Although there is little NH_2-terminal homology among the four rat liver cytochromes P-450, they are very similar in having extremely hydrophobic NH_2-terminal amino acid sequences. Possibly a much more hydrophilic sequence lies just beyond the hydrophobic NH_2-terminal amino acid sequence. Rat liver cytochromes P-450b (Botelho et al., 1979) and P-450d and rabbit liver cytochrome P-450_{LM2} (Haugen et al., 1977) have the sequence Arg-Gly (not in registry) which may mark the beginning of a hydrophilic region in these sequences. A greater amino acid sequence homology could exist among the various isozymes of cytochrome P-450 beyond the hydrophobic NH_2-terminal sequence. The length of the hydrophobic stretch may be variable among the isozymes, and it may be more informative to align the amino acid sequences when sequence data are available for the interior regions of the proteins. Nevertheless, the present structural studies in conjunction with biochemical and immunological comparisons of rat liver microsomal cytochromes P-450a, P-450b, P-450c, and P-450d (Ryan et al., 1980) definitively establish cytochrome P-450d as an isozyme (Kenney, 1974) rather than a posttranslationally modified form of cytochrome P-450a, P-450b, or P-450c.

Acknowledgments

We thank D. Hawke for operation of the microsequencer and Ann Marie Williams for her assistance in the preparation of the manuscript.

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Purification and Properties of Type 1 Topoisomerase from Chicken Erythrocytes: Mechanism of Eukaryotic Topoisomerase Action†

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ABSTRACT: A simple method for the purification of the major topoisomerase (topoisomerase 1) from chicken erythrocytes is described. Because of the generally repressed state of the chromatin from these nuclei, the heterogeneity of the nonhistone proteins is reduced, and it is possible to purify this enzyme from a nuclear extract by a single chromatographic step. The chicken erythrocyte topoisomerase appears to be similar to previously described eukaryotic type I topoisomerases with respect to its physical and enzymological properties. The pattern of intermediate products generated during the action of chicken erythrocyte topoisomerase on a supercoiled closed circular DNA substrate has been examined quantitatively and has been shown to be consistent with a mechanism in which the enzyme closes its substrate DNA molecule after the removal of each superhelical turn and in which dissociation of the enzyme substrate complex may, but does not necessarily, occur after each cycle of the reaction.

DNA topoisomerases comprise a ubiquitous and varied class of enzymes that change the linking number of closed circular DNA molecules through the production of transient breaks in the phosphodiester backbones of the molecules. Although these enzymes were originally sought in order to overcome the perceived problems of unwinding DNA at a sufficient rate ahead of a replication fork, they are now believed to participate in several other cellular processes involving DNA. These include facilitation and possible control of RNA transcription (Falco et al., 1978; Yang et al., 1979), participation in both site-specific (Kikuchi & Nash, 1979) and nonspecific (Witkin, 1976) recombination events, and disentanglement of catenated DNA circles (Liu et al., 1979; Kreuzer & Cozzarelli, 1980;

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0006-2960/82/0421-1155$01.25/0

Mizuuchi et al., 1980; Baldi et al., 1980). Other reactions that may or may not have physiological significance that are catalyzed by topoisomerases in vitro include the knotting and unknotting of single-stranded DNA circles (Liu et al., 1976) and the linking of complementary single-stranded DNA circles to form topologically closed double-stranded DNA circles (Champoux, 1977).

Three distinct classes of topoisomerases have been studied extensively although more have been recognized. Prokaryotic topoisomerases of type I have been shown to selectively but incompletely remove negative superhelical turns from closed circular supercoiled DNA. The prototype of this class of enzymes is the ω enzyme described originally by Wang (1971). Prokaryotic topoisomerases of type II, the DNA gyrases, can both remove negative superhelical turns and, in the presence of ATP, drive negative superhelical turns into a nonsupercoiled closed circular DNA substrate (Gellert et al., 1976). A third class consists of those topoisomerases found associated with the chromatin of eukaryotic nuclei. For the purposes of the present work, these will be referred to as type 1 topoisomerases. These enzymes act on both positively and negatively supercoiled circular DNA substrates (Champoux, 1977) to produce a family of nonsupercoiled topological isomers at thermal equilibrium with the environment (Pulleyblank et al., 1975). In addition to type 1 topoisomerases, there have been recent reports that a fourth type of topoisomerase is present in eukaryotes which requires ATP as a cofactor and resembles bacterial type II topoisomerases in their capacity to reversibly catenate closed circular DNA molecules (Baldi et al., 1980).

