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Inhibition of calf thymus type II DNA topoisomerase by poly(ADP-ribosylation)

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The effect of poly(ADP-ribosylation) on calf thymus topoisomerase type II reactions has been investigated. Unknotting of phage P4 head DNA, and relaxation and catenation of supercoiled PM2 DNA are inhibited. We conclude that the inhibition results from poly(ADP-ribosylation) on the following grounds. Firstly, the enzyme poly(ADP-ribose) (PADPR) synthetase and NAD are required, secondly, the competitive synthetase inhibitor nicotinamide abolishes topoisomerase inhibition, and thirdly, the polymer alone is not inhibitory. The mechanism of inhibition appears to be disruption of the strand cleavage reaction. A topoisomerase-DNA complex can be formed that upon treatment with protein denaturant at low ionic strength results in strand cleavage. The amount of DNA present in such a cleavable-complex progressively decreased following pretreatment of topoisomerase type II with PADPR synthetase and increasing concentrations of NAD. Treatment of the pre-formed complex with NAD and PADPR synthetase had no effect on its salt-induced dissociation. This suggests that either poly(ADP-ribosylation) has no influence on dissociation of topoisomerase, in contrast to association, or topoisomerase is not accessible to the synthetase when bound to DNA. Similar data were obtained with calf thymus type I topoisomerase.

Key words: eukaryotic topoisomerase type II/eukaryotic topoisomerase type I/poly(ADP-ribose)/topoisomerase inhibition

Introduction

Covalent modification is a common mechanism for the regulation of molecular interactions and activities in biology. One extensively studied example of post-translational protein modification in eukaryotes is the addition of a polymer of ADP-ribose. This reaction is carried out by an ubiquitous nuclear protein, poly(ADP-ribose) synthetase (for reviews see, Mandel *et al.*, 1982; Hayaishi and Ueda, 1983). Transfer of the first ADP-ribose unit, usually to a carboxyl group, elongation and branching are performed by the synthetase (Ueda *et al.*, 1980). Removal of all but the last ADP-ribose unit is achieved with a glycohydrolase, and the final residue is cleaved from the protein by ADP-ribosyl protein lyase (Oka *et al.*, 1984). Known acceptors of poly(ADP-ribose) *in vitro* include the synthetase itself (Ogata *et al.*, 1981), histone H1 (Nishizuka *et al.*, 1968; Wong *et al.*, 1977), rat liver Ca^{2+},Mg^{2+}-dependent endonuclease (Yoshihara *et al.*, 1975), high mobility group proteins (Poirier *et al.*, 1982), DNA ligase II (Creissen and Shall, 1982) and SV40 large T antigen (Goldman *et al.*, 1981). Recent evidence has shown major acceptors *in vivo* following DNA damage to be histone H2B and two proteins of higher mol. wt. (100 000 – 116 000 and 170 000) (Adamietz and Rudolph, 1984). Modification of histone H1 *in vivo* has been demonstrated (Ueda *et al.*, 1975). The significance of poly(ADP-ribosylation) *in vivo* is far from understood. It has been implicated in many genetic functions, for example, DNA replication (Mandel *et al.*, 1982) and repair (Berger and Sikorski, 1981), transcription (Slattery *et al.*, 1983; Kurl and Jacob, 1985), and cell differentiation and transformation (Ohashi *et al.*, 1984; Hirai *et al.*, 1983).

The DNA topoisomerases have been similarly implicated in many nuclear processes such as replication, recombination, transcription (for reviews, see Gellert, 1981; Liu, 1983; Vosberg, 1985) and chromatin assembly (Glikin *et al.*, 1984). The basic reaction of a topoisomerase is the concerted cleavage and reunion of DNA. This allows not only the solution of topological problems such that occur during replication, but also, in principle, an influence on aspects of gene expression that are dependent on chromatin architecture. Two types of topoisomerase can be distinguished on a mechanistic basis: type I enzymes break only one DNA strand and are independent of an external energy source, whereas type II enzymes are ATP-dependent and cleave both DNA strands. Recently, in two independent reports, the co-purification of calf thymus poly(ADP-ribose) synthetase with topoisomerase I was described (Ferro *et al.*, 1983; Jongstra-Bilen *et al.*, 1983). Both reports demonstrated that topoisomerase I relaxing activity was inhibited as a result of poly(ADP-ribosylation).

Here we present evidence that the unknotting, relaxing and catenating activities of calf thymus type II topoisomerase are inhibited by poly(ADP-ribosylation). Analysis of the formation and dissociation of DNA-topoisomerase II complexes reveals the basis for inhibition to be disruption of the strand cleavage reaction. This observation is also confirmed for calf thymus type I topoisomerase.

