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ATP inhibits nuclear and mitochondrial type I topoisomerases from human leukemia cells

(nucleotide-binding site/mammalian type I topoisomerase/acute lymphoblastic leukemia cells)

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ABSTRACT **Type I topoisomerases have been purified from nuclei and mitochondria of human acute lymphoblastic leukemia cells. Both of these ATP-independent enzymes are actually found to be inhibited by ATP at physiologically significant concentrations. Other adenine nucleotides showed varying effects: ADP inhibited only at high concentrations; AMP had no effect on either topoisomerase. Both enzymes were also inhibited by dATP. The importance of the adenine ring structure was confirmed by the lack of an inhibitory effect observed with equivalent levels of GTP, UTP, CTP, or their deoxy counterparts. Assays performed in the presence of nonhydrolyzable analogs of ATP suggest that hydrolysis of ATP does not accompany this enzyme inhibition. This was supported by direct determination of the ATPase activity of the purified enzymes. Type I topoisomerases from calf thymus and HeLa cells were also found to be sensitive to ATP. These results suggest that mammalian type I topoisomerases in general may possess a nucleotide-binding site that may be involved in regulation of enzyme activity.**

Topoisomerases are enzymes that interconvert topologically equivalent forms of DNA by means of mechanisms involving concerted breaking and rejoining of DNA strands. Type I topoisomerases create a single-strand nick and pass an intact strand through the break before resealing. This process, which changes the linking number of the DNA in steps of one, is ATP independent. Type II topoisomerases create a double-strand cleavage and pass an intact double strand through the break before resealing. This duplex breaking and rejoining changes the linking number in steps of two.

This class of enzymes has been found throughout nature; topoisomerases have been isolated from bacteria, viruses, lower and higher eukaryotes, and organelles such as mitochondria and chloroplasts (1–4). We have reported the occurrence of four topoisomerases from human acute lymphoblastic leukemia (HALL) cells—type I and type II enzymes from nuclei and mitochondria (5–7). The partially purified type I enzymes from these cells were found to be somewhat sensitive to ATP (7). Although relaxation of supertwisted DNA could proceed in the absence of ATP, this activity was actually reduced by the addition of ATP. An ATP-inhibitory effect has also been observed with the type I topoisomerase from *Ustilago maydis* (8).

Recently it was reported that another type I enzyme—the eukaryotic virus-encapsidated topoisomerase isolated from vaccinia virus—is stimulated by added ATP (9). We have investigated the effects of ATP (and other mononucleotides) on the purified type I topoisomerases prepared from HALL cells to elucidate the mechanisms of action as well as to determine differences in behavior between the nuclear and mitochondrial enzymes.

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MATERIALS AND METHODS

Materials. Mononucleoside triphosphates, diphosphates, and monophosphates were from Sigma as were adenosine 5′-[α,β-methylene]triphosphate (pp[CH_2]pA) and adenosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppA). [^{3}H]ATP was from New England Nuclear. Polyethyleneimine thin-layer plates were from MCB Chemical (Norwood, OH).

Superhelical pBR322 was prepared from *Escherichia coli* HB100 according to published procedures (10). Purified HeLa and calf thymus type I topoisomerases and calf thymus type II topoisomerase were gifts from Leroy Liu, and kinetoplast DNA was the gift of Paul Englund, both of The Johns Hopkins School of Medicine. Leukemia cells were generously provided by David Poplack, Pediatrics Oncology Division of the Clinical Center, National Institutes of Health.

Isolation of Topoisomerases. Centrifugations were performed at 4°C for 10 min in a Sorvall SS-34 rotor. HALL cells (3×10^7) were suspended in isolation buffer (0.25 M sucrose/10 mM Tris·HCl, pH 7.0/1 mM EDTA) and blended in a Waring blender (20 sec, low power). This homogenate was centrifuged at 1500 rpm. After decanting the supernatant (subsequently used to prepare mitochondria) the pellet was resuspended in 150 ml of isolation buffer containing 2.5 mM $MgCl_2$ and filtered through cheesecloth, and the nuclei were collected by centrifugation at 2000 rpm.