Fundamental differences between the mode of action of the three well-studied classes of topoisomerase are indicated by marked differences in the spectra of intermediates generated during the course of their respective reactions. Prokaryotic type 1 topoisomerases cause a gradual reduction in the number of superhelical turns in all molecules of a negatively supercoiled DNA substrate population until a limit product is reached in which each plasmid molecule retains a significant number of negative superhelical turns (Wang, 1971). By comparison, the most closely analogous eukaryotic type of topoisomerase (the type 1 enzymes) catalyzes the complete relaxation of a fraction of the population of supercoiled DNA molecules soon after initiation of the reaction. This relaxed population gradually increases at the expense of supercoiled substrate molecules as the reaction goes to completion. A simple model of topoisomerase activity in which the enzyme first nicks the DNA, allowing free rotation of the two ends of the broken strand about the complementary unbroken strand, and then at some later time reseals the broken strand (the free wheeling model) can be ruled out since a significant fraction of partially supercoiled DNA molecules is present at all intermediate stages of the reaction (Keller & Wendel, 1975; Shure & Vinograd, 1976). Two forms of an alternative model in which the enzyme must reseal the break after the release of a finite number of superhelical turns are considered in the present work. If the resealing reaction does occur after the release of a finite number of superhelical turns, this change in the linking number cannot be greater than one since there is direct experimental evidence that the type 1 topoisomerase can cause a 1-unit change in the topological linking number (Pulleyblank et al., 1975). This result is in contrast to the situation that has been described for a prokaryotic type II topoisomerase where the enzyme has been shown to cause a 2-unit change in the topological winding number in each cycle of the reaction (Brown & Cozzarelli, 1979). The latter result is consistent with a mechanism in which both strands of the DNA are nicked and transferred past another part of the same DNA molecule before religation occurs. The latter mode of action also permits the efficient linking (and unlinking) of closed circular DNA molecules to form catenated species (Kreuzer & Cozzarelli, 1980; Mizuuchi et al., 1980).

Although there have been several reports of successful purifications of type 1 topoisomerases [see, for example, Champoux & McConaughty (1976), Keller (1975), and Tang (1978)], all of the reported methods require several steps which in our own hands invariably give poor yields. A simple method for the purification of an enzyme of this category should greatly facilitate enzymological studies of this important class of enzymes.

Avian erythrocyte nuclei are a uniquely attractive system for the study of chromosomal proteins since they can be prepared in large amounts. The transcriptionally repressed state of the chromatin in these nuclei is reflected in a restricted variety of nonhistone chromosomal proteins. Following reports from other laboratories that despite this repressed state, both chicken and duck erythrocyte nuclei contain an active type 1 topoisomerase (Camerini-Otero & Felsenfeld, 1977; Bina-Stein et al., 1976), we have investigated methods of purification and the properties of topoisomerase from chicken erythrocytes.

Materials and Methods

Purification of Chicken Erythrocyte Topoisomerase. All steps were carried out at 0–4 °C. EDTA[1] (10 mM) was used to inhibit the clotting of fresh chicken blood (700 mL). Erythrocytes were pelleted and washed twice in 500 mL of 0.15 M NaCl–10 mM Tris-HCl–1 mM EDTA, pH 8.0 (S buffer; 5000 rpm for 10 min in a Sorvall GSA rotor), and were then resuspended in 500 mL of S buffer and lysed by the addition of Triton X-100 to a final concentration of 1%. Nuclei were pelleted by centrifugation at 10000 rpm for 10 min in the GSA rotor, then resuspended in S buffer, and centrifuged through a layer of 10% sucrose in S buffer twice. The nuclear pellet was a light buff color. Nuclei were washed twice with 100 mL of 0.35 M NaCl–20 mM potassium phosphate, pH 7.0, and the supernatants containing weakly bound chromosomal nonhistone proteins were saved. The supernatants were filtered under pressure through a Whatman GF/A filter and applied under pressure (1.5-m head) to a 2.4 × 20 cm column of hydroxylapatite (Bio-Rad; HTP). The subsequent elution of the enzyme from this column is described in the legend to Figure 1. Enzymatic activity eluted as the late peak in the 0.2 M potassium phosphate–0.6 M sodium chloride wash. Although a significant fraction of the enzymatic activity was also found in the 0.4 M potassium phosphate wash, this fraction was discarded because of the high level of contamination with other proteins (Figure 1B). The yield of enzyme is highly dependent upon the rapidity with which the elution from hydroxylapatite is carried out since the enzyme is rapidly inactivated during the time in which it remains bound to the column.

Topoisomerase Assays. An electrophoretic method has been used to monitor the purification of the enzyme. Ten microliters of an enzyme sample diluted with 0.2 M NaCl–10 mM Tris-HCl (pH 8.0)–1 mM EDTA (assay buffer) was added to 20 μL of a solution containing 5 μg/mL supercoiled pBR322 DNA substrate dissolved in the same buffer and incubated for 30 min at 23 °C. The reaction was terminated by the addition of 10 μL of a 30% sucrose solution containing 5

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

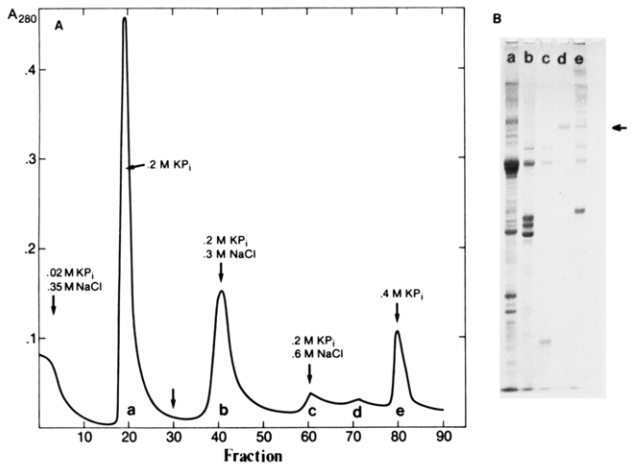


FIGURE 1: Purification of chicken erythrocyte topoisomerase 1 on hydroxylapatite. 400 mL of the 0.35 M NaCl nuclear wash containing chicken erythrocyte topoisomerase 1 was applied under pressure to a 2.5 × 20 cm column of hydroxylapatite (Bio-Rad; Bio-Gel HTP). The column was eluted in succession with (a) 70 mL of 0.35 M NaCl–0.02 M potassium phosphate, pH 7.0 (KP_i), (b) 100 mL of 0.2 M KP_i, (c) 100 mL of 0.2 M KP_i–0.3 M NaCl, (d) 100 mL of 0.2 M KP_i–0.6 M NaCl, and (e) 0.4 M KP_i. Fraction volumes were 5 mL. Activity appears in peaks d and e [see (B)]. The single protein band in fraction d (B) was shown to migrate with a mobility corresponding to a molecular weight of 62 000 by $NaDodSO_4$ gel electrophoresis against a series of known molecular weight standards (not shown).

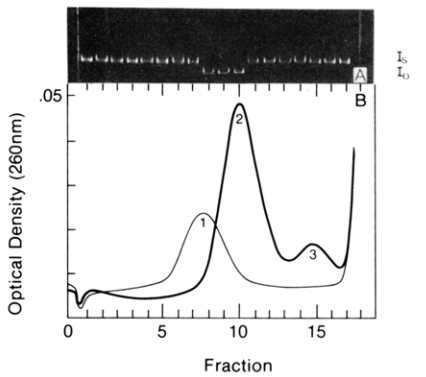


FIGURE 2: Sucrose density gradient centrifugation of topoisomerase 1. 200-μL aliquots containing purified topoisomerase 1 and bovine serum albumin (BSA) or ovalbumin were sedimented at 4 °C for 20 h at 50 000 rpm through a 5–20% sucrose gradient containing 0.3 M NaCl and 20 mM Tris, pH 8.0, in a Beckman SW50.1 rotor. The sucrose gradient fractions containing BSA were diluted 20 times with 0.2 M NaCl–20 mM Tris, pH 8.0, and assayed for topoisomerase activity by the electrophoretic technique described under Materials and Methods (A). (B) shows the optical density profiles of ovalbumin (43 000 daltons; peak 1), BSA monomer (67 500 daltons; peak 2), and BSA dimer (135 000 daltons; peak 3). The molecular weight of topoisomerase 1 determined from these internal standards is 60 000 uncorrected for molecular asymmetry.

μg/mL Pronase, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.05% Bromphenol Blue. The stopped reaction mixture (15 μL) was applied to a sample well of a 1.2% agarose gel prepared with 40 mM Tris–acetate (pH 8.0)–5 mM sodium acetate–1 mM EDTA–0.25 μg/mL ethidium bromide and electrophoresed at 5 V/cm for 1–2 h. Gels were photographed as described previously (Pulleyblank et al., 1977). Under the above electrophoresis conditions, supercoiled substrate DNA migrates between the relaxed product and nicked, circular molecules, and a clear distinction can be made between topoisomerase relaxed closed circular DNA molecules and DNA molecules nicked by endonuclease activity (see Figure 2). The activity of the enzyme is calculated from the dilution at which the intensities of the relaxed (I_0) and supercoiled (I_s) bands are equal.

The fluorometric assay used in the enzyme kinetic studies has been modified from the alkaline ethidium fluorescence assay described previously (Morgan et al., 1979). Five microliters of diluted enzyme was added to 100 μL of assay buffer containing 50 μg/mL supercoiled DNA substrate and 1.5 mM *p*-nitrophenol. At 1-min intervals starting at zero time, 12-μL samples of this reaction mixture were pipetted into 1-mL samples of the alkaline fluorescence assay solution containing 20 mM trisodium phosphate (pH 12.0), 1 mM EDTA, and 0.5 μg/mL ethidium bromide. The samples were heated at 97 °C for 2 min in order to eliminate any contribution to the fluorescence by nicked circular DNA and cooled to 15 °C for fluorometric assay in a thermostated 1-mL cuvette in a Gilson Spectra-Glo fluorometer. Excitation with 520-nm light was achieved by the use of an interference filter, and the emitted light (peak emission 600 nm) was measured after passage through three layers of a Wratten 23A gelatin filter. Stability of this instrument when used under the conditions described is sufficient that measurements can be determined with an accuracy of ±0.2%. Sample dilution variations, which are the major residual source of error, are corrected by subsequent measurement of the absorbance at 400 nm due to *p*-nitrophenol. Each series of readings included a DNA sample fully relaxed with an excess of topoisomerase to provide the fluorescence end point. One unit is defined as the amount of enzyme required to relax half the molecules in 1 μg of DNA in 30 min of incubation (Vosberg et al., 1975).