Results

Inhibition of topoisomerase type II activities by poly(ADP-ribosylation)

The effect of poly(ADP-ribosylation) on topoisomerase II activities was determined with two different assays. The unknotting of phage P4 DNA (Liu *et al.*, 1981) is a highly specific assay for type II activity by virtue of an obligatory mechanistic requirement for a double-strand break. In view of the reported co-purification of poly(ADP-ribose) synthetase (Jongstra-Bilen *et al.*, 1983) with calf thymus topoisomerse I and the number of common steps employed in our purification of the two topoisomerases, a control experiment was first performed to determine endogenous synthetase activity. Topoisomerase II was pre-incubated with increasing concentrations of NAD, the reaction

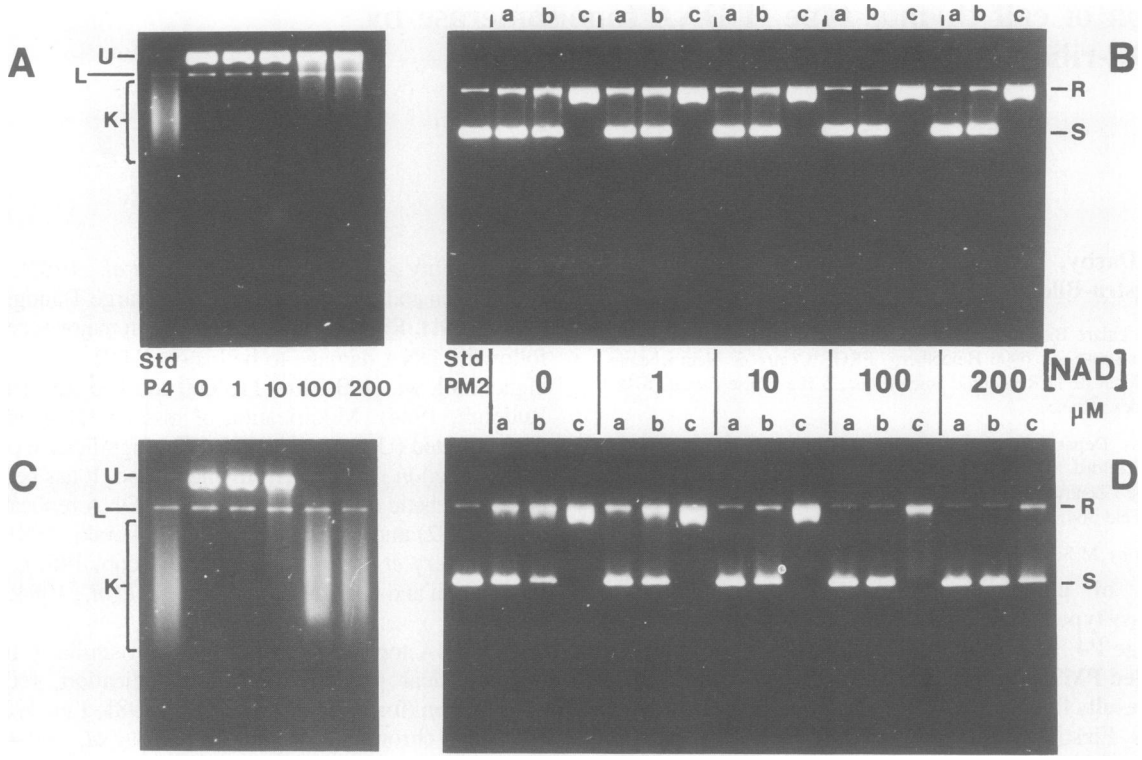

Fig. 1. Inhibition of topoisomerase II catalyzed relaxing and unknotting activities. Calf thymus topoisomerase type II (2.0 μg) was incubated in a volume of 10 μl without (**A** and **B**) and with (**C** and **D**) poly(ADP-ribose) synthetase (0.66 μg) for 16 min at 23°C in the presence of the indicated concentration of NAD. In (**A**) and (**C**) after 16 min a 1.0 μl aliquot (0.2 μg) was taken from each incubation for assay of unknotting activity with P4 head DNA. In (**B**) and (**D**) after 16 min each preincubation was diluted and 1.0 μl was used in the PM2 relaxation assay; (**a**) 0.02 μg, (**b**) 0.04 μg, and (**c**) 0.2 μg topoisomerase II. The positions of unknotted (U), knotted (K) and linear (L) P4 DNA and relaxed (R) and supercoiled (S) PM2 DNA are indicated. Std. is DNA incubated without topoisomerase.