Nuclear Topoisomerase. The purified nuclei were suspended in 60 ml of lysing buffer (150 mM KP_i, pH 7.0/1.0 M NaCl/20% glycerol/10 mM 2-mercaptoethanol) and made 1 mM phenylmethylsulfonyl fluoride and 1% Triton X-100. The nuclei were lysed for 30 min at 0°C and treated with 0.5 vol of 18% PEG 6000 in 1 M NaCl/solution A, pH 7.0 (solution A is 20% glycerol/10 mM 2-mercaptoethanol). After gentle stirring for 30 min, the mixture was centrifuged at 10,000 rpm.

The PEG supernatant (1.36×10^6 units) was diluted with 2 vol of solution A (all column buffers are made in solution A and are at pH 7.0) and loaded onto a hydroxyapatite column (2.5 × 6.5 cm) equilibrated in 0.33 M NaCl/50 mM KP_i. After washing with 0.2 M KP_i, the column was developed with a linear gradient from 0.20 to 0.90 M KP_i. The topoisomerase I eluted between 0.56 and 0.69 M phosphate. Active fractions were combined (19.5 mg; 1.43×10^6 units), diluted with solution A to 0.2 M phosphate, and loaded onto a phosphocellulose column (0.8 × 3.5 cm) equilibrated in 0.2 M KP_i. The column was washed with and developed with 10-ml steps of 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 M KP_i. The enzyme eluted at 0.4 M KP_i.

The active fractions were combined (2.64 mg; 3.2×10^5 units), diluted with 1 vol of solution A, and loaded onto a Bio-Rex 70 column (1-ml bed volume) in 0.2 M KP_i. After washing in, the column was developed with 7.5-ml steps of 0.3, 0.4, 0.5, 0.6, and 0.7 M KP_i. Active fractions eluted at 0.5

Abbreviations: pp[CH_2]pA, adenosine 5′-[α,β-methylene]triphosphate; p[CH_2]ppA, adenosine 5′-[β,γ-methylene]triphosphate; HALL, human acute lymphoblastic leukemia.

M (860 μg; 1.9 × 10^5 units). Protein determinations of column fractions were made by the method of Bradford (11) with ovalbumin as standard. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (12) indicated that the enzyme had a molecular weight in agreement with that of the topoisomerase isolated from HeLa cells (13).

Mitochondrial Topoisomerase. The supernatant from the first slow spin was centrifuged 30 min at 10,000 rpm. The mitochondrial pellet was washed once, resuspended in 170 ml of isolation buffer, and loaded on gradients of 25%/42.5% sucrose in isolation buffer. The samples were centrifuged 1 hr at 19,000 rpm in a Beckman SW 27 rotor at 4°C. The mitochondrial band at the interface of the 25% and 42.5% sucrose was removed by pipet, diluted with an equal volume of 10 mM Tris·HCl, pH 7.0/0.1 mM EDTA, and collected by centrifugation at 12,000 rpm for 30 min. The pellets were resuspended in isolation buffer (7.5 ml), the protein content was determined by biuret analysis (14) (41.6 mg), and the mitochondrial outer membrane was stripped off as described (7) using digitonin (1 mg of digitonin/8 mg of mitochondrial protein) according to the procedure of Schnaitman and Greenawalt (15). The mitoplast pellet was washed once, resuspended in 5 ml of lysing buffer, lysed with 1% Triton X-100 for 15 min at 0°C, and precipitated with PEG as above.

The PEG supernatant (27.3 mg; 2.3 × 10^2 units/mg) was loaded onto a hydroxyapatite column (2.5 × 6.5 cm) in 0.33 M NaCl/6% PEG/50 mM KP_i. The column was washed with 0.2 M KP_i and eluted with 5-ml steps of 0.3, 0.4, 0.6, 0.8, and 0.9 M KP_i. The topoisomerase I eluted in the 0.6 M step.

Active fractions were combined (1.4 mg; 6300 units), diluted with solution A to 0.2 M KP_i, and loaded on a Bio-Rex 70 column (1-ml bed volume). After washing with 0.2 M KP_i, the column was developed with 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 M KP_i. The topoisomerase I eluted with 0.6 M phosphate (67 μg; 4000 units). Denaturing polyacrylamide gel electrophoresis showed a major polypeptide band at 60,000 daltons, as reported (5).

