Volume 10 Number 21 1982 Nucleic Acids Research

The binding of gyrase to DNA: analysis by retention by nitrocellulose filters

N.P.Higgins[1,2] and N.R.Cozzarelli[1,3]

Department of [1]Biochemistry and [3]Biophysics and Theoretical Biology, University of Chicago, Chicago, IL 60615 and [2]Department of Biochemistry, University of Wyoming, Laramie, WY 82071, USA

Received 6 July 1982; Revised and Accepted 27 September 1982

ABSTRACT

Three distinct Escherichia coli DNA gyrase complexes with DNA can be identified using a nitrocellulose filter-binding assay. One complex consists of an ensemble of two subunit A and two subunit B protomers bound non-covalently to specific sequences of DNA. High levels of each subunit alone are inactive but a single gyrase molecule binds DNA to a filter. At 23°, the complex has a dissociation constant of approximately 10^{-10} M and a half-time of decay of about 60 h. It is sufficiently stable that it can be purified by gel filtration and retain full supercoiling activity. Gyrase binds preferentially to relaxed DNA over supercoiled DNA by a factor of about 10. On addition of oxolinic acid, a second complex is formed that is distinguished by its stability in high ionic strength solutions and by efficient conversion to a third form upon addition of protein denaturants. The first and second complexes require Mg^{++} for optimal formation. The third form has been shown previously to contain denatured A protomers covalently linked to DNA that is broken at the site of attachment.

INTRODUCTION

Topoisomerases are enzymes that interconvert DNA isomers differing in topological properties such as linking, interlocking, and knotting (reviewed in 1-3). DNA gyrase is unique among topoisomerases in its ability to increase the negative superhelical content of covalently closed DNA molecules; it shares the capacity to remove (relax) negative superhelical turns with all other topoisomerases. Gyrase is an essential cellular activity and is responsible for the supercoiling of bacterial DNA. It is important in many DNA related processes including replication, phage λ DNA integration into the host chromosome, DNA repair, transformation, and transcription (1-3).

Gyrase is composed of two subunits which can be independently purified and then mixed to reconstitute activity (4,5). In E. coli the A subunit is coded by the gyrA (formerly nalA) gene that controls the sensitivity of cells and gyrase to oxolinic acid and nalidixic acid (4, 6 & 7). Subunit B, the product of the gyrB (formerly cou) gene, is responsible for sensitivity to

0305-1048/82/1021-6833$ 2.00/0

novobiocin and coumermycin A1 (4, 8). The active enzyme contains two copies of each protomer (9, 10).

There are two components to gyrase reactions: double-strand DNA breakage-and-reunion and coupled energy transduction. Double strand cuts in DNA are the sites at which topological changes occur (11) and result from covalent attachment of the A subunit to DNA. Subunit B, the target for novobiocin and coumermycin, contains the binding site for ATP (12,13).

We have studied the interactions between gyrase and DNA using a filter binding assay. Nitrocellulose membrane filters efficiently retain an ensemble of gyrase subunits A and B and DNA, thereby providing a quantitative measure for the first step of the reaction sequence. These complexes form 10-fold more readily on relaxed as compared to native supercoiled DNA and are quite stable. At 23°, the half-life of a gyrase complex is roughly two days and the dissociation constant is 10^{-10}M. These stable complexes can be isolated by gel filtration chromatography and retain full activity. Upon addition of the inhibitor oxolinic acid, a different DNA enzyme complex is stablized that is resistant to high salt concentrations. As previously demonstrated (9), this complex is converted upon denaturation to a cleaved DNA substrate with covalent attachment of subunit A protomers.†

Materials and Methods

Nucleic acids. Unlabeled and ^{3}H-labeled ColE1 DNA (2.5 X 10^{4} cpm/μg) was prepared from the thy⁻ *E. coli* strain, JC411, as described (14).

Enzymes. Gyrase subunits A and B were purified as described through the phosphocellulose and Sephacryl S-200 chromatography steps respectively (4, 15). The concentration of gyrase subunit A was determined by the method of Lowry et al. (16) using bovine serum albumin as the standