DNA Topoisomerase II from Drosophila melanogaster

RELAXATION OF SUPERCOILED DNA*

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In order to study the double-strand DNA passage reaction of eukaryotic type II topoisomerases, a quantitative assay to monitor the enzymic conversion of supercoiled circular DNA to relaxed circular DNA was developed. Under conditions of maximal activity, relaxation catalyzed by the Drosophila melanogaster topoisomerase II was processive and the energy of activation was 14.5 kcal·mol⁻¹. Removal of supercoils was accompanied by the hydrolysis of either ATP or dATP to inorganic phosphate and the corresponding nucleoside diphosphate. Apparent $K_m$ values were 200 μM for pBR322 plasmid DNA, 140 μM for SV40 viral DNA, 280 μM for ATP, and 630 μM for dATP. The turnover number for the Drosophila enzyme was at least 200 supercoils of DNA relaxed/min/molecule of topoisomerase II. The enzyme interacts preferentially with negatively supercoiled DNA over relaxed molecules, is capable of removing positive superhelical twists, and was found to be strongly inhibited by single-stranded DNA. Kinetic and inhibition studies indicated that the $\beta$ and $\gamma$ phosphate groups, the $2'$-OH of the ribose sugar, and the $\text{C}_2\text{NH}_2$ of the adenine ring are important for the interaction of ATP with the enzyme. While the binding of ATP to Drosophila topoisomerase II was sufficient to induce a DNA strand passage event, hydrolysis was required for enzyme turnover. The ATPase activity of the topoisomerase was stimulated 17-fold by the presence of negatively supercoiled DNA and approximately 4 molecules of ATP were hydrolyzed/supercoil removed. Finally, a kinetic model describing the switch from a processive to a distributive relaxation reaction is presented.

Type II topoisomerases are enzymes which catalyze changes in the topological structure of DNA by the generation of transient double-stranded breaks in the DNA backbone (1-6). Activity has been detected in many diverse sources. Among these are bacteria (7-11), bacteriophages (12, 13), yeast (14), insects (15), amphibians (16), and mammals (17-21). Since the first type II enzyme, DNA gyrase, was isolated from Escherichia coli in 1976 (7), a large body of information concerning the properties, activities, and functions of prokaryotic type II topoisomerases has accumulated (1-6). Unfortunately, comparatively little is known about the eukaryotic proteins.

Although the topological state of DNA in bacteria has been shown to affect replication, repair, recombination, and transcription (4-6, 22), the role of DNA topology in eukaryotic species is not as yet understood. Clearly, before the cellular functions of eukaryotic type II topoisomerases can be properly determined, their in vitro activities must be well characterized. The recent purification of these enzymes from eukaryotic species (14, 20, 23) has made such characterizations possible.

Fundamental to all the in vitro interactions which have been ascribed to type II topoisomerases is their ability to resolve interlocking constraints of DNA by the double-strand passage mechanism (1-6). The most straightforward reaction which can be employed to monitor this central function is the interconversion between superhelical and relaxed forms of circular DNA. This paper describes the development of a quantitative assay which has been used to follow the ATP-dependent relaxation of supercoiled circular DNA by topoisomerase II from the fly, Drosophila melanogaster. Reaction parameters, kinetic constants, requirements for the interaction of DNA and ATP with the enzyme, and the relationship between ATP hydrolysis and DNA relaxation are discussed. A kinetic model describing the processive versus distributive nature of the reaction is also presented. Results are compared with those obtained for the supercoiling-relaxation and ATPase reactions of E. coli DNA gyrase (24, 25), the prototypical type II enzyme found in bacteria.

EXPERIMENTAL PROCEDURES

Drosophila melanogaster DNA topoisomerase II was purified from the nuclei of 6- to 18-h-old embryos by the procedure of Shelton et al. (23). Bacterial plasmid DNAs were prepared by a Triton X-100 lysis followed by double banding in cesium chloride-ethidium bromide gradients (26). SV40 viral DNA was prepared by the procedure of Hirt (27) followed by centrifugation through a cesium chloride-ethidium bromide density gradient and a neutral sucrose gradient (28). ATP, ADP, AMP, GTP, CTp, UTP, ITP, XTP, dATP, dGTP, dCTP, and dTPP were obtained from P-L Biochemicals; coumarinyl Ac, novobiocin, nalidixic acid, Tris, APP(CH₂)P, AP(CH₃)PP, PPP₃, and bovine serum albumin (fraction V) from Sigma; adenine, adenosine, and ethidium bromide from Calbiochem; APP(NH₄)PP and ATPγS from Boehringer Mannheim; PP, from J. T. Baker Chemical Co.; poly-L-glutamic acid from Miles-Yeda (Israel); proteinase K from Beckman; adenosine 5'-[γ-P³²P] or 5'-[β-P³²P] triphosphate and cytidine 5'-[β-P³²P] triphosphate from Amersham. Oxolinic acid was a gift from J. D. Stein (Warner-Lambert Research Institute, Morris Plains, N.J.).