The degree of mitochondrial purification was assessed by following certain marker enzymes. In particular, assays of DNA polymerase α (a nuclear enzyme) and γ (the mitochondrial polymerase) were performed at various stages as described (7). Typically, the γ/α activity ratio was >20:1 when assays were performed using mitoplasts from gradient-purified mitochondria. This ratio is comparable to values published by investigators in other mitochondrial systems (16, 17).

Topoisomerase Assays. Type I topoisomerase was measured by the relaxation of superhelical pBR322. The 10-μl assay mixture contained 40 mM Tris·HCl (pH 7.0), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, bovine serum albumin (30 μg/ml), pBR322 (20 μg/ml), and varying amounts of enzyme. After 30 min at 37°C, the reactions were terminated by addition of 1.5 μl of 5% $NaDodSO_4$/25% (wt/vol) sucrose/0.025% bromophenol blue. The products were separated by electrophoresis and visualized as described (18). One unit of activity is the amount of topoisomerase that relaxes 50% of the substrate DNA under the specified conditions.

ATPase Assays. The enzyme preparation was tested for ATPase activity essentially according to Foglesong and Bauer (9). The [^{3}H]ATP was added to the standard topoisomerase assay and unlabeled nucleotide was added to the final desired concentration—e.g., 1 mM. After 30 min an aliquot was removed and spotted on a fluorescent indicator-impregnated polyethyleneimine plate. A mixture of unlabeled ATP/ADP/AMP was also spotted and the plate was developed with 0.5 M sodium phosphate (pH 7.0). After drying, the nucleotides were visualized by UV illumination, scraped off the plate, and assayed for radioactivity in a Packard scintillation counter.

Calculation of Percent Inhibition. The photographic negative of each gel was traced by using a Quick Scan Jr. densitometer (Helena Laboratories, Beaumont, TX). The area under each peak was computed with a Hewlitt–Packard digitizer. From the control (without enzyme) sample, the fraction of open circular DNA and fraction of supercoiled DNA were calculated essentially as described by Osheroff *et al.* (19). The percent relaxed DNA for the enzyme-treated sample in the absence or presence of inhibitor, normalized for any variation in total DNA loaded in any gel lane, is given by the following equation. Activity = % relaxed = [(area relaxed/total area) − fraction open circular/fraction supercoiled] × 100.

RESULTS

Effects of ATP on Nuclear and Mitochondrial Type I Topoisomerase. Topoisomerase assays using gel electrophoresis to monitor relaxation of supercoiled pBR322 were performed in the absence and presence of increasing concentrations of ATP. Fig. 1 and Table 1 show the results of such assays. There was a marked reduction of relaxation as the ATP concentration was increased from 0 to 10 mM (Fig. 1*A*). The nuclear topoisomerase activity was reduced 50% at 1.0 mM ATP, and relaxation was completely inhibited by 5.0 mM ATP. The amount of enzyme was adjusted so that ≈2 units of relaxation were obtained in the absence of ATP.