Table I. Poly(ADP-ribose) *per se* does not inhibit topoisomerase type II

	Incubation conditions[a]	Total NAD incorporated[b]	Topoisomerase activity (units/mg)[c]
A	Topoisomerase II	–	3×10^5
B	Topoisomerase II + poly(ADP-ribose) synthetase	1.30 nmol	$<3 \times 10^3$
C	Poly(ADP-ribose) synthetase, followed by nicotinamide and topoisomerase II	1.56 nmol	3×10^5

[a]Experimental details are given in Materials and methods.
[b]Acid-insoluble radioactivity derived from [^{3}H]NAD.
[c]Determined by relaxation of PM2 DNA, one unit relaxes 100 ng of supercoiled PM2 DNA by 50% in 30 min at 30°C.

was quenched by addition of nicotinamide, and the reaction mix was diluted appropriately for topoisomerase assay. As the concentration of NAD in the pre-incubation mix was increased, the residual topoisomerase activity decreased slightly (Figure 1A), indicative of a minor contamination with synthetase. If additional purified calf thymus synthetase was provided (Figure 1C), then the inhibition at a given NAD concentration was much more marked, the onset of inhibition was observed at 10 μM NAD rather than 100 μM. Nicotinamide is a competitive inhibitor of NAD in the ADP-ribosylation reaction (Niedergang *et al.*, 1979). The inclusion of nicotinamide in the pre-incubation significantly reduces the inhibition of topoisomerase activity, such that at 2.0 mM nicotinamide and 200 μM NAD no inhibition was observed (data not shown).

The relaxation of supercoiled phage PM2 DNA is similarly inhibited in an NAD- and poly(ADP-ribose) synthetase-dependent manner (Figure 1B, D). Topoisomerase II was again pre-incubated in the presence of NAD with (D) or without (B) the synthetase. After quenching the reaction with nicotinamide the enzyme was serially diluted and an aliquot from each dilution was used to relax supercoiled DNA. As the NAD concentration in the pre-incubation was increased the amount of enzyme required to relax 50% of the supercoiled DNA progressively increased. Compare the lane (b) of each block of three in Figure 1B or lane c of each block in Figure 1D. The onset of inhibition was observed at 1 μM NAD with exogenous synthetase, in comparison with 10 μM in the unknotting assay. The apparent discrepancy between the two values probably reflects the greater sensitivity of the relaxation assay.

The poly(ADP-ribose) polymer itself is not an inhibitor of the type II topoisomerase, as demonstrated by the following experiment. Poly(ADP-ribose) synthetase was incubated with ^{3}H-labelled NAD, which enables the synthesis of poly(ADP-ribose) by virtue of the automodification reaction (Ogata *et al.*, 1981). After quenching the reaction with nicotinamide, topoisomerase II was added and topoisomerase activity in the presence of the synthetase-bound polymer was measured in a DNA relaxation assay. As controls, firstly both topoisomerase II and synthetase were coincubated with [^{3}H]NAD, essentially as in the experiment of Figure 1, and secondly topoisomerase II was incubated

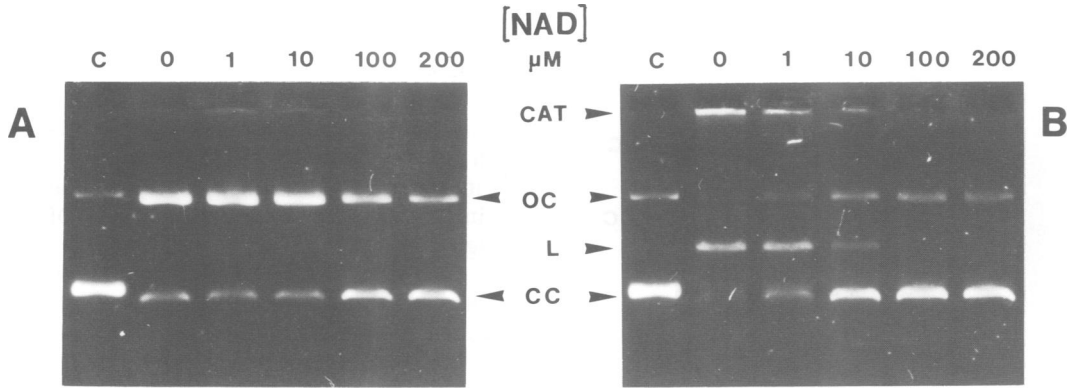

Fig. 2. Inhibition of the topoisomerase nicking reaction. DNA topoisomerase type I (1.5 μg) (**A**) or type II (0.64 μg) (**B**) was incubated for 10 min a 25°C with poly(ADP-ribose) synthetase (60 ng) and the indicated concentration of NAD in a volume of 10 μl. Supercoiled PM2 DNA (30 ng) and nicontinamide to 2.0 mM were simultaneously added to this reaction mixture and incubation was continued at 37°C for 5 min. The reaction was stopped with SDS to 0.5% (w/v) final concentration and protein digested with proteinase K to 100 μg/ml for 30 min at 37°C. Gel electrophoresis was in the presence of ethidium bromide. The lane marked C is a control incubation without topoisomerase. CAT, OC, L and CC are catenated, open circular, linear, and closed circular (supercoiled and relaxed) PM2 DNA respectively.