High-Resolution Gel Electrophoresis of Supercoiled Closed Circular DNA. Relaxed and partially relaxed closed circular DNA species were separated by electrophoresis in 1.5% agarose gels containing 7.5 μg/mL chloroquin phosphate in 50 mM Tris–phosphate, pH 7.0, for 18 h at 3 V/cm (Shure et al., 1977). Quantitation of DNA in the agarose gels was performed by scanning photographs with an Optronics P-1000 scanning densitometer to give a digitized optical density tracing. Since the photographic response is strongly curvilinear (Pulleyblank et al., 1977), a transfer function was calculated from the step images of a calibrated optical density wedge photographed alongside the sample gel, and this was used to convert the digitized optical density tracing to relative fluorescence intensities before integration of the peaks was performed.

Results and Discussion

Purification and Stability of the Chicken Erythrocyte Topoisomerase. Eukaryotic topoisomerases of class 1 that have been examined appear to be highly conserved since all exhibit similar chromatographic and enzymological properties. The success of the simplified purification procedure for the chicken erythrocyte enzyme described above depends upon the selection of a source for the enzyme in which the usual mixture of nonhistone proteins is reduced in complexity. The key steps in the procedure are (1) the specific elution of weakly bound nonhistone proteins from chromatin by the 0.35 M NaCl wash of the nuclei and (2) the specific elution of the topoisomerase from hydroxylapatite under carefully controlled conditions. It is of interest to note that although 0.35 M potassium phosphate buffer without added sodium chloride elutes the chicken erythrocyte topoisomerase from hydroxylapatite, other proteins contaminate the enzyme when it is prepared in this way. Although complex buffer systems have not generally been used in the purification of enzymes using hydroxylapatite, the present example suggests that this modification of the basic technique may prove to be generally useful. The elution properties of the calf thymus type 1 topoisomerase from

chromatin and its chromatographic properties on hydroxylapatite are identical with those observed for the chicken erythrocyte type 1 topoisomerase. However, the greater complexity of the nonhistone chromosomal proteins in calf thymus results in preparations of the enzyme that are still heterogeneous after the hydroxylapatite chromatography step. $NaDodSO_4$ gel electrophoresis of the chicken erythrocyte enzyme reveals a single component of 62000 molecular weight in the most active fractions; a band of the same molecular weight is also present in the less pure 0.4 M potassium phosphate eluate (see Figure 1). This molecular weight is similar to previously reported molecular weights of enzymes isolated from other sources including KB cells [60000 (Keller, 1975)], rat liver [66000 (Champoux & McConaughty, 1976)], and mouse LA9 cells [68000 (Tang, 1978)]. There have been two recent reports that larger, 110000-dalton forms of the type 1 topoisomerase can be isolated from different eukaryotic sources providing precautions are taken to ensure that proteolytic degradation of the enzyme does not occur (Liu, 1980; Dynan et al., 1981). We have not obtained any evidence to date for the presence of a larger form of the enzyme in chicken erythrocytes. The 62000-dalton form of the enzyme is the only one that we have been able to detect when either crude or purified samples are analyzed by sedimentation velocity on sucrose gradients (see Figure 2), and the addition of the protease inhibitors phenylmethanesulfonyl fluoride and/or bovine lung aprotinin to the nuclear extraction buffers does not alter either the form or the yield of topoisomerase obtained. If proteolytic processing of a larger form of the chicken erythrocyte topoisomerase occurs, it is probably an in vivo reaction. It is of interest that *Xenopus* oocytes contain a nucleoplasmic stored form of the topoisomerase which is also of lower molecular weight (Attardi et al., 1981).

The chicken erythrocyte topoisomerase in the pure state exhibits poor storage properties, losing activity with a half-life of 1 week when stored at 4 °C in the hydroxylapatite elution buffer. We have observed that the vanadate (V) ion confers enhanced thermostability to the enzyme. At 42 °C in the absence of this inhibitor, the enzyme loses activity with a half-life of 5 min, while in the presence of 10^{-4} M vanadate, its half-life is extended to 60 min. We currently store the enzyme at 4 °C in the presence of 10^{-4} M sodium metavanadate, a practice which increases the storage life of the enzyme approximately 3-fold.