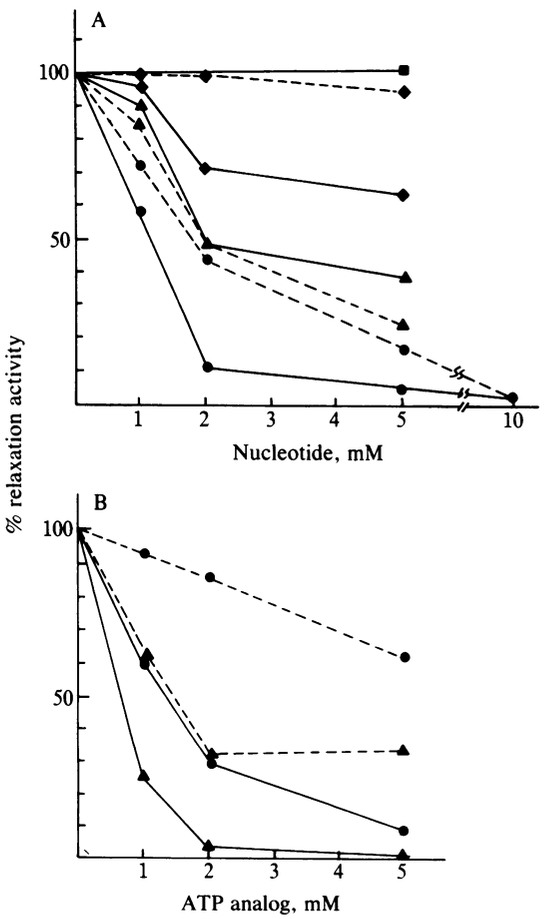

FIG. 1. Effect of nucleotides upon the relaxation activity of nuclear and mitochondrial type I topoisomerases. (*A*) Ribo- and deoxyribonucleotides. ●, ATP; ▲, dATP; ◆, ADP; ■, UTP, CTP, GTP, dCTP, dGTP, and AMP. (*B*) Nonhydrolyzable analogs of ATP. ●, pp[CH_2]pA; ▲, p[CH_2]ppA. —, Nuclear enzyme; – – –, mitochondrial enzyme.

Table 1. Concentrations of nucleotides that inhibit DNA topoisomerase relaxation activity by 50%

Nucleotide	Topoisomerase I, mM	
	Nuclear	Mitochondrial
ATP	0.60	0.90
dATP	1.0	1.0
ADP	5.0	ND
AMP	ND	ND
GTP, CTP, UTP	ND	ND
dGTP, dCTP	ND	ND
pp[CH_2]pA	0.70	3.5
p[CH_2]ppA	0.30	0.80

ND, no significant inhibition detectable at concentrations up to 10 mM.

After correcting for the actual amount of enzyme activity used in these assays, it was found that 0.60 mM ATP was sufficient to inhibit 1 unit of nuclear topoisomerase I by 50%. Similar results were obtained with the mitochondrial enzyme; in this case, 50% inhibition was obtained with 1.8 mM ATP. After normalization, it was found that 0.90 mM ATP inhibits 1 unit by 50%.

To determine whether this enzyme–nucleotide interaction is specific for ATP, we tested the effects of other adenine nucleotides. The substitution of dATP for ATP resulted in similar patterns of inhibition for both enzymes (Fig. 1*A*): 1 unit of the mitochondrial topoisomerase was inhibited 50% by 1.0 mM dATP, which is close to the value obtained for ATP; 1 unit of the nuclear relaxing activity was also reduced 50% by 1.0 mM dATP, which is slightly higher than the value obtained with ATP.

The nuclear enzyme was inhibited by ADP but only at more than an 8-fold higher concentration (1 unit of activity was decreased 50% by ≈5.0 mM ADP), whereas ADP was essentially ineffective in reducing relaxation by the mitochondrial topoisomerase. No inhibitory effect on either enzyme was observed with AMP. Phosphate ion alone at pH 7.0 showed no inhibitory effect up to 5.0 mM KP_i (data not shown). The ribose triphosphates GTP, CTP, and UTP as well as the deoxyribose triphosphates dGTP and dCTP exerted no inhibitory effect on either enzyme.

ATP Inhibition Does Not Involve Hydrolysis of ATP. To determine if ATP hydrolysis is required for topoisomerase inhibition, we performed assays in the presence of the nonhydrolyzable ATP analogs pp[CH_2]pA and p[CH_2]ppA. Results of assays in the presence and absence of the nonhydrolyzable ATP analogs are shown in Fig. 1*B*. After normalization, it was found that the nuclear enzyme was inhibited 50% by 0.70 mM pp[CH_2]pA and by 0.30 mM p[CH_2]ppA. These values are roughly equivalent to the value obtained with ATP. On the other hand, the mitochondrial topoisomerase was somewhat more sensitive, relative to ATP, to inhibition by p[CH_2]ppA (0.80 mM) but much less sensitive to inhibition by pp[CH_2]pA (3.5 mM).

