Proc. Natl. Acad. Sci. USA
Vol. 75, No. 12, pp. 5960–5963, December 1978
Biochemistry

DNA gyrase: Subunit structure and ATPase activity of the purified enzyme

(DNA supercoiling/*Escherichia coli*/novobiocin/oxolinic acid)

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Communicated by Gary Felsenfeld, September 25, 1978

ABSTRACT **DNA gyrase has been purified to near homogeneity from *Escherichia coli*. The enzyme consists of two subunits of molecular weights 90,000 and 100,000 present in roughly equimolar amounts. The subunits can be identified as the products of two genes, determining resistance to coumermycin A_1 and novobiocin (*cou*) and to nalidixic acid and oxolinic acid (*nal*A), respectively. These antibiotics were previously shown to be specific inhibitors of DNA gyrase. The ATPase activity of DNA gyrase is stimulated by double-stranded DNA and strongly inhibited by novobiocin but is relatively insensitive to oxolinic acid. Covalent attachment of an ATP derivative to the smaller (coumermycin-specific) subunit is also inhibited by novobiocin, suggesting that this drug interferes with the energy-coupling aspect of the DNA supercoiling reaction by blocking the access of ATP to the enzyme.**

DNA gyrase is an enzyme that catalyzes the ATP-dependent supercoiling of covalently circular DNA (1). The enzyme is one of the simpler mechanochemical systems known; it causes the storage of mechanical strain energy in the superhelical turns of DNA at the expense of ATP hydrolysis. Previous work (2–4) has identified two genetic loci as responsible for controlling the enzyme activity in *Escherichia coli:* the *cou* gene, which determines resistance to coumermycin A_1 and novobiocin, and the *nal*A gene, which determines resistance to nalidixic acid and oxolinic acid. Both groups of antibiotics inhibit the supercoiling reaction; DNA gyrase from the appropriate genetically resistant strain is resistant to the action of that group of antibiotics.

DNA gyrase has been purified from *E. coli* (1–6) and from *Micrococcus luteus* (7). In the latter case, DNA gyrase activity was reconstituted from two purified proteins; in the former case, such a reconstitution has also been reported and the proteins have been identified as the products of the *nal*A and *cou* genes (4, 6).

In this paper, we report the purification to near homogeneity of DNA gyrase. The enzyme is an equimolar complex of two subunits plausibly identified with the *nal*A and *cou* gene products. A binding site for ATP is found to exist on the Cou subunit, and the DNA-dependent hydrolysis of ATP is shown to be blocked by novobiocin. Thus, the Cou subunit appears to be specifically involved in the energy transduction aspect of the supercoiling reaction.

MATERIALS AND METHODS

Chemicals. [γ-^{32}P]ATP and [α-^{32}P]ATP (>100 Ci/mmol), and [2,8-^{3}H]ATP (25 Ci/mmol) were obtained from New England Nuclear. The [^{3}H]ATP was purified by chromatography on DEAE-cellulose (8). Sources of other materials have been described (1–3).

Preparation of Substrates. Colicin E1 plasmid (ColE1) DNA and its relaxed form were prepared as described (1). Highly supercoiled ColE1 DNA was made by treating ColE1 DNA with a nicking-closing extract prepared from chicken reticulocytes (9) in the presence of ethidium bromide (20 μg/ml). The DNA was purified by two cycles of CsCl/ethidium bromide equilibrium centrifugation, which also provided a measure of the superhelix density. The final sample had superhelix density of −0.16 and contained about 5% nicked DNA.

Linear ColE1 DNA was generated by cleavage with restriction endonuclease *Eco*RI. Single-stranded ϕX174 DNA was a gift from Sue Wickner.

Methods. The reaction conditions for assay of DNA gyrase activity (1) have been modified. The modified reaction mixture (70 μl) contained 35 mM Tris·HCl (pH 7.5), 6 mM $MgCl_2$, 1.8 mM spermidine·HCl, 24 mM KCl, 0.14 mM Na_3EDTA, 5 mM dithiothreitol, 6.5% (wt/vol) glycerol, 1.4 mM ATP, 9 μg of *E. coli* tRNA (Calbiochem) per ml, 0.36 mg of bovine serum albumin (Armour, crystalline) per ml, and 0.4 μg of relaxed covalently closed circular ColE1 DNA. For assays of fraction II, the tRNA concentration was increased to 90 μg/ml. Enzyme was diluted into 50 mM Tris·HCl, pH 7.5/0.2 M KCl/1 mM Na_3EDTA/5 mM dithiothreitol/50% (wt/vol) glycerol containing 3.6 mg of bovine serum albumin per ml. The assay procedure was as described (1); units were defined as before (3) but in terms of the revised reaction conditions. The apparent DNA gyrase activity of wild-type enzyme was about twice as high under these assay conditions as under those previously described. Conditions for assay of the nicking–closing activity of DNA gyrase and the oxolinic acid-dependent DNA cleavage reaction have been described (3).

ATP hydrolysis by DNA gyrase was measured under two sets of conditions. In experiment 1 of Table 2, the reaction conditions were similar to those of the assay described above, except that tRNA was omitted, the KCl concentration was increased to 40 mM, and the ATP concentration was decreased to 0.20 mM, providing 500,000 cpm of [γ-^{32}P]ATP per assay volume of 20 μl. In experiment 2 of Table 2, reaction conditions (adapted from ref. 1) were chosen to minimize the nicking–closing activity of the enzyme and to display the dependence of ATP hydrolysis on DNA species. The reaction mixture (20 μl) contained 35 mM Tris·HCl (pH 7.5), 1.6 mM $MgCl_2$, 18 mM potassium phosphate (pH 7.5), 20 mM KCl, 5 mM spermidine·HCl, 30 ng of DNA, and the same concentrations of bovine serum albumin and ATP described above. Incubation in both cases was for 3 hr at 25°C. Hydrolysis products were resolved by thin-layer chromatography on polyethyleneimine-cellulose plates, with 0.8 M acetic acid/0.8 M LiCl as developing buffer.

For covalent labeling of the ATP-binding site, the 2′,3′-dialdehyde derivative of [α-^{32}P]ATP (o[^{32}P]ATP) was prepared

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Abbreviations: ColE1, colicin E1 plasmid; oATP, 2′,3′-dialdehyde derivative of ATP.

and purified by a standard method (10). DNA gyrase (250 ng) was incubated with 2.5 μM α[^{32}P]ATP in 0.125 M *N*-ethylmorpholine acetate, pH 8.3/6 mM $MgCl_2$, in the absence or presence of novobiocin (20 μg/ml), for 5 min at 25°C. The reaction volume was 20 μl. After chilling, solid sodium borohydride (1 mg) was added, and the solution was kept at 0°C for 1 hr. After acid precipitation, the protein was electrophoresed on a sodium dodecyl sulfate/polyacrylamide gel and the wet gel was subjected to autoradiography.

Enzyme Purification. A 300-liter culture of *E. coli* N99 *rec* B_{21} was grown and the cells were stored as described (1). All of the steps (Table 1) were carried out at 0–4°C. Centrifugation was at 15,000 × *g* for 10 min unless otherwise specified. All solutions contained 1 mM dithiothreitol unless noted.

Step I: The frozen cell suspension (750 ml) was thawed in a 25°C water bath and chilled to 0°C. Cells were lysed in 12 centrifuge tubes for the Beckman 45 Ti rotor (62.5 ml each). To each tube were added sequentially 0.31 ml of 0.2 M dithiothreitol, 3.1 ml of a freshly made solution (20 mg/ml) of lysozyme (Calbiochem) in 0.25 M Tris·HCl (pH 7.5), and 0.63 ml of 0.2 M Na_3EDTA. After gentle mixing, the solution was incubated at 0°C for 30 min and centrifuged at 35,000 rpm for 3 hr in a Beckman 45 Ti rotor. The supernatant extract (fraction I; 477 ml; 3.53 g of protein) was frozen in liquid nitrogen and stored at −70°C. This fraction was not routinely assayed for DNA gyrase activity.