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The abbreviations used are: APP(CH₃)P, adenosine 5'-β,γ-methylene-triphosphate; AP(CH₃)PP, adenosine 5'-β, γ-methylene-triphosphate; APP(NH₄)PP, adeny 5'-yl-imidodiphosphate; ATPγS, adenosine 5'-O-(3-thiotriphosphate); Pipes, 1,4-piperazinediethanesulfonic acid.
Relaxation of Supercoiled DNA by Topoisomerase II

The standard reaction mixture contained 0.1 pmol (0.3 μg) of supercoiled plasmid DNA, 0.5 to 4.0 pmol of Drosophila topoisomerase II (35 to 280 units), and 1 mM ATP (NH)P in a total of 20 μl of relaxation buffer. Samples were incubated at 30 °C for 6 min, followed by the addition of 3 μl of 0.77% sodium dodecyl sulfate, 77 mM EDTA. Proteinase K (2 μl of a 625 μg/ml solution in 50 mM Tris-Cl, pH 7.9, 1 mM CaCl2) was added and digestion was at 37 °C for 30 min. Reaction products were resolved by electrophoresis on agarose gels as described above. When cofactors other than ATP(NH)P were employed, their concentrations were at least 2-fold and usually 10-fold higher than their Kᵦ values (as reported in Table II).

ATPase Activity of Topoisomerase II—Prior to assay, topoisomerase II was freshly diluted to a concentration of 5 units/μl in the dilution buffer described above. The standard reaction mixture contained 5 units of enzyme, 15 μg of DNA (1.15 × 10⁸ M base pairs of double-stranded or 2.30 × 10⁸ M bases of single-stranded molecules), and 1 mM γ-[³²P]ATP (0.02 Ci/mmol) in a total of 20 μl of relaxation buffer. Reaction was at 30 °C. Samples (2.5 μl) were removed 2 or 4 min intervals up to 16 min, spotted onto thin layer cellulose plates impregnated with polyethyleneimine (Polygram CEL 300 PEI, Brinkmann) and chromatographed in freshly made 0.4 M NH₄HCO₃, ATP, ADP, and AMP standards were visualized by fluorescence, while ³²P standards and reaction products were visualized by autoradiography with Kodak XAR film. Radioactive areas corresponding to reaction products were cut out of the chromatograms and the rate of ATP hydrolysis was quantitated employing a Beckman LS-230 liquid scintillation counter and toluene-based scintillation fluid. When necessary, the corresponding rate of relaxation of supercoiled DNA was determined as described above.

RESULTS

The time course for the relaxation of 0.3 μg of negatively supercoiled circular pBR322 plasmid DNA (2.3 × 10⁸ M base pairs) by 1 unit of D. melanogaster topoisomerase II is shown in Fig. 1. The velocity is linear for the first 8 min of the reaction, and for at least that long at higher DNA concentrations. All subsequent initial velocities were calculated for time points up to 6 min, well within the linear range for every condition examined. The fact that as much as 75% of the supercoiled DNA is relaxed during the linear period indicates that the enzyme interacts more strongly with its substrate, supercoiled DNA, than with relaxed DNA, which is the product of the reaction.

Under optimal conditions, the relaxation reaction is extremely processive. As can be seen in the agarose gel presented in Fig. 1, substrate and final product distributions predominate and virtually no reaction intermediates are visible. In contrast, distributive reactions where DNA intermediates are prevalent were observed only under suboptimal conditions (see below). Thus, under the assay conditions employed, one measurable event is the complete relaxation of the bacterial plasmid, pBR322. This corresponds to the removal of approximately 30 supercoils/molecule of DNA (12).

Requirements for Relaxation

Ionic Strength Dependence—The ionic strength dependence of the topoisomerase II reaction is shown in Fig. 2A. The Drosophila enzyme exhibited a broad maxima which was centered between salt concentrations of 100 and 150 mM. The reaction proceeded in a processive manner below 150 mM ionic strength, but became markedly distributive at 175 mM.