without synthetase in the preincubation buffer. The results are summarised in Table I. The type II topoisomerase is inhibited only when incubated with both synthetase and NAD. Synthetase-bound polymer, the presence of which is indicated by the incorporation of [^{3}H]NAD into an acid insoluble form, is insufficient for inhibition. It has been previously shown that synthetase-bound polymer is not an inhibitor of the type I enzyme from calf-thymus (Ferro and Olivera, 1984). The dependence of the observed inhibition on NAD and exogenous poly(ADP-ribose) synthetase and the counteraction by nicotinamide, together with the observation that the synthetase-bound polymer is non-inhibitory, strongly suggests that inhibition is a consequence of covalent modification of topoisomerase with poly(ADP-ribose).

Strand cleavage is inhibited by poly(ADP-ribosylation)

Essential to understanding the mechanism of inhibition of topoisomerase activity is the determination of the point in the reaction cycle that is affected by poly(ADP-ribosylation). In a generalised scheme for a topoisomerase reaction cycle, binding of the enzyme to DNA is followed by cleavage of DNA strand(s), rotation or transport of strands to effect a topological change, and finally by DNA reclosure and, in principle, enzyme dissociation. In the assays above, the reaction cycle was slowed but not aborted, that is no nicked or linear material was generated. Thus inhibition probably resulted from blocking of the binding or cleavage steps of the reaction cycle. The data presented in Figure 2 demonstrate that the inhibited step of both type I (panel A) and type II (panel B) topoisomerases indeed lies before strand cleavage.

If a topoisomerase is incubated with DNA under conditions of low ionic strength and enzyme excess, a complex is formed that when disrupted by addition of protein denaturants, such as SDS, results in the breakage of the DNA strand(s). This complex has been termed the 'cleavable complex' by Liu *et al.* (1983) and probably represents a trapped covalent intermediate of the normal reaction cycle. We have used the formation of the cleavable-complex as an assay for the efficacy of the initial nicking reaction. Topoisomerase was incubated with NAD and poly(ADP-ribose) synthetase and the reaction quenched with nicotinamide. Supercoiled PM2 DNA was added, a complex was allowed to form and was subsequently treated with SDS. Protein bound to the DNA was digested with proteinase K to allow migration of the DNA in an agarose gel. Lanes marked (0) in Figure 2(A) and (B) show that if no NAD was provided in the pre-incubation then in the case of topoisomerase I >90% of the DNA was nicked subsequent to SDS treatment. The incubation with the type II enzyme generated some catenanes, but much of the DNA was linearised. As the concentration of NAD was increased, progressively less DNA was nicked by topoisomerase I. Similarly, fewer double-strand breaks or catenanes were formed by topoisomerase II. Thus in both cases, less enzyme was able to form a covalent DNA-enzyme intermediate as poly(ADP-ribosylation) became more extensive, strongly suggesting that the nicking reaction is inhibited. This result has been confirmed for topoisomerase I reaction with single-strand DNA at 10−200 μM NAD, as measured by filter retention of the topoisomerase-DNA complex (Prell and Vosberg, 1980). The formation of filter-retainable complexes is reduced by 50% following pre-incubation with poly(ADP-ribose) synthetase and 40 μM NAD (B.Schmitt, unpublished).

Dissociation of the cleavable-complex is not inhibited

The question as to whether the inhibition of topoisomerase activity by poly(ADP-ribosylation) is restricted to the strand cleaving stage of the reaction cycle assumes particular importance when considering the potential regulatory role of poly(ADP-ribosylation) *in vivo*. Immediate inactivation of those topoisomerase molecules engaged in reaction at the moment of poly(ADP-ribosylation) could prevent resealing of the DNA and lead to the generation of undesirable protein-associated strand breaks. We have exploited salt-induced dissociation of the cleavable-complex as an assay for the effect of poly(ADP-ribosylation) on the enzyme-DNA complex.