Step II: Fraction I was thawed and diluted to 6.9 mg of protein per ml with 10% sucrose/0.05 M Tris·HCl, pH 7.5, followed by addition of 1/20 vol of 4 M NaCl. Precipitation with polymin P, successive extractions with 0.5 M and 1 M NaCl, and ammonium sulfate precipitation were then carried out as described (1). The precipitate was collected by centrifugation, resuspended in 15 ml of 0.1 M KCl/0.05 M Tris·HCl, pH 7.5/1 mM Na_3EDTA, and stored at −70°C (fraction II; 17.5 ml; 99 mg of protein).

Step III: Fraction II was thawed and dialyzed for 4 hr against 1 liter of 0.1 M KCl/0.05 M Tris·HCl, pH 7.5/1 mM Na_3EDTA/10% (wt/vol) glycerol. Insoluble material was removed by centrifugation. The sample was applied to a column (bed volume, 20-ml) of hydroxyapatite (Bio-Gel HTP, Bio-Rad) previously equilibrated with 0.1 M KCl/0.05 M Tris·HCl, pH 7.5/0.02 M potassium phosphate, pH 7.5/10% (wt/vol) glycerol. The column was washed with 60 ml of 0.05 M potassium phosphate, pH 7.5/0.1 M KCl/10% (wt/vol) glycerol and eluted with a 200-ml linear gradient of 0.05–0.5 M potassium phosphate (pH 7.5) containing 0.1 M KCl and 10% (wt/vol) glycerol. Active fractions, which were eluted between 0.1 and 0.16 M phosphate, were pooled (fraction III; 20 ml; 2.7 mg of protein).

Step IV: Fraction III was dialyzed for 2½ hr against 2 liters of 0.05 M potassium phosphate, pH 6.8/1 mM Na_3EDTA/10% (wt/vol) glycerol and was loaded onto a column (bed volume, 2.3 ml) of Bio-Rex 70 (Bio-Rad) previously equilibrated with the same buffer. The column was washed with 4.6 ml of 0.025 M potassium phosphate, pH 6.8/0.1 mM Na_3EDTA/10% (wt/vol) glycerol and developed with a 25-ml linear gradient of 0.0–0.5 M KCl containing 0.025 M potassium phosphate, pH 6.8/0.1 mM Na_3EDTA/10% (wt/vol) glycerol. Fractions were quickly assayed (agarose gel electrophoresis for 2 hr at 175 V). Active fractions, eluted between 0.14 and 0.21 M KCl, were pooled and immediately carried on to the next purification step (fraction IV; 3.5 ml; 0.19 mg of protein).

Step V: Fraction IV was applied to a column (bed volume, 0.2 ml) of hydroxyapatite previously equilibrated with 0.025 M potassium phosphate, pH 6.8/0.1 M KCl/5 mM dithiothreitol/20% (wt/vol) glycerol. The column was washed with 0.55 ml of 0.05 M potassium phosphate, pH 6.8/0.1 M KCl/5 mM dithiothreitol/30% (wt/vol) glycerol and developed with a 2.5-ml gradient of 0.05–0.5 M potassium phosphate (pH 6.8) containing 0.1 M KCl/5 mM dithiothreitol/30% (wt/vol) glycerol. Active fractions were pooled and brought to 1 mM Na_3EDTA before freezing in liquid nitrogen and storage at −70°C (fraction V; 0.25 ml; 90 μg of protein).

This preparation, and all intermediate fractions except fraction IV, were stable in storage at −70°C for at least 1 month.