Magnesium Dependence—Purified Drosophila topoisomerase II has an absolute requirement for magnesium and showed optimal activity between 5 and 10 mM (Fig. 2B). Relaxation was processive at concentrations lower than 10 mM, but increased levels of magnesium resulted in a distributive reaction. Clearly, the need for magnesium ions reflects more than the requirement for a Mg₂⁺ ATP substrate, since at a concentration of 2.5 mM magnesium, approximately 97% of

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2 Segraves, W. A., personal communication.
the ATP should be complexed (32), but the enzymic activity is only 50% that of the optimal rate. Over the concentration range examined (2.5 to 25 mM), manganese and calcium showed no activity.

**pH Dependence**—No relaxation of DNA was observed below pH 6.5 in acetate, Pipes, or Tris buffers. In Tris, activity increased rapidly between pH 6.5 and 7.5, and plateaued up to pH 10, the highest pH examined (Fig. 2C). No distributive reaction was observed. The activity profile in glycine buffer was much different, showing no activity below pH 8 or above pH 10.5, with a sharp maximum at pH 9.5. Relaxation became distributive at pH 10.

**Temperature Dependence and Thermal Stability**—Maximal rates of activity were obtained at 30 °C (Fig. 2D) and the reaction remained processive over the entire active temperature range. The subsequent Arrhenius plot (30) (not shown) was linear from 15 to 30 °C and indicated an energy of activation for the topoisomerase II relaxation reaction of 14.3 kcal·mol⁻¹.

The thermal stability of the *Drosophila* enzyme in the absence of DNA was studied at temperatures ranging from 15 to 45 °C (Fig. 3). The energy for the thermal inactivation of the topoisomerase II is 13.4 kcal·mol⁻¹. Over the course of a 6-min incubation at 30 °C (standard assay conditions), less than 4% of enzymic activity was lost. The low rates of relaxation obtained at elevated temperatures cannot be entirely explained by thermal inactivation. Indeed, at 35 and 40 °C, enzyme activity dropped by 30 and 80%, respectively, (Fig. 2D), as compared to the maximal reaction rate obtained at 30 °C, while the thermal stability study (Fig. 3) indicated that at these two temperatures, only 6 and 12% of the topoisomerase II should have been inactivated over the course of a 6-min assay. Therefore, either the lowered rates of relaxation at high temperatures are due to a change in the catalytic mechanism or thermal inactivation is greatly exacerbated by the enzymic reaction.

**Kinetics of Relaxation of Supercoiled DNA**

Since the relaxation of supercoiled circular DNA by the *Drosophila* topoisomerase II is coupled to the hydrolysis of ATP (15) (see below), the effects of both substrates on the kinetics of the reactions were studied. Results are displayed in Eadie-Hofstee single reciprocal plots (Figs. 4 and 5), in which the negative reciprocal slope of the line is the apparent

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Michaelis constant ($K_m$) and the intercept with the abscissa is the maximal velocity ($V_{max}$) (30).

**Effect of DNA Concentration**—The kinetics of relaxation of negatively supercoiled circular DNA by the *Drosophila* topoisomerase II are shown in Fig. 4. The apparent $K_m$ for the bacterial plasmid pBR322 was $2.0 \times 10^{-4}$ M and the $V_{max}$ was approximately $2 \times 10^{-9}$ M base pairs of supercoiled DNA relaxed/min/unit of enzyme. This corresponds to a turnover number of about 200 supercoils relaxed/min/molecule of topoisomerase II. As this number is based on the assumption that every molecule of topoisomerase II is active, it must be considered a lower limit for the activity of the enzyme.

Under standard assay conditions, with $4.6 \times 10^{-5}$ M base pairs of pBR322 plasmid DNA and 1 unit of topoisomerase II, the enzyme:plasmid ratio is at least 1:15, well within the limits of the steady state assumption (i.e. enzyme << substrate) (30). In addition, Michaelis-Menten kinetics were observed at ratios as low as 1:7. However, at an enzyme:plasmid proportion of about 1:4, the kinetics of relaxation diverged from linearity and exhibited initial velocities approximately 2-fold higher than predicted.

The relaxation of negatively supercoiled circular DNA from the eukaryotic virus SV40 by the *D. melanogaster* type II topoisomerase was also examined (Fig. 4). The enzyme showed a somewhat higher affinity for the viral DNA (apparent $K_m = 1.4 \times 10^{-4}$ M) as compared to that for the prokaryotic pBR322 plasmid DNA. Whether this results from an interaction between the enzyme and a specific site on the eukaryotic DNA, or simply an effect of general base composition is unknown. However, in regard to the latter, SV40 DNA has a much higher A-T content (59.2%) (33, 34) than pBR322 DNA (46.5%) (35).

**Effect of Nucleoside Triphosphate Concentration**—The ATP and dATP dependence of the kinetics of relaxation of negatively supercoiled DNA is presented in Fig. 5. A low DNA concentration was employed for this study in order to overcome the difficulties of quantitating the small percentage of total DNA which is relaxed under conditions of high DNA and low ATP concentrations. This accounts for the relatively low maximal velocities which are observed. Although the *Drosophila* enzyme is able to utilize either ATP or dATP as substrate (15), the affinity of ATP for the topoisomerase is approximately twice that of dATP, showing an apparent $K_m$ value of $2.8 \times 10^{-4}$ M as compared to $6.3 \times 10^{-4}$ M. Thus, the 2’-OH of the ribose ring imparts some specificity to the interaction of the high energy cofactor with the enzyme. Clearly, the absence of the 2'-OH does not affect the rate of hydrolysis of the bound nucleotide, as the kinetic plots for both ATP and dATP extrapolate to the same $V_{max}$ value. Substrate inhibition was observed for both the ribo- and deoxyribonucleotides above 1.5 mM. In the absence of ATP or dATP, no relaxation was observed even with a 300-fold increase in enzyme concentration. The purified topoisomerase could not be activated with any other nucleoside triphosphate tested, including GTP, CTP, UTP, XTP, dGTP, dCTP, and dTTP. Moreover, no catalytic activity was found with analogues of ATP that have nonhydrolyzable δ-γ phospho-analogue bonds or improper conformations about their δ and/or γ-phosphatate bonds or improper conformations about their δ and/or γ-phosphatate groups, such as APP(NH)P, ATPγS, APP(CH3)P, and AP(CH3)PP. This is despite the fact that many of these nucleotides interact strongly with the ATP binding site of the *Drosophila* enzyme (see Table I).

**Effect of DNA Length**—To ascertain the effect of DNA length on the relaxation reaction, two deleted forms of the bacterial plasmid pBR322 (35), pBR327 (36), and pAG12 were employed. It should be noted that at any given DNA concentration, irrespective of plasmid size, the total number of supercoils and DNA base pairs present in the assay mixture was always constant. However, decreases in plasmid length resulted in corresponding increases in the number of molecules present.

Three major observations resulted from this study (Fig. 5 and Table I). First, within the homologous series employed, plasmid size had no effect on the apparent $K_m$ of the relaxation reaction. Second, the maximal velocity of the reaction was found to be directly proportional to the length of the DNA being assayed. Finally, the turnover number of the *D. melanogaster* topoisomerase II, expressed as the number of plasmid molecules relaxed/min/molecule of enzyme, remained constant, despite the fact that the smaller DNAs have correspondingly fewer supercoils/molecule. Therefore, when the enzyme operates processively, the velocity of relaxation must...
FIG. 6. The effect of DNA length on the topoisomerase II relaxation reaction. Bacterial plasmids pBR322 (4362 bp) (), pBR327 (3273 bp) (C), and pAG1 (2299 bp) (•) were employed. Reaction conditions are described in the legend to Fig. 4. bp, base pairs.

**TABLE I**

| Plasmid | Size  | Relative length | Relative \( V_{max} \) | Turnover number* |
|---------|-------|----------------|-------------------------|-----------------|
| pBR322  | 4362  | 100            | 100                     | 6.3             |
| pBR327  | 3273  | 75             | 77                      | 6.5             |
| pAG1    | 2299  | 53             | 51                      | 6.1             |

*Expressed as the number of plasmid molecules relaxed/min/molecule of topoisomerase II. Turnover number = \( V_{max} \) (plasmid length in base pairs - enzyme concentration).

be related to the rate of dissociation of the topoisomerase II-relaxed DNA complex. Since smaller plasmids obviously contain fewer base pairs of DNA/molecule, this accounts for the observed proportional decrease in \( V_{max} \) values.

Relaxation of Positively Supercoiled DNA—In the presence of ethidium bromide, fully relaxed circular DNA can be induced to form positive superhelical turns (31). If this positively supercoiled DNA is subsequently relaxed with a topoisomerase and the ethidium bromide is removed, the resulting structure will show a net gain of negative supercoils. This reaction is reflected by an increased electrophoretic mobility of the DNA when applied to agarose gels.

By employing the strategy described above, the Drosophila topoisomerase II was found to relax positive supercoils (not shown). Moreover, at low concentrations of DNA, the initial velocities of relaxation were comparable to those found for negatively supercoiled molecules. Unfortunately, the highest DNA concentration which could be employed for this study was \( 6.0 \times 10^{-5} \) M, as increased levels required the inclusion of prohibitively high amounts of ethidium bromide.

**Inhibition of Relaxation**

Table II lists the \( K_i \) values for a number of inhibitors of the Drosophila type II topoisomerase. The site of inhibition was determined by the ability of the compound to compete with either ATP or DNA as judged by Eidg-Hofstee analysis (30).

Coumermycin A\(_1\) and novobiocin, both potent inhibitors of the ATPase reaction of the prokaryotic type II topoisomerase, DNA gyrase (24, 37), inhibited the Drosophila topoisomerase II, but at concentrations 3 to 4 orders of magnitude higher (Table II). Oxolinic acid and nalidixic acid, which inhibit the interaction of DNA with gyrase (38-40), also affect the eukaryotic enzyme, but once again much higher levels are required. It was originally reported (15) that the Drosophila enzyme was insensitive to nalidixic acid. However, in that study the maximal concentration of inhibitor employed was less than one-third of the determined \( K_i \) value, making inhibition difficult to detect.

Relaxation of DNA was strongly inhibited by micromolar levels of the ATP analogues ATP\(_y\)S and APP(NH)P (Table II), both of which have conformations about their \( \beta-\gamma \) phosphate bonds which are nearly identical to that of ATP (41, 42). Inhibition by ADP was approximately one order of magnitude less efficient and no inhibition was observed with AMP, adenosine, or adenine. These results indicate that the majority of binding interactions between ATP and the enzyme involve the \( \beta \) and \( \gamma \) phosphate groups of the nucleotide, with little or no contribution from the \( \alpha \) phosphate. This was supported by the finding that PPP, had a \( K_i \) value which was comparable to those of PP, and imidodiphosphate.

Steric requirements for the interaction of phosphate groups with the nucleoside triphosphate binding site of Drosophila topoisomerase II were analyzed by using the \( \beta-\gamma \) and \( \alpha-\beta \) methylene analogues of ATP as inhibitors of the relaxation reaction (Table II). The spatial arrangements of the carbon-phosphate bonds in these derivatives differ from those of the corresponding phosphate linkages in ATP, since the bond angle of \( P-C-P \) (117°) is more acute than that of \( P-O-P \) (129°) (42). Thus, the \( K_i \) of APP(CH\(_2\))P, which has an altered conformation about the \( \beta-\gamma \) bond, was similar to that of ADP and the \( K_i \) for AP(\( CH_2\))PP, in which the spatial orientations of both the \( \beta \) and \( \gamma \) phosphates are affected by the \( \alpha-\beta \) methylene bond, was an order of magnitude higher.

**Table II**

| Inhibitors of the topoisomerase II relaxation reaction | \( K_i \) | Site of inhibition |
|------------------------------------------------------|---------|------------------|
| Drugs                                                 | \( \mu M \) |                  |
| Coumermycin A\(_1\)                                   | 10      | ATP              |
| Novobiocin                                            | 210     | ATP              |
| Oxolinic acid                                         | 1300    | DNA              |
| Nalidixic acid                                        | 2700    | DNA              |
| ATP analogues                                         |         |                  |
| ATP\(_y\)S                                            | 8       | ATP              |
| APP(NH)P                                              | 10      | ATP              |
| APP(CH\(_2\))P                                        | 140     | ATP              |
| AP(\( CH_2\))PP                                       | 1200    | ATP              |
| Nucleotides                                           |         |                  |
| ADP                                                   | 120     | ATP              |
| AMP, Adenosine, Adenine                               |        |                 |
| ITP                                                   | 360     | ATP              |
| XTP, GTP                                              |        |                 |
| CTP                                                   | 100     | ATP              |
| UTP                                                   | 1500    | ATP              |
| Polyphosphates                                        |         |                  |
| PPP                                                   | 1400    | ATP              |
| PP                                                     | 2000    | ATP              |
| P(NH)P\(^b\)                                          | 1500    | ATP              |
| Single-stranded DNA:                                   |         |                  |
| \( \delta X 174 \)                                     | 0.8     | DNA              |
| M13-KB \( H_{mot} \)                                  | 0.9     | DNA              |
| Polyampholyptides                                     |        |                 |
| Poly-L-glutamic acid                                   |        |                 |

*No observable inhibition at concentrations up to \( 3 \) mM.