The cleavable-complex can be dissociated with salt such that the DNA is no longer cleaved upon SDS treatment (Liu *et al.*, 1983). This observation presumably results from release of the enzyme from a complex with DNA, possibly with concomitant reclosure of DNA strand breaks. A control experiment that serves to illustrate this dissociation/reclosure phenomenon is shown in Figure 3A. Topoisomerase II was incubated with closed, circular PM2 DNA in low salt. After 5 min an aliquot was added directly to SDS (lane b), to assess the proportion of DNA in a cleavable complex. NAD was added to aliquots of the remaining incubation at concentrations between 10 and 200 μM. (ADP-ribosylation) of complex and free enzyme was allowed for 5 min, after which each aliquot was split into halves. To one SDS was

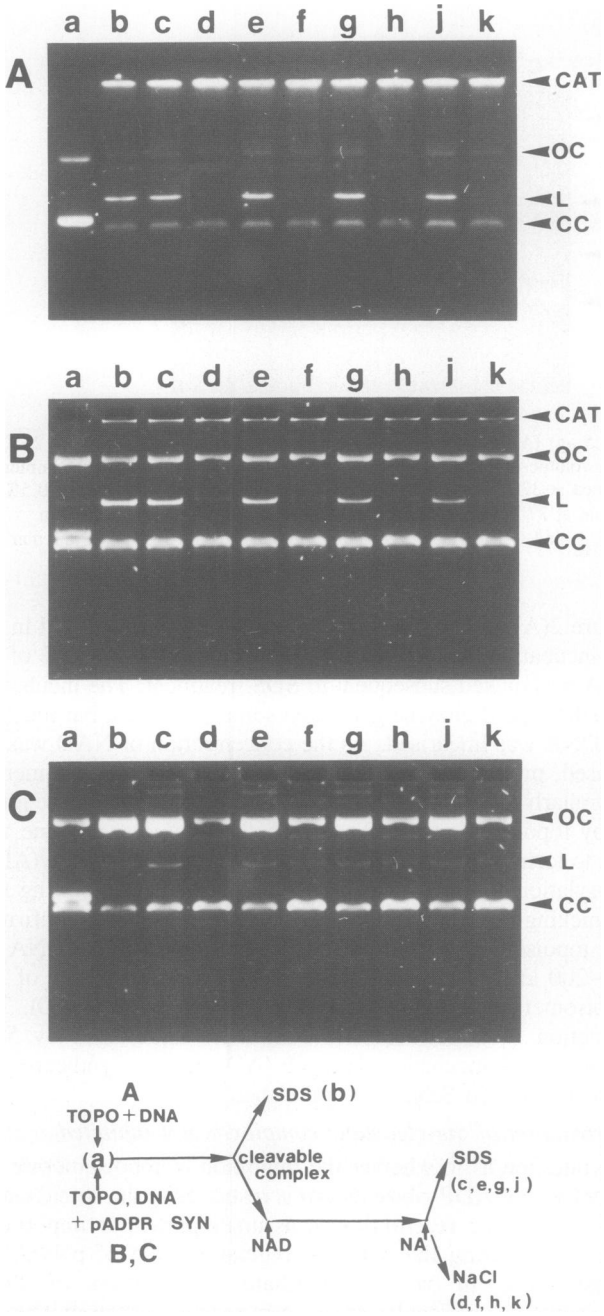

Fig. 3. Effect of poly(ADP-ribosylation) on DNA-bound topoisomerase. (**A**) Topoisomerase II in the absence of exogenous poly(ADP-ribose) synthetase. Topoisomerase II (5.1 μg) was incubated with PM2 DNA (0.51 μg) for 5 min at 37°C in a volume of 105 μl, an aliquot (10 μl) was removed and added to SDS [final concentration 0.5% (w/v)] to stop further reaction (**lane b**). The remaining incubation mixture was divided equally into four aliquots and each was supplemented with NAD (**lane c** and **d** none, **e** and **f** 10 μM, **g** and **h** 100 μM, **j** and **k** 200 μM) and incubation was continued for 5 min at 37°C, the ADP-ribosylation reaction was then quenched with 2.0 mM nicotinamide. The four aliquots were then divided into two equal portions, to one SDS was immediately added (**lanes c, e, g** and **j**), to the other NaCl to 0.5 M was added followed 2 min later by SDS (**lanes d, f, h** and **k**). All samples were subsequently treated with proteinase K before analysis of products by electrophoresis. **Lane a** is an incubation for 12 min at 37°C without topoisomerase. (**B**) Topoisomerase II in the presence of exogenous poly(ADP-ribose) synthetase. As (**A**) except for the inclusion of poly(ADP-ribose) synthetase (0.66 μg) in the initial incubation. (**C**) Topoisomerase I. Experimental as (**B**), the initial incubation contained calf thymus topoisomerase type I (12.0 μg) and poly(ADP-ribose) synthetase (0.66 μg). Right-hand abbreviations are defined in legend to Figure 2. A schematic representation of the experiment is given at the bottom of the figure.

added, to the other NaCl followed two minutes later by SDS. The samples stopped with SDS (lanes c, e, g and j) give a measure of the proportion of DNA remaining in the cleavable-complex after the incubation with NAD, as indicated by the amount of linear DNA. Samples treated with NaCl before SDS (lanes d, f, h and k) determine whether the complex is dissociable, which is indicated by the absence of strand breaks.