For use in experiments on ATP hydrolysis, fraction V was passed through a column of Agarose A1.5 M (Bio-Rad) to remove contaminating ATPases. The column (bed volume, 0.5 ml) was washed first with 1 vol of bovine serum albumin (4 mg/ml in water) and then with several volumes of 0.3 M KCl/0.05 M potassium phosphate, pH 7.5/1 mM Na_3EDTA/5 mM dithiothreitol/25% (wt/vol) glycerol. A 30-μl sample of fraction V enzyme was applied to the column and eluted with the same buffer. This step was carried out at room temperature. The enzyme was stored at −70°C.

Table 1. Purification of *E. coli* DNA gyrase

Step	Vol, ml	Protein, mg/ml	Specific activity, units/mg	Total activity, units
I. Extract	477	7.4	—	—
II. Polymin P	17.5	5.7	1,600	160,000
III. Hydroxyapatite	19	0.14	48,000	125,000
IV. Bio-Rex 70	3.5	0.055	450,000	85,000
V. Hydroxyapatite	0.25	0.36	550,000	50,000

RESULTS

Subunit Structure. On sodium dodecyl sulfate/polyacrylamide gel electrophoresis, the DNA gyrase preparation gave two principal bands whose molecular weights were estimated as 100,000 and 90,000 by calibration against the subunits of *E. coli* RNA polymerase (Fig. 1). As judged by photometry of the stained gel, these two chains were present in roughly equimolar amounts and made up 90% of the protein in the sample. When successive fractions from the final hydroxyapatite gradient elution were electrophoresed similarly, these two protein bands in constant ratio accompanied the peak of DNA gyrase activity (data not shown).

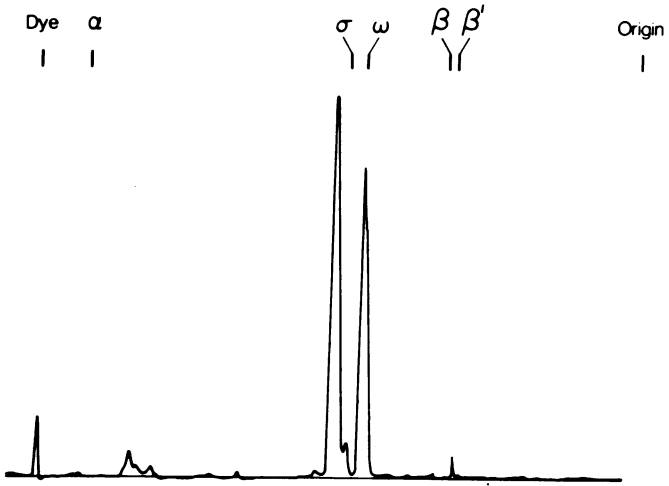

FIG. 1. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of DNA gyrase (fraction V). The figure shows a densitometer scan of the Coomassie blue-stained gel. Molecular weight reference markers were the subunits of *E. coli* RNA polymerase (β', β, σ, and α) and *E. coli* ω protein.

DNA gyrase activity was eluted from a column of Agarose A5M (Bio-Rad) only slightly behind *E. coli* RNA polymerase activity, indicating a molecular weight of at least 350,000 for the native enzyme.

The subunit of molecular weight 90,000 was identified as related to the *cou* gene by labeling it with an ATP derivative and by showing that this labeling was strongly inhibited by novobiocin. Coupling of the derivative o[^{32}P]ATP to the enzyme resulted in labeling mainly of the smaller subunit (Fig. 2*A*). This labeling was much decreased in the presence of novobiocin (Fig. 2*B*). The molecular weight of this subunit was similar to that of the protein identified as the *cou* gene product by Higgins *et al.* (6). A weaker reaction with the large subunit was relatively unaffected by novobiocin.

The larger subunit of DNA gyrase comigrated on sodium dodecyl sulfate/polyacrylamide gel electrophoresis with a sample of purified *E. coli* ω protein (kindly supplied by J. Wang). It has been shown previously that the NalA protein, although distinct from ω protein, comigrates with it on such gels (4). [The DNA-relaxing activity of DNA gyrase was also shown to be distinct from that of ω protein by several tests (3, 4).] By this criterion, the larger subunit of the DNA gyrase complex is presumably to be identified as the NalA protein.

Catalytic Properties. The DNA gyrase preparation (fraction V) was purified more than 300-fold relative to fraction II and by an unknown additional factor relative to the crude extract, which could not be assayed reliably. The specific activity was about 10 times that of our original preparation (1). The nicking-closing activity (3, 4) was purified together with the DNA-supercoiling activity, as demonstrated by cochromatography on the final hydroxyapatite column (data not shown). Incubation with 40 units of enzyme was sufficient to relax completely 0.4 μg of ColE1 DNA. The oxolinic acid-dependent DNA-cleaving activity (3, 4) was also copurified with the other two activities of the enzyme.

DNA gyrase works catalytically, in that 1 equivalent of enzyme can lead to the supercoiling of many equivalents of DNA.

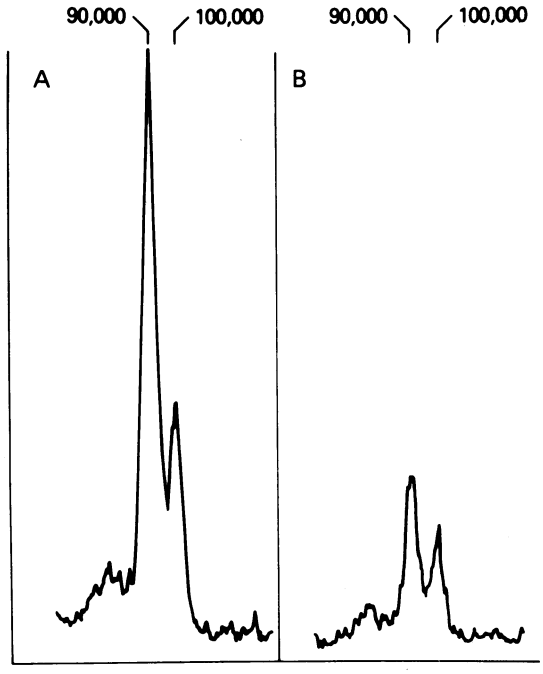

FIG. 2. Coupling of o[^{32}P]ATP to DNA gyrase. After electrophoresis, the sodium dodecyl sulfate/polyacrylamide gel was subjected to autoradiography, and the film was scanned in a densitometer. (*A*) Incubation without novobiocin; (*B*) incubation with novobiocin added (20 μg/ml).

Incubation of 4 μg of relaxed ColE1 DNA with 1.1 ng of enzyme for 24 hr led to the supercoiling of 1 μg of DNA, which corresponds to about 40 molecules of supercoiled DNA per enzyme heterodimer of molecular weight 190,000.

Hydrolysis of ATP. ATP hydrolysis by DNA gyrase was strongly stimulated by double-stranded DNA (Table 2). Under conditions close to those of the supercoiling assay (with a lower ATP concentration), relaxed closed circular ColE1 DNA stimulated the ATPase activity by a factor of 7. This activity was drastically inhibited by novobiocin, at concentrations similar to those that block the supercoiling reaction. Even the low level of ATP hydrolysis seen in the absence of DNA was largely sensitive to novobiocin, implying that most of this hydrolysis also was caused by DNA gyrase rather than by contaminating enzymes. Oxolinic acid, by contrast, caused little inhibition of the ATPase activity (either with or without DNA present) at a concentration that blocks the supercoiling reaction.