\(^b\) Molarities are expressed in nucleotide equivalents.
of the adenine base is important for the interaction of ATP with the Drosophila type II topoisomerase. This conclusion is based on the following two findings. 1) ITP, which is identical to ATP except for the replacement of the C6 amino on the purine ring with a ketone, had a $K_i$ value of 360 $\mu$M, approximately 40-fold higher than those observed for APP(NH)$_2$P and ATP$\gamma$S. 2) CTP, which has an amino group at the C4 position of the pyrimidine ring (corresponding to the C6 position of purine rings), had a $K_i$ value of 100 $\mu$M, 15 times lower than that for UTP, which carries a C4 ketone.

The relaxation of supercoiled molecules is strongly inhibited by single-stranded DNA (Table II). Nucleic acids from bacteriophage $\phi$X174 and M13-KB H$_{su}$ (29) (a derivative of bacteriophage M13 which contains a cloned 740-base pair fragment of human mitochondrial DNA including the heavy strand origin of replication) were utilized for this study. These single-stranded DNAs both competed with supercoiled double-stranded molecules for the same site on the Drosophila enzyme. Moreover, they were the most potent inhibitors found, exhibiting $K_i$ values of 0.8 and 0.9 $\mu$M, respectively. Poly-L-glutamic acid did not inhibit the relaxation reaction at concentrations as high as 3 mM. Thus, the DNA binding site on topoisomerase II requires more than a polyanion for proper interaction.

**Stoichiometric Relaxation of Supercoiled DNA**

As demonstrated above and in the following section, both the binding and hydrolysis of ATP by the D. melanogaster topoisomerase II are necessary for the catalytic conversion of supercoiled DNA to relaxed structures (Figs. 5 and 8, Tables II and III). It has been shown for E. coli DNA gyrase (24) and bacteriophage T4 topoisomerase II (12) that ATP binding alone is sufficient to induce a strand passage event, while hydrolysis of the cofactor is required for enzyme turnover. To see if this was also the case for the Drosophila enzyme, the relaxation of negatively supercoiled pBR322 plasmid DNA by stoichiometric amounts of topoisomerase II was examined. Since pBR322 DNA contains approximately 30 supercoils/molecule (12) and type II topoisomerases remove 2 supercoils/strand passage, if every DNA-bound enzyme catalyzes a single event, approximately 15 molecules of enzyme would be required to completely relax 1 molecule of DNA. It should be noted that native Drosophila topoisomerase II is a homodimer (23).

As can be seen in Fig. 7, in the absence of a nucleoside triphosphate cofactor, no relaxation was observed, even at an enzyme:plasmid ratio of 20:1. Moreover, this ratio could be increased to 40:1 (not shown) with no effect on the topological state of the DNA. However, when APP(NH)$_2$P was present, relaxation was evident at ratios as low as 7.5:1 (Fig. 7). Similar results were found with ATP$\gamma$S. Thus, even in the absence of hydrolysis, the binding of ATP to the Drosophila enzyme is sufficient to induce a DNA strand passage event. Moreover, the presence of the adenine ring, the $\gamma$-phosphate group, and the correct spatial arrangement about the $\beta$-$\gamma$ phosphonate bond are all required for strand passage, as APP(CH$_3$)$_2$P, AP(CH$_3$)$_2$PP, ITP, CTP, ADP, and PPP, were not functional as cofactors.

**ATPase Activity of Topoisomerase II**

Topoisomerase II hydrolyzes ATP to ADP and P, as determined from reactions containing ATP labeled with $^{32}$P at either the $\beta$ or $\gamma$ phosphate. No other reaction products could be detected, even during prolonged incubation times (up to 1 h). As can be seen in Table III, the ATPase activity of the Drosophila enzyme was DNA-dependent and was stimulated 17-fold by the presence of negatively supercoiled plasmid DNA. Moreover, the topoisomerase was sensitive to the topological state of the DNA substrate, as supercoiled DNA yielded a reaction velocity which was 4-fold higher than generated by either relaxed circular or linear molecules (Table III). Together with the kinetic data discussed earlier (Fig. 1), this strongly suggests that the affinity of the enzyme for its supercoiled substrate is higher than for its relaxed product. Although single-stranded DNA interacts very tightly with the DNA site on topoisomerase II (Table II), it serves as a poor

