mM potassium phosphate). *W* = start of 1.5 M potassium phosphate wash. The units of enzyme activity refer to total units/fract.

TABLE III

Reactivation of DNA topoisomerase by treatment with mild alkali

Preincubation mixtures (100 μl) contained 100 mM Tris-Cl, pH 8.0, 1 mM DTT, 10 mM $MgCl_2$, 100 μg/ml of calf thymus DNA, 0.2 mM NAD, [^{3}H]NAD (final NAD specific activity = 45,000 cpm/nmol), and poly(ADP-ribose) synthetase preparation 1. After 30 min at 23 °C, reaction mixtures were divided into two 45-ml aliquots and the buffer quickly exchanged by using a basket centrifugation technique (18). The macromolecular components of the preincubation mixtures were thereby treated with 25 mM $NaHCO_3$ at pH 6.5 or 10, containing 0.2 M NaCl, 10% glycerol, 2mM DTT. The solutions were stood 1 h at 23 °C. DNA topoisomerase activity was then assayed as described under "Experimental Procedures," except the Tris-Cl, pH 8.0, buffer concentration was increased to 70 mM, and 2.5 mM nicotinamide was included in the reaction mixture.

Pre-incubation reaction	NAD incorporated during pre-incubation	Buffer treatment	DNA topoisomerase
	nmol/ml	*pH*	*units/ml*
Complete	120	6.5	≤50
		10	750
−NAD		6.5	2000
		10	1000

A more direct test for poly(ADP-ribosylation) of the topoisomerase was carried out. A preparation of poly(ADP-ribose) synthetase and the copurifying DNA topoisomerase was incubated with NAD. The resultant poly(ADP-ribosylated) mixture was then chromatographed on a hydroxylapatite column. Any poly(ADP-ribosylated) protein will change its chromatographic behavior, since the polymer has a high affinity for hydroxylapatite (19). As shown in Fig. 3*B*, both the topoisomerase and the synthetase activities were drastically shifted in their chromatographic behavior after the poly(ADP-ribosylation) reaction (radioactive poly(ADP-ribose) co-eluted with the enzyme activities; results not shown). Within the limits of detection, no topoisomerase activity remained at the original position of elution (Fig. 3*A*). We have previously shown that poly(ADP-ribose) synthetase, which has been inhibited by auto-poly(ADP-ribosylation), can be reactivated by dialysis at pH 10 because of the release of poly(ADP-ribose) chains from the enzyme (7). As shown in Table III, dialysis at pH 10 after preincubation in the presence of NAD similarly releases the topoisomerase from inhibition. If the topoisomerase eluted at high salt on hydroxylapatite after poly(ADP-ribosylation) (Fig. 3*B*) was dialyzed at pH 10 and then rechromatographed on hydroxylapatite, topoisomerase activity was eluted at the position of unmodified enzyme (results not shown). The most straightforward interpretation of these results is that the topoisomerase was poly(ADP-ribosylated) by the synthetase, but the enzyme-poly(ADP-ribose) linkage was hydrolyzed by the mild alkaline treatment.

These initial observations raise numerous questions, some of which are presently being investigated. The biological significance of the poly(ADP-ribosylation) of a DNA topoisomerase is unknown. Our first priority is to characterize the DNA topoisomerase that copurifies with the poly(ADP-ribose) synthetase and clarify its relationship to previously characterized topoisomerases from calf thymus.

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Note Added in Proof—We have recently found that the major Topoisomerase I activity of calf thymus is similarly poly(ADP-ribosylated). A large Topoisomerase I, similar to the preparation of Liu and Miller (PNAS, 1978, 3487-3491), was obtained.

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