Kinetics of Topoisomerase I Action. Because there is no simple method for determining the concentration of relaxed closed circular product DNA molecules in the presence of a large excess of supercoiled substrate DNA molecules, the initial rate of topoisomerase activity cannot be determined, and it is necessary to include assay points where up to 90% of the initially supercoiled DNA substrate has been converted to product. Previous results have shown that the relaxed closed circular DNA product is itself a substrate for a type I topoisomerase (Pulleyblank et al., 1975), and competitive inhibition of the enzyme by product molecules must be considered when formulating the overall course of the reaction (eq 1).

$$\frac{-d[S]}{dt} = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]} \quad (1)$$

In the present example, the sum of the product, [I], and substrate, [S], concentrations is equal to the initial substrate concentration, $[S_0]$. If as an initial estimate we assume that the binding affinities of type I topoisomerases for DNA are unaffected by torsional strain in the substrate molecule (see below), we may make the equality $K_i = K_m$ where K_i and K_m are inhbition and Michaelis constants, respectively, and eq 1 can be reduced to

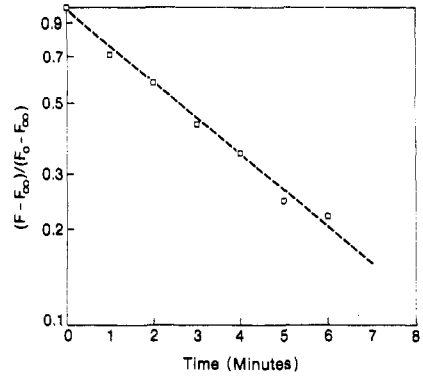


FIGURE 3: Exponential decay in the number of superhelical turns in a closed circular DNA sample catalyzed by chicken erythrocyte topoisomerase 1. pBR322 DNA, 5 μg in 100 μL of 0.2 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8, and 1.5 mM *p*-nitrophenol, was relaxed by the addition of 10 units of chicken erythrocyte topoisomerase for fluorometric assay at the time points indicated. The fluorescence increment of a DNA sample is proportional to the total number of superhelical turns in the DNA sample (Pulleyblank et al., 1975).

$$\frac{-d[S]}{dt} = \frac{V_{max}[S]}{K_m + [S_0]} \quad (2)$$

which upon rearrangement and integration becomes

$$[S]/[S_0] = e^{-V_{max}t/(K_m + [S_0])} \quad (3)$$

Exponential decay in the amount of supercoiled substrate is observed experimentally for the chicken erythrocyte topoisomerase (see Figure 3), and the above assumption of equality between K_i and K_m is therefore consistent with our observations. Further support for this equality is provided by the observations that type 1 topoisomerases are equally effective in relaxing positive and negative superhelical turns (Champoux & Dulbecco, 1972). This behavior is in marked contrast to that of the prokaryotic type I and II topoisomerases. In the latter case it has been stated that the enzyme binds strongly to a nonsupercoiled DNA substrate molecule but only weakly to a negatively supercoiled substrate molecule (Higgins, Morrison, and Cozzarelli, unpublished observation).

As noted in the introduction, DNA molecules with superhelix densities intermediate between those of substrate and product are generated during the course of eukaryotic topoisomerase activity (see also Figure 4). We now consider the kinetic consequences of reaction mechanisms in which reclosure of the DNA helix must occur after the release of each superhelical turn. Two alternative models for such a mechanism can be formulated (Figure 5). In reaction scheme A, the enzyme molecule is obliged to dissociate from its substrate after each cycle of the reaction. Reaction scheme B is processive in that the enzyme molecule may, but does not necessarily, dissociate from its substrate DNA molecule before initiating the next cycle of the reaction. The ratio of the forward (k_τ) and reverse ($k_{-\tau}$) rate constants for the sequence of elementary reactions that is shared by both models (i.e., nicking of the DNA followed by rotation about the unbroken strand and closure of the nick) can be calculated from the free energy decrease associated with the removal of one negative superhelical turn.

$$k_\tau/k_{-\tau} = K_{eq} = e^{-B[\tau^2 - (\tau + 1)^2]/(2RT)} = e^{-B(2\tau + 1)/(2RT)} \quad (4)$$

K_{eq} is the equilibrium ratio of species having τ and $\tau + 1$ superhelical turns, respectively, and $-B/(2RT)$ is the molecular

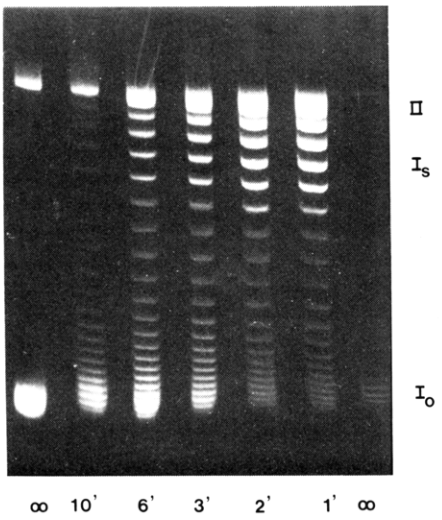
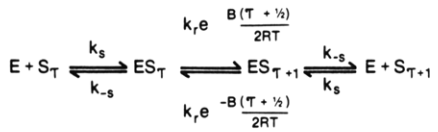