The nucleotide product of ATP hydrolysis was ADP. This was shown in reactions parallel to experiment 1 of Table 2 but with [^{3}H]ATP as substrate. More than 98% of the hydrolysis product in the presence of DNA was [^{3}H]ADP. Half-maximal hydrolysis of ATP was obtained at approximately the ATP concentration used here (0.2 mM). However, hydrolysis at low levels of ATP was not proportional to ATP concentration, making the estimation of a Michaelis constant uncertain.

In order to display the dependence of ATP hydrolysis on the type of DNA used as cofactor (Table 2, experiment 2), it was useful to change the reaction conditions (see *Materials and Methods* for details). The DNA concentration was decreased, and the ion concentrations were altered to decrease the nicking–closing activity of DNA gyrase while leaving the supercoiling activity relatively high. Otherwise, the part of the experiment dealing with supercoiled DNA could have been defeated by partial relaxation of the DNA during the reaction.

There were clear differences among the DNA species tested. Single-stranded φX174 DNA gave no stimulation of ATP hydrolysis. Linear and nicked-circular forms of ColE1 DNA gave a small but consistent enhancement above the level found with the relaxed closed circular form, possibly because the free

Table 2. ATP hydrolysis by DNA gyrase

	P_i, pmol
Experiment 1:	
−DNA	80
−DNA + novobiocin	30
−DNA + oxolinic acid	70
+ relaxed ColE1 DNA	570
+ relaxed ColE1 DNA + novobiocin	40
+ relaxed ColE1 DNA + oxolinic acid	420
Experiment 2:	
−DNA	10
+ single-strand φX174 DNA	10
+ relaxed ColE1 DNA	330
+ nicked ColE1 DNA	380
+ linear ColE1 DNA	530
+ supercoiled ColE1 DNA	80

Inorganic phosphate (P_i) was determined by thin-layer chromatography. The two experiments were carried out under different reaction conditions as described in *Materials and Methods*. Each reaction contained 5 units of DNA gyrase. The supercoiled ColE1 DNA was a sample with a high negative superhelix density ($\sigma = -0.16$). When added, novobiocin was present at 20 μg/ml and oxolinic acid at 200 μg/ml. The amount of P_i found after incubation without enzyme (≈20 pmol) has been subtracted.

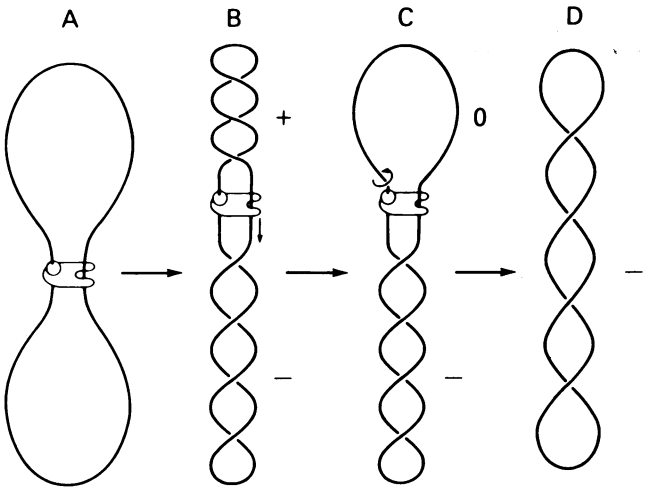

FIG. 3. Possible model for the catalysis of DNA supercoiling by DNA gyrase. ATP-driven translocation of DNA past the bound enzyme, combined with rotation of the helix to keep the same structural features facing the enzyme, partitions the DNA into overwound (+) and underwound (−) loops (*B*). The overwound loop is then relaxed by the nicking-closing activity (*C*). Removal of enzyme liberates a negatively supercoiled DNA molecule (*D*).

swiveling of DNA strands accessible to the former species avoids the necessity for ATP hydrolysis to be coupled to DNA supercoiling. A more drastic effect of the state of the DNA was seen with the highly supercoiled sample of ColE1 DNA, which was several-fold less effective than the other ColE1 DNA types in supporting the ATPase activity.