Thus, the inhibition of topoisomerase activity by ATP does not involve hydrolysis. This conclusion was supported by another experiment. Fig. 2 shows the results of electrophoresis in 0.7% agarose of aliquots of nuclear topoisomerase assays. In the absence of ATP (lane b), 2 units of enzyme activity completely relaxed the substrate DNA. In the presence of ATP, however, there was an increasing amount of inhibition: at 1.0 mM ATP there was ≈50% activity and at 5.0 mM ATP there was essentially complete inhibition. When aliquots of these same assays were fractionated by thin-layer chromatography, there was no evidence that inhibition required ATP hydrolysis (Table 2). In the control sample, 88.1% of the total radioactivity was found in the ATP, with 8.4% and 3.5% found in ADP and AMP, respectively, after

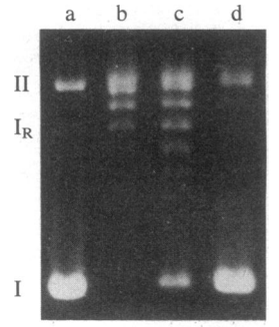

FIG. 2. Agarose gel electrophoresis of nuclear topoisomerase I reaction products in the presence and absence of ATP. Lane a, control pBR322 with no enzyme added; lane b, pBR322 with topoisomerase I in the absence of ATP; lane c, same as lane b but with 1 mM ATP added; lane d, same as lane b but with 5 mM ATP added. The DNA band assignments are as follows: I, supercoiled; II, open circular and/or completely relaxed; I_R, relaxed closed circular DNA containing only a few supertwists.

incubation under standard assay conditions. In the presence of enzyme, there was still no change in this distribution. With 1 and 5 mM ATP, still 88.5% and 88.3%, respectively, of the radioactivity were found in the triphosphate and there were no increases in the radioactivity associated with ADP or AMP. These experiments confirm that hydrolysis of ATP is not necessary for inhibition of topoisomerase I.

Although no enzyme with DNA-gyrase-like supercoiling activity has been isolated from eukaryotic cells, the above ATPase and nonhydrolyzable ATP analog experiments argue against the faint possibility that such an enzyme might be contaminating our preparations. If this had been the case, supercoiling of any relaxed DNA would require ATP hydrolysis. This was not observed in the first case and could not occur in the latter case. Furthermore, as described below, purified type I enzymes from another laboratory gave similar results.

Other Type I Topoisomerases May Be Regulated by ATP. Highly purified type I topoisomerases from calf thymus and HeLa cell nuclei were also tested for ATP sensitivity. Serial dilutions of these homogeneous enzymes were performed in the absence and presence of 5 mM ATP or GTP. Results, shown in Fig. 3, indicate that the HeLa cell enzyme was completely inhibited by 5.0 mM ATP. The same result was obtained with the calf enzyme (data not shown). As a negative control, GTP was found to have no inhibitory effect (lanes f and g). These results are in agreement with the results obtained with the leukemia cell nuclear and mitochondrial enzymes.

Table 2. Distribution of radioactivity among ATP hydrolysis products after topoisomerase assays

Added nucleotide, mM	Radioactivity, cpm			Total cpm
	ATP	ADP	AMP	
Control*	19,950 (88.1)	1899 (8.4)	795 (3.5)	22,644
ATP				
1	13,765 (88.5)	1287 (8.3)	495 (3.2)	15,547
5	11,439 (88.3)	1100 (8.5)	422 (3.2)	12,961

The number in parenthesis is the % of total cpm that was present in each species. This normalizes for differences in total cpm loaded onto the polyethyleneimine plate.

*This sample had no enzyme added but contained pBR322 and 1.0 mM ATP. This final ATP concentration was the result of mixing 1 mM ATP (unlabeled) and 1.68 μM [^{3}H]ATP (specific activity, 29.7 Ci/mol).

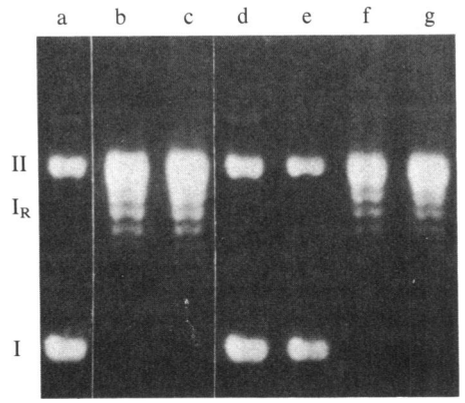

FIG. 3. Assays in the presence of ATP of topoisomerase I from HeLa cells. Lane a, control (without enzyme); lane b, 1 μl of 1:50 topoisomerase; lane c, 1 μl of 1:100 topoisomerase; lane d, same as lane b with 5 mM ATP; lane e, same as lane c with 5 mM ATP; lane f, same as lane b with 5 mM GTP; lane g, same as lane c with 5 mM GTP. See Fig. 2 for DNA band assignments.