FIGURE 4: Intermediates generated by the action of chicken erythrocyte topoisomerase on supercoiled pBR322 DNA. 50 μg of pBR322 DNA in 1 mL of topoisomerase assay buffer was treated with 500 units (15 μL) of chicken erythrocyte topoisomerase at 23 °C. 200-μL aliquots taken at the time points indicated were added to 5 μL of trypsin, 1 mg/mL, in order to terminate the topoisomerase reaction. The percent of the initial supercoiling remaining in each of the samples was determined by fluorometric assay: 1 min, 79%; 2 min, 70%; 5 min, 57%; 6 min, 45%; 10 min, 35%. Samples containing 200 ng of DNA were applied to the wells of an agarose electrophoresis gel containing chloroquin phosphate. In the presence of this intercalative ligand, nonsupercoiled DNA (I_0) migrates more rapidly than nicked circular (II) and negatively supercoiled substrate species (I_s).

A. Non Processive Model



B. Processive Model

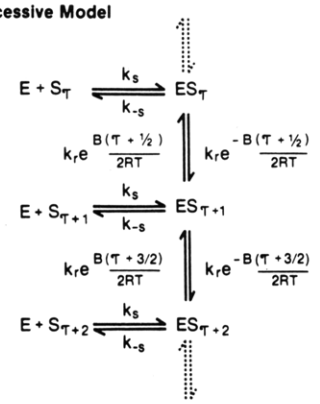


FIGURE 5: Kinetic mechanisms of topoisomerase action.

weight dependent free energy coefficient defined previously (Pulleyblank et al., 1975). The ratio $k_\tau/k_{-\tau}$ can be further dissected into its separate components by the introduction of the intrinsic rate constant k_r for the nicking, rotation, and closing cycle in the absence of contributions from the superhelix free energy.

$$k_\tau = k_r e^{-B(\tau + 1/2)/(2RT)} \quad (5a)$$

$$k_{-\tau} = k_r e^{B(\tau + 1/2)/(2RT)} \quad (5b)$$

k_r could in principle be determined experimentally by measuring the rate of interconversion of two closed circular DNA species having $\tau = +^1/_2$ and $-^1/_2$ superhelical turn, respectively. However, it will become apparent from the following discussion that knowledge of its value, as well as the values of the rate constants for enzyme substrate binding (k_s) and dissociation (k_{-s}), is not required in order to discriminate between the reaction schemes outlined in Figure 5.

Although true steady-state conditions are never attained during the course of either of the reaction schemes illustrated in Figure 5, a conventional steady-state kinetic approach can be used to estimate the ratio of intermediates having $\tau - 1$ and τ superhelical turns, ($[S_{\tau-1}]/[S_\tau]$), that would result from the processive reaction scheme illustrated in Figure 5B. When steady-state conditions are assumed

$$\frac{d[S_\tau]}{dt} = 0 = k_s[E][S_\tau] - k_{-s}[ES_\tau] \quad (6)$$

$$\frac{d[ES_\tau]}{dt} = 0 = k_{-s}[ES_\tau] - k_s[E][S_\tau] - [ES_\tau][k_r e^{B(\tau - 1/2)/(2RT)} + k_r e^{-B(\tau + 1/2)/(2RT)}] + [ES_{\tau-1}]k_r e^{-B(\tau - 1/2)/(2RT)} + [ES_{\tau+1}]k_r e^{B(\tau + 1/2)/(2RT)} \quad (7)$$

By substituting eq 6 into eq 7 and imposing the further condition that τ be sufficiently large that rate terms having negative exponents do not contribute significantly to the overall rate of reaction, eq 7 can be simplified to

$$\frac{d[ES_\tau]}{dt} = 0 = [ES_{\tau-1}][k_r e^{-B(\tau - 1/2)/(2RT)}] = [ES_\tau](k_r e^{-B(\tau + 1/2)/(2RT)}) \quad (8)$$

Rearrangement of the right-hand equality gives

$$\frac{[ES_{\tau-1}]}{[ES_\tau]} = e^{-B/(2RT)} \quad (9)$$

Since under steady-state conditions

$$[ES_\tau] \propto [S_\tau]$$

$$[S_{\tau-1}]/[S_\tau] = e^{-B/(2RT)} \quad (10)$$

For pBR322 DNA $-B/(2RT)$ is 0.25 and the expected ratio of adjacent intermediates is 0.8. At this point it should be noted that any processive model of topoisomerase action in which the enzyme can release more than one superhelical turn in each nicking–closing cycle (e.g., the free-wheeling model mentioned in the introduction or a distributive model in which a random number of superhelical turns is released) will result in an increased ratio of adjacent intermediates when compared to the single-turn model under steady-state conditions.