In the absence of exogenous poly(ADP-ribose) synthetase (Figure 3A), as the NAD concentration was increased there was no observable change in the amount of linear PM2 DNA generated subsequent to SDS treatment. Thus neither NAD nor incubation conditions alone lead to permanent dissociation of the complex over this time period. At each NAD concentration, NaCl prevented strand cleavage. The quantity of covalently closed DNA remaining, however, is reduced. Addition of salt under these reaction conditions possibly promotes incorporation of DNA into the catenated network that can be seen at the top of the gel. In contrast, when exogenous synthetase was provided, Figure 3B, the amount of linear material generated by SDS (lanes c, e, g and j) decreased progressively in comparison to the control value (lane b) as the NAD concentration was increased. Again, NaCl addition at all NAD concentrations prevented strand cleavage. These data suggest a NAD and poly(ADP-ribose) synthetase-dependent process is leading to the dissociation of the cleavable-complex. All of the remaining complex can be dissociated by NaCl, demonstrating that under conditions in which the poly(ADP-ribosylation) system is highly active (i.e., at 200 μM NAD) the DNA cannot be trapped in a non-dissociable complex with topoisomerase II. Separate experiments demonstrated that under these conditions the synthesis of poly(ADP-ribose) was at least as extensive as in the preincubation conditions used in the experiment shown in Figure 2, which was sufficiently high to totally inhibit the nicking activity of the topoisomerase.

Similar observations were made when the experiment was repeated with topoisomerase type I, Figure 3C. As the NAD concentration was increased during the period allowed for ribosylation, the amount of cleavable complex remaining decreased slightly, as demonstrated by the decrease in nicked DNA (lanes c, e, g and j). Those complexes remaining could be dissociated such that strand cleavage was prevented, as evidenced by the constant ratio of nicked to closed circular molecules in lanes d, f, h and k.

Together, these data indicate that the dissociation of the enzyme and reclosure of DNA breaks are not inhibited by poly(ADP-ribosylation), or that no or a non-inhibitory amount of poly(ADP-ribose) is attached to the topoisomerase when DNA-bound.

Discussion

We have obtained evidence suggesting calf thymus topoisomerase type II, like the type I enzyme, is inhibited as a result of poly(ADP-ribosylation). Although we have not directly demonstrated the covalent attachment of the polymer, the requirements for active poly(ADP-ribose) synthetase and NAD for inhibition and the non-inhibitory nature of the poly(ADP-ribose) polymer *per se* provide compelling evidence that inhibition is related to covalent modification with poly(ADP-ribose). It has previously been demonstrated that the type I enzyme co-purifies with poly(ADP-ribose) synthetase (Ferro *et al.*, 1983; Jongstra-Bilen *et al.*, 1983). The type II enzyme used in these studies also has a minor contamination of synthetase. In early experiments, the endogenous synthetase was sufficiently active to completely

inhibit topoisomerase II at 200 μM NAD. This activity was lost more rapidly than the topoisomerase activity and, as illustrated in Figure 1, exogenous synthetase was eventually required to effect full inhibition.

All tested topoisomerase II activities; unknotting, relaxation and catenation, were inhibited by poly(ADP-ribosylation). Although catenation was not systematically investigated, inspection of Figure 2B shows progressively less catenation as the NAD concentration is increased. The onset of inhibition lies in the range 1 – 10 μM NAD, similar to that previously observed for calf thymus topoisomerase I, but maximal inhibition required at least ten-fold more NAD. The reported K_m for poly(ADP-ribose) synthetase is dependent on DNA concentration and lies in the range 43 – 130 μM (Niedergang *et al.*, 1979). Since estimates of the average intracellular NAD concentration are between 0.1 and 1.0 mM (Hilz and Stone, 1976), the inhibition of topoisomerase could be of physiological relevance.