![Fig. 7. Relaxation of negatively supercoiled circular DNA by stoichiometric amounts of Drosophila topoisomerase II. Reaction conditions and electrophoresis methodologies employed to generate the agarose gel seen above are described under "Experimental Procedures." The numbers above the lanes represent the ratio of enzyme homodimers to plasmid DNA molecules in the reaction mixture. When employed, the concentration of APP(NH)$_2$P was 1.0 mM.](image-url)
substrate for the ATPase reaction (Table III). Therefore, either double-stranded DNA is necessary to promote hydrolysis of ATP, or the single-stranded substrate binds strongly but incorrectly to the enzyme.

Despite the fact that CTP binds tightly to the ATP site on the topoisomerase, it is not hydrolyzed by the enzyme. This was demonstrated by substituting [α-32P]CTP for ATP in the ATPase assay.

The effects of coumermycin A1 and oxolinic acid on the ATPase reaction were examined. A concentration of 20 μM coumermycin A1 was found to inhibit the reaction by 50%. This value is similar to the $K_i$ (16 μM) determined for the drug-induced inhibition of the relaxation reaction (Table II). Although oxolinic acid inhibits relaxation with a $K_i$ of 1.3 mM (Table II), levels of 2 mM had almost no effect on the ATPase activity of the *Drosophila* enzyme. A similar resistance was previously found with DNA gyrase (25). Thus, oxolinic acid appears to be a preferential inhibitor of relaxation over ATPase activity.

Since the catalytic relaxation of supercoiled DNA by *Drosophila* topoisomerase II is coupled to the hydrolysis of ATP (Fig. 5, Tables II and III) (15), the ratio of ATP molecules hydrolyzed for every supercoil removed was determined (Fig. 8). It was found that approximately 4.1 molecules of ATP are hydrolyzed/supercoil relaxed. A different preparation of enzyme yielded 3.8 ATP molecules/supercoil. These values are higher than those obtained for the supercoiling reaction of DNA gyrase (~0.4 ATP hydrolyzed/supercoil induced) (25) and the relaxation reaction of bacteriophage T4 topoisomerase II (~1 ATP hydrolyzed/supercoil removed) (12). However, since the *Drosophila* enzyme acts processively and can turn over ATP molecules even on fully relaxed DNA substrates, the calculated ATP/supercoil ratio may be inflated by hydrolysis occurring in the complex between the topoisomerase and relaxed DNA.

**Discussion**

**Processive versus Distributive Nature of the Relaxation Reaction**

Under conditions which yield optimal rates (Fig. 2), the enzymic conversion of negatively supercoiled circular DNA to fully relaxed circular DNA by *D. melanogaster* topoisomerase II proceeds in a processive manner. Thus, the enzyme forms a complex with the supercoiled DNA, catalyzes several successive rounds of relaxation, and does not dissociate until the DNA has been completely relaxed. The kinetic scheme depicting this reaction is shown in Fig. 9. Therefore, under processive conditions, for any enzyme-bound DNA intermediate, the rate of relaxation ($k_{rel}$) must be considerably faster than the rate of dissociation of the complex ($k_{off}$). This leads to two conclusions. First, since the topoisomerase is present in catalytic amounts and must dissociate from fully relaxed molecules in order to interact with other supercoiled molecules, the velocity of the relaxation reaction must be related to the rate of dissociation of the E·R complex (see Fig. 6 and Table I). Second, the processive versus distributive nature of the relaxation reaction is controlled by the term $k_{rel}/k_{off}$, which is the ratio of the rate of relaxation to the rate of dissociation for any given enzyme-bound DNA intermediate. When this ratio is high, relaxation takes precedence over dissociation and the reaction is processive. However, conditions which decrease $k_{rel}$ and/or increase $k_{off}$ such that their ratio approaches unity, lead to a distributive reaction in which the enzyme catalyzes only a few or as little as one round of relaxation before dissociating from the DNA.