DISCUSSION

Although type I topoisomerases do not require an energy source to relax supercoiled DNA, we have found that ATP serves to inhibit enzyme-mediated relaxation. Nuclear and mitochondrial activities were reduced to comparable extents—0.60 mM ATP and 0.90 mM ATP inhibited the respective enzymes by 50%. ADP was also inhibitory, albeit at severalfold higher concentrations; AMP up to 10 mM did not reduce topoisomerase relaxing activity. These results suggest that the topoisomerase has a nucleotide-binding site that can accommodate ATP. Because it possesses one less phosphate group the ADP fits more loosely or less frequently into this putative binding site and the change in enzyme conformation brought about by the nucleotide occurs less often or completely and only at higher ADP concentrations. AMP, possessing two fewer phosphates, would fit so loosely into this site that it is completely ineffective in inducing this conformational change. Such a conformational change is specific for the adenine nucleotides since GTP, CTP, UTP, dGTP, and dCTP did not inhibit the topoisomerases to any extent, even at high concentrations.

Generally the nuclear and mitochondrial enzymes behaved similarly except for one instance. Whereas the nuclear enzyme was inhibited 50% by 5.0 mM ADP, a concentration eight times greater than that of ATP, the mitochondrial enzyme was insensitive to as much as 10 mM ADP. This difference suggests that an interaction between the γ phosphate in ATP and the mitochondrial enzyme may be important in effecting the correct conformational changes leading to decreased enzyme activity. This is further supported by the results with p[CH_2]ppA and pp[CH_2]pA. The presence of the methylene group in these analogs alters the bond angle about the grouping of atoms from 129° for P-O-P to 117° for P-C-P (20). This altered conformation about the β,γ bond seems not to affect the enzyme–nucleotide interaction for either enzyme, but the change in the α,β bond in pp[CH_2]pA (which also alters the β,γ bond) quite strongly affects the interaction for the mitochondrial enzyme. The level of analog necessary to inhibit 50% (3.5 mM) almost approaches that of ADP (5.0 mM), which lacks the γ phosphate group altogether.

The inhibition by ATP appears to require only binding of ATP by the enzyme. Hydrolysis of ATP is apparently not necessary since nonhydrolyzable analogs of ATP inhibit topoisomerase activity as effectively as ATP. In addition, from the same assays shown by electrophoresis to be strongly inhibited by added ATP, we find no evidence of ATP hydrolysis products (Table 2). Further experiments are necessary to determine where on the enzyme this ATP-binding site may be.

Finally, these experiments implicate a role for ATP in regulating the activity of DNA topoisomerase *in vivo*. The concentration of ATP in mitochondria has been reported to range from 0.6 to 6.0 mM (21–23), so that the values found to be inhibitory to the mitochondrial topoisomerase in this study are certainly within the physiological range. Thus, variations in ATP concentration within the mitochondrion could turn on or off topoisomerase activity. Analogously, ATP levels in the nucleus could help regulate the activities of the type I topoisomerase in that organelle: the enzyme being turned on at low concentrations and then being increasingly shut down as ATP levels become elevated. This form of modulation by ATP would suggest that the two topoisomerases possess nucleotide-binding sites through which conformational changes that affect protein–DNA interactions, and thereby catalytic activity, are transmitted.

It was been observed recently that ATP (10 mM) reduces DNA binding by topoisomerase I from chicken erythrocytes (24). On the other hand, yeast topoisomerase I activity has been reported to be unaffected by up to 5 mM ATP (25). Our observations that the leukemia cell topoisomerases as well as the purified HeLa and calf thymus enzymes are ATP-sensitive suggest that this phenomenon may be a general feature of the mammalian enzymes, possibly responsible for regulation of competing intracellular topoisomerase activities. Our ATPase result, as well as the fact that purified enzymes from different laboratories were used, argues against the possibility that impurities in the enzyme preparation could be responsible for some indirect process—e.g., protein phosphorylation, which could mediate topoisomerase activity. Further studies to elucidate this ATP regulatory effect are necessary.

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