As judged by the inhibition of cleavable complex formation (Figure 2), it appears to be prevention of the initial nicking reaction of the topoisomerases that is responsible for the inhibition of catalytic activities. Inhibition of this step occurs in the same range of NAD concentration as does inhibition of unknotting and relaxation. The release of DNA from the cleavable complex does not appear to be inhibited (Figure 3). The relationship of the cleavable complex to the putative enzyme-DNA intermediate of the topoisomerase reaction has not been unequivocably established. However, the steps involved in forming this complex are blocked as a consequence of poly(ADP-ribosylation), whereas the release of intact DNA upon disruption of the complex is not. We conclude from the data in Figure 3 that as poly(ADP-ribosylation) is increased less complex remains cleavable. Two simple mechanisms would be consistent with this conclusion. If only free enzyme were modified, then those molecules that dissociate from the complex during the period allowed for poly(ADP-ribosylation) would become modified and thus unable to re-react, since the strand cleavage reaction is blocked. Alternatively, if the enzyme were modified whilst in the complex and dissociation were still possible, then re-reaction would again be blocked. The present data does not resolve between these two possibilities. Both mechanisms, however, would be compatible with the simple model of charge repulsion previously invoked for topoisomerase I by Ferro and Olivera (1984). Association of free enzyme with DNA would be hindered, whereas additional negative charge on the enzyme in the enzyme-DNA complex could actually enhance release. In these very simple mechanisms only association and dissociation rates would be affected rather than the nicking/closing activities *per se*, thus the reaction is not trapped mid-cycle. A further alternative would be covalent modification at the active sites of the enzymes, that is, poly(ADP-ribosylation) of the complex cannot occur because of steric hinderance by the bound DNA. For the type I enzyme at least, this model seems less likely since it has been previously shown that modification occurs at more than one site and inhibition is proportional to the number of ADP-ribose units (Ferro and Olivera, 1984).

Topoisomerases are present in high copy number in eukaryotic nuclei, estimates falling between one topoisomerase every 10 – 15 nucleosomes, depending on the source. Thus if poly(ADP-ribosylation) were to be of significance *in vivo*, one would expect these proteins to be major acceptors. In a recent study of poly(ADP-ribosylation) *in vivo* following dimethyl sulphate-induced DNA damage (Adamietz and Rudolph, 1984), in addition to histone H2B two other major ADP-ribose acceptors were species of 100 000 – 116 000 and 170 000 mol. wt. These mol. wts. correspond well to typical reported values for eukaryotic topoisomerase types I and II respectively.

DNA damage is associated with a depletion of cellular NAD and extensive poly(ADP-ribosylation), and it has been suggested that the significance of blocking topoisomerase activity in these circumstances is to avoid strand transfer reactions or protein-associated DNA strand breaks (Ferro and Olivera, 1984). The inhibition of topoisomerase II revealed by our data is equally compatible with this hypothesis. However, this particular aspect of topoisomerase inhibition may only be required in the special instance of DNA repair. Of the two types of topoisomerase, the type II in particular is supposed to be involved in higher organisation of chromatin structure by virtue of its ability to concertedly break and reseal both DNA strands. Although any proposal for the significance of the inhibition is clearly speculative, inhibition of topoisomerase II at times of chromatin relaxation resultant from histone H1 poly(ADP-ribosylation) (Aubin *et al.*, 1983; Niedergang *et al.*, 1985) might be necessary to restrict the introduction of topological changes in the chromatin. Topoisomerase II activity may be continuously required to maintain a condensed structure, thus relaxation of chromatin could be enhanced by blocking topoisomerase II activity.

Although the type I and II topoisomerases may be expected to differ in their DNA-related physiological roles (for discussion see Vosberg, 1985), an analysis of phenotypic properties of temperature sensitive *top* 1 and *top* 2 mutants of yeast has concluded that at least in this organism topoisomerase II can replace a deficient topoisomerase I, but not vice versa (Uemura and Yanagida, 1984). If this applies for eukaryotic topoisomerases in general, it may be expected that effective negative regulation of topoisomerase I by poly(ADP-ribosylation) requires a comparable and simultaneous reduction in topoisomerase II activity. Our data suggest that poly(ADP-ribosylation) could be a means by which eukaryotic cells achieve concerted regulation of topoisomerase activity.

Phosphorylation is the only other hitherto documented covalent modification of topoisomerases. It has recently been reported that the *Drosophila* type II topoisomerase has a tightly associated serine kinase activity (Sander *et al.*, 1984) for which the topoisomerase itself is a substrate *in vitro*. This may be of general significance *in vivo*, since a type I topoisomerase from Novikoff cells has been isolated as a serine phosphoprotein (Durban *et al.*, 1983). Viral and cellular tyrosine kinases have been shown to phosphorylate and inactivate type I topoisomerases from both pro- and eukaryotic sources *in vitro* (Tse-Dinh *et al.*, 1984).

In this report we have demonstrated the potential for poly(ADP-ribosylation) as a regulatory mechanism for calf thymus topoisomerase II, but as with the other *in vitro* modifications above, further study is required to confirm its significance as a control mechanism *in vivo*.