Reaction scheme A in Figure 5 is not amenable to analysis using steady-state assumptions. We have therefore performed computer simulations of both reaction schemes A and B by iterative procedures. Figure 6 shows the results of two such simulations conducted by using the conditions that the rate-limiting step is the dissociation of the enzyme–substrate complex. Providing that this assumption is maintained, the computed pattern of intermediates is relatively insensitive to the assumed enzyme/substrate ratio and to the relative values of k_r and k_s. The predictions of the two models are clearly different: model A gives a monomodal distribution to which the median superhelix density declines until the completely relaxed limit product is reached, while model B, in agreement with the steady-state calculations shown above, results in an almost constant ratio of adjacent intermediates between the distributions of nonrelaxed substrate molecules and totally relaxed product molecules. Comparison of the results of these simulations with the experimental observations (Figure 6A) shows that the predictions of the processive model (B) conform

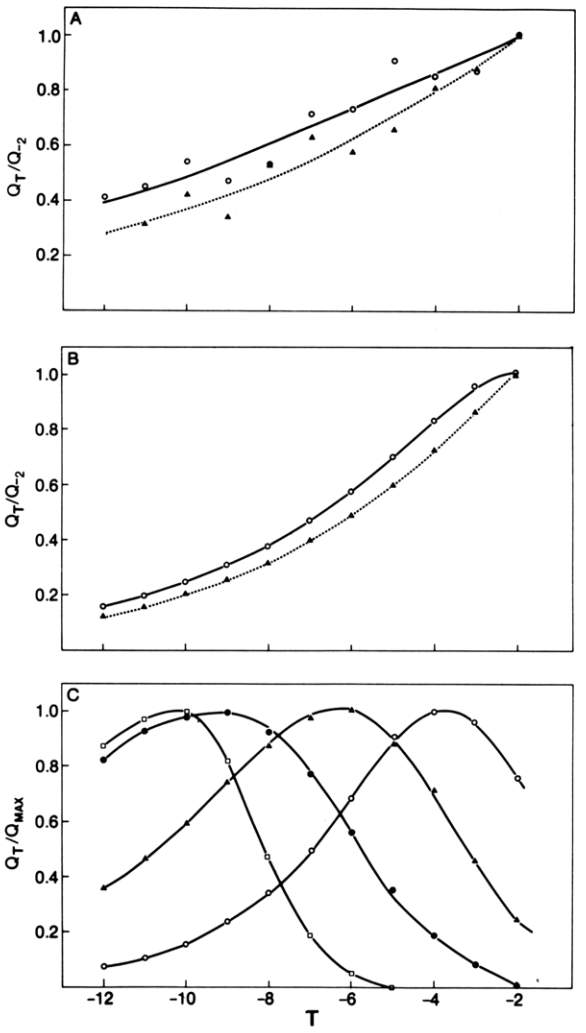


FIGURE 6: (A) Experimental determination of the amounts of the intermediate species generated by the action of the eukaryotic topoisomerase. Quantities of DNA in the bands displayed in Figure 4 were calculated by the method described in the test and normalized with respect to the amount of DNA in the species having $\tau = -2$ superhelical turns. The points displayed were determined for the 2-min [30% relaxed (○)] and the 6-min [55% relaxed (▲)] pBR322 DNA samples. Since negatively supercoiled substrate species overlap with the most highly supercoiled intermediates of relaxation, species migrating in the region I_s were not included in the analysis shown. (B) Predicted patterns of intermediates generated by computer simulation using the processive model (Figure 5B) of topoisomerase action. (○) 35% relaxation and (▲) 60% relaxation of a pBR322 DNA substrate initially having 18 superhelical turns. Kinetic parameters used were $-B/(2RT) = 0.25$ (Pulleyblank et al., 1975) and $k_s/k_{-s} = 1.0$. (C) Predicted patterns of intermediates generated by computer simulation using the nonprocessive model (Figure 5A) of topoisomerase action. (□) 40%, (●) 50%, (▲) 70%, and (○) 85% relaxation of a pBR322 substrate initially having 18 superhelical turns. Kinetic parameters used in the simulation were $-B/(2RT) = 0.25$, $k_s/k_{-s} = 20$, and $k_r/k_s = 1.0$.