DISCUSSION

From the present experiments as well as the reconstitution studies of Higgins *et al.* (6), it appears that DNA gyrase activity is produced by a complex of two protein subunits specified by the *cou* and *nal*A genes; no other protein factors have been found to be required. Both in the *E. coli* enzyme and in the corresponding system from *M. luteus* (7) it seems that the subunits can exist either separately or as a complex. The intracellular state of the proteins is not known in either case.

The ATPase activity of DNA gyrase has been shown to be inhibited by novobiocin but not significantly by oxolinic acid. In addition, the predominant ATP-binding site has been localized on the Cou subunit, and this binding was also inhibited by novobiocin. These results indicate that novobiocin interferes with the DNA-supercoiling activity of DNA gyrase by blocking the access of ATP to the Cou subunit. In keeping with this interpretation, the DNA-relaxing and oxolinic acid-dependent DNA-cleaving activities of DNA gyrase, which are not sensitive to novobiocin (3, 4), are reactions that occur in the absence of ATP.

ATP hydrolysis by DNA gyrase is quite dependent on binding to double-stranded DNA and seems to be sensitive to the conformational possibilities of the DNA cofactor, with linear and nicked DNA being more effective than closed circular DNA and highly negatively supercoiled DNA being a poor cofactor. It is not clear whether the ineffectiveness of this last DNA is due to weaker binding of the enzyme or to inability of the enzyme to hydrolyze ATP once bound to such a DNA. Either mechanism would produce a type of coupling between ATP hydrolysis and DNA supercoiling.

It was previously suggested (3, 4) that the activities of DNA gyrase might be divided between its subunits, with the Nal subunit handling the nicking–closing function and the Cou subunit involved in energy transduction, coupling ATP hydrolysis to the production of a twisting stress on the DNA. The present results agree with the latter assignment, at least in regard to the location of the ATP binding site and to the novobiocin sensitivity of the ATPase activity.

It is possible to develop a class of models for DNA gyrase action that incorporates the functional separation of the nicking–closing reaction from the imposition of a twisting stress on the DNA. A common feature of such models is a partitioning of the DNA molecule into overwound and underwound regions, followed by relaxation of the overwound segment. One way to achieve such a partitioning is to have the enzyme sit at a crossing of two DNA duplex segments, binding to both and restricting their rotation with respect to each other. One speculative model of this type that has seemed attractive to us involves an ATP-driven translocation of a DNA double helix past such a junction (Fig. 3). The enzyme is considered to bind tightly at a site on DNA and to attach to a second segment which is then translocated past the junction in such a way that the DNA helix always presents the same structural feature to the enzyme. The helix thus rotates as it passes the junction, with helical turns being unable to pass the enzyme and being accumulated in the upstream loop (upper loop of Fig. 3*B*), while helical length, without the accompanying turns, accumulates in the downstream (lower) loop. This aspect of the model is schematized in Fig. 3 by drawing the translocating element of the enzyme as a slot. The DNA molecule thus becomes partitioned into overwound and underwound loops. If the polarity of the enzyme specifies that the nicking–closing activity can act to relax only the overwound (upstream) loop (Fig. 3*C*), then there will be a net accumulation of underwound (negative) superhelical turns in the DNA, which will spread over the whole molecule when the enzyme is removed. This model allows the enzyme to act iteratively, with one enzyme molecule catalyzing the accumulation of many superhelical turns in a DNA molecule. A model similar in several respects has been proposed by Liu and Wang (7). In their proposal, emphasis is placed on the coiling of DNA around the enzyme, with the transfer of superhelical turns from one loop to the other being achieved by means of this coiling. Both models require that translocation occurs without transient release of DNA from the enzyme. The participation of multiple enzyme sites is thus implied.

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