Materials and methods

Materials

β-NAD and nicotinamide were purchased from Sigma Chemical Corp. (München, FRG). Radiolabelled NAD ([adenine-2,8-^{3}H]NAD) was from New England Nuclear (Dreieich, FRG). Dithiothreitol and proteinase K were from Merck (Darmstadt, FRG). Bovine serum albumin was from Miles Scientific (Bayer AG, München, FRG).

Preparation of enzymes and DNA. Topoisomerase I was prepared from calf thymus as previously described by Schmitt *et al.* (1984). Topoisomerase II was located in the earlier eluting fractions of the final Bio-Rex chromatography. Calf thymus poly(ADP-ribose) synthetase was prepared as described (Niedergang *et al.*, 1979) and was a gift of Dr. M.-E.Ittel (Centre de Neurochemie, CNRS, Strasbourg).

Two synthetase preparations were used during the course of this work. The first, used for data in Figures 1 and 2, required no additional DNA for activity, whereas the second, used for Figure 3 and Table I, required s-DNA at 0.5 μg/ml (Niedergang *et al.*, 1979).

Knotted phage P4 DNA was prepared as described by Liu *et al.* (1981). Supercoiled phage PM2 DNA was prepared according to Espejo, *et al.* (1969).

Poly(ADP-ribosylation) reactions

Topoisomerase and poly(ADP-ribose) synthetase were incubated at a ratio of 5 – 10:1 (w/w) for the time indicated in the figure legends. The buffer used for ADP-ribosylation prior to unknotting or relaxing assays was 50 mM Tris-HCl, pH 7.9, 90 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 μg/ml bovine serum albumin. For strand cleavage experiments NaCl was omitted from the preincubation.

Topoisomerase assays

Knotted phage P4 DNA (100 ng) was incubated with topoisomerase (amount given in figure legends) for 30 min at 30°C in 50 mM Tris-HCl pH 7.7, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 1.0 mM ATP, 2.0 mM nicotinamide, 30 μg/ml bovine serum albumin (total volume 10 μl). The reaction was terminated by the addition of SDS to a final concentration of 0.5% (w/v). Relaxation of supercoiled PM2 DNA (100 ng) was assayed with topoisomerase (diluted in the reaction buffer below, but without $MgCl_2$ or ATP) for 30 min at 30°C in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 6.0 mM $MgCl_2$, 0.5 mM EDTA, 1.0 mM ATP, 2.0 mM nicotinamide, 50 μg/ml bovine serum albumin. Reaction was terminated as above. Products of both assays were analysed by agarose gel electrophoresis.

Strand cleavage reactions

Strand cleavage reactions carried out with topoisomerase I were performed in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 20 μg/ml bovine serum albumin 2.0 mM nicotinamide. Type II cleavage reactions were carried out in the same buffer including 1.0 mM ATP. Reactions were stopped with SDS to a final concentration of 0.5% (w/v) and digested with proteinase K (100 μg/ml) for 30 min at 37°C.

Experimental details for Table I

(A) Topoisomerase II was serially diluted in doubling steps such that 1 μl contained between 1.8 and 1.6 ng and assayed for relaxation of 100 ng of PM2 DNA. (B) Poly(ADP-ribose) synthetase (0.38 μg) and topoisomerase II were incubated for 10 min at 23°C with 0.2 mM [^{3}H]NAD (specific activity 0.5 mCi/μmol) in a volume of 25 μl. The reaction was quenched by addition of 2 μl of 50 mM nicotinamide. Two 5 μl aliquots were removed, added to 5 μl 5 mg/ml BSA and acid precipitated with 1 ml ice-cold 10% TCA. After standing on ice for 15 min the precipitate was collected on a Whatman GF/C filter, washed three times with 5 ml TCA, dried, and counted in a toluene-based scintillant. The remaining incubation mixture was diluted as in (A) and assayed for relaxation of PM2 DNA. (C) Poly(ADP-ribose) synthetase (0.38 μg) was incubated for 10 min at 23°C with 0.2 mM [^{3}H]NAD in a volume of 25 μl. Nicotinamide (2 μl of 50 mM solution) was added to quench the reaction followed by topoisomerase II (1.92 μg, 3.0 μl). Aliquots were removed to determine acid-insoluble counts and enzyme activity as in (B).

Gel electrophoresis

Electrophoresis was carried out in all cases in 0.8% (w/v) horizontal agarose gels. Electrophoresis buffer was 40 mM Tris-acetate, pH 7.8, 5.0 mM Na acetate, 1.0 mM EDTA. For the strand cleavage experiments buffer additionally included 0.5 μg/ml ethidium bromide.

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