Three different conditions have been found to promote a distributive relaxation reaction. They are high ionic strength (≥175 mM), high magnesium concentration (≥15 mM), and high pH (≥10) in glycine buffer. Although it has not been directly demonstrated, two lines of evidence imply that increased ionic strength acts by increasing $k_{rel}$: 1) When the ionic strength dependence of the relaxation reaction was analyzed by a modified form of the Bronsted equation (43) (not shown), it was found that electrostatic interactions are important for the binding of *D. melanogaster* topoisomerase II to DNA. Therefore, increased levels of salt should decrease binding between the two (i.e. decrease $k_{rel}$ and increase $k_{off}$) by shielding charged groups. 2) Direct measurements have shown that increased levels of salt decrease the binding between DNA and the type I topoisomerase from rat liver (44), an interaction which can also be shown to depend on the involvement of electrostatically charged groups (43). Whether the effects of magnesium concentration and pH in glycine buffer manifest themselves through changes in $k_{rel}$ or $k_{off}$ remains to be determined.

**Comparison of *D. melanogaster* Topoisomerase II with *E. coli* DNA Gyrase**

**Interaction with Nucleoside Triphosphates**—The ATPase sites of *D. melanogaster* topoisomerase II and *E. coli* DNA gyrase differ by several criteria. First, although the drugs novobiocin and coumermycin A1 compete with ATP for the eukaryotic enzyme (Table II), they are far more effective inhibitors of DNA gyrase, showing $K_i$ values which are 3 and 4 orders of magnitude lower, respectively (24). Second, gyrase has a far more stringent requirement for ATP than *dATP*. While the absence of the 2′-OH on the ribose ring results in a 2-fold increase in the apparent $K_m$ for the interaction of the nucleotide with the eukaryotic enzyme (Fig. 5), it raises the apparent $K_m$ for the prokaryotic enzyme by a factor of at least 30 (7, 24, 25). This demarcation between eukaryotic and prokaryotic type II topoisomerases also extends to other species. Whereas *dATP* is reported to be fully capable of substituting for ATP with the protein from human HeLa cell nuclei (20), it cannot function as a cofactor for the bacteriophage T4 enzyme (12). Third, the nucleoside triphosphate site of DNA gyrase has a much stricter requirement for the adenine base than does the *Drosophila* enzyme. Although the ATPase reaction of gyrase is unaffected by the addition of CTP, UTP, or GTP (25), the relaxation reaction of *Drosophila* topoisomerase II is effectively inhibited by CTP (Table II). Therefore, while the Cα amino group of adenine seems to be responsible for a large degree of the specificity for the nucleotide site of *D. melanogaster* topoisomerase II, additional or different interactions are necessary to confer specificity in gyrase. Fourth, whereas the α, β, and γ phosphates of ATP all contribute to binding with gyrase, only the β and γ phosphates appear to interact with the eukaryotic enzyme. This follows...
from the demonstration that ATP analogues, ADP, and AMP all inhibit reactions of DNA gyrase (24, 25) while only ATP analogues and ADP affect the D. melanogaster topoisomerase II (Table II).

Interaction with DNA—A major difference between the DNA sites of E. coli gyrase and Drosophila topoisomerase II is reflected by their sensitivity toward single-stranded molecules. Whereas single-stranded DNA is a potent inhibitor of the topoisomerase II relaxation reaction (Table II), it has no effect on the supercoiling reaction of gyrase (25, 39). Despite the fact that the Drosophila enzyme is able to accommodate single-stranded molecules, such structures do not appear to be intermediates in the relaxation process. This was concluded from experiments demonstrating that: 1) the rate of relaxation of positively supercoiled DNA, which is overwound, is comparable to that of underwound, negatively supercoiled DNA and 2) single-stranded DNA is a poor activator of ATPase activity (Table III), even at concentrations 2500 times higher than its $K_v$ value (Table II).

Finally, both DNA gyrase and the Drosophila topoisomerase II are able to distinguish between different topological isomers of DNA. Moreover, both appear to interact preferentially with the form of DNA which serves as substrate for their supercoiling-relaxation reactions. Thus, DNA gyrase, which induces supercoils in relaxed DNA, has a 4-fold higher kinetic and binding affinity for relaxed, nicked, or linear DNA over supercoiled forms (25). Conversely, the Drosophila enzyme, which relaxes supercoiled DNA, shows an increased specificity for supercoiled DNA over relaxed linear molecules. This was deduced from the unusually long linear phase of the ATPase course for relaxation (Fig. 1) and the increased velocity of the ATPase reaction observed with supercoiled DNA (Table III). Since the type I enzyme from chicken erythrocytes appears to accommodate DNA molecules, such structures do not appear to be intermediates in the relaxation process. This was concluded from the demonstration that ATP analogues, ADP, and AMP all inhibit reactions of DNA gyrase (24, 25) while only ATP analogues and ADP affect the D. melanogaster topoisomerase II (Table II).

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