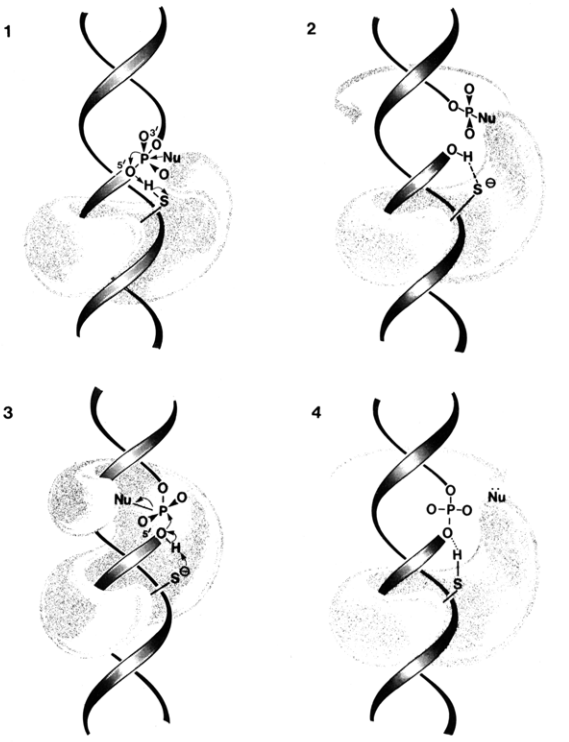


FIGURE 7: Proposed mechanism of eukaryotic topoisomerase action. The reaction is envisioned as proceeding through four stages: (1) The first stage is nucleophilic attack to displace oxygen from the 5′ P–O bond with participation by a cysteine residue which donates a proton to the displaced 5′-oxygen atom. (2) The second stage is rotation of the 3′ enzyme–phosphate bond about the axis of the DNA helix with continued noncovalent attachment of the enzyme to the 5′ side of the single-stranded break. A strong hydrogen bond from the 5′-OH group to the charged cysteine residue helps to stabilize this binding. (3) The third stage is reclosure of the DNA helix by a reversal of the initial attack reaction but with the orientation of the covalent enzyme–DNA bond reversed. The charged cysteine residue reaccepts the proton initially donated to the 5′-oxygen atom. (4) The fourth stage is isomerization of the enzyme–substrate complex with the nucleophilic residue of the enzyme returning to the site of initial attack.

closely to the experimental observations, while the nonprocessive model (A) predicts a pattern of intermediates reminiscent of prokaryotic type I topoisomerases.

By introduction of the minor modification to the nonprocessive model (A) that the enzyme shows preferential binding to negatively supercoiled substrate molecules, the additional characteristics of prokaryotic type I topoisomerases (i.e., specificity for negative superhelical turns and their incomplete removal) can be reproduced by the computer simulation procedure (D. E. Pulleyblank, unpublished observations).

Our conclusion, that a reaction pathway which requires an obligatory resealing step following each cycle of eukaryotic topoisomerase action is consistent with the observed kinetics of action, imposes constraints on the variety of possible mechanisms of enzyme action. In particular, there is a requirement that the geometry of the enzyme–DNA complex during the resealing step must be distinct from that existing during the nicking step. One way in which this may occur is illustrated in Figure 7. It should be noted that although the reaction is illustrated as proceeding in a unique direction, the mechanism is reversible and both positive and negative superhelical turns could be relaxed by reversal of the same pathway. A feature of the mechanism illustrated is the need for a conformational transition at the phosphorus atom in the covalent enzyme–substrate complex during the DNA isomerization step. This may be achieved either by a 180° rotation about the O3′–P–O5′ bond or by stereochemical inversion at the phosphorus atom. In the former case, the configuration of the two oxygen atoms would be retained after completion of the reaction cycle, while in the latter, the positions of these atoms would be inverted. The reaction pathways with and without inversion can in principle be distinguished by the use of DNA labeled stereospecifically at the phosphorus atom. Both the processive and nonprocessive mechanisms illustrated in Figure 5 share the first three steps of the reaction cycle. The two mechanisms differ in that for the processive mechanism, the isomerization of the enzyme can occur while the enzyme remains bound to the substrate molecule (step 4) whereas for the nonprocessive mechanism, the isomerization

can occur only after the enzyme has dissociated from its substrate molecule.

Other studies of the chicken erythrocyte topoisomerase (D. E. Pulleyblank, unpublished experiments) have indicated the presence of an essential sulfhydryl residue which appears to be at or near the active site. In the hypothetical mechanism illustrated in Figure 7, this sulfhydryl residue has been tentatively assigned the role of the proton donor–acceptor required for the strand breakage and resealing reactions.

Recently several biological factors have been identified which can mediate the assembly of histone and DNA into nucleosome cores at physiological salt concentrations (0.15 M NaCl) in vitro (Laskey et al., 1978; Germond et al., 1979; Nelson et al., 1979, 1981). The most notable of these from the point of view of this paper is the observation that a purified topoisomerase 1 from rat liver can assemble nucleosome cores in vitro when present in stoichiometric amounts (Germond et al., 1979). We have examined the ability of the chicken erythrocyte topoisomerase 1 to mediate core particle assembly by determining the amount of core particle length DNA produced when histone–DNA complexes, assembled in the presence or absence of topoisomerase at 0.15 M NaCl, were digested with micrococcal nuclease. In addition, the average number of negative supercoils introduced into relaxed closed circular DNA upon nucleosome core assembly in the presence of catalytic and stoichiometric amounts of topoisomerase was determined. We have found that even a 5-fold stoichiometric excess of topoisomerase over nucleosome cores fails to stimulate core particle assembly as judged by the above two criteria. This failure suggests that a topoisomerase-mediated nucleosome assembly pathway may not be universal among eukaryotes. This conclusion is supported by the recent finding that the nucleosome assembly activity present in rat liver extract appears to be due to an RNA contaminant and not topoisomerase I as originally reported (Nelson et al., 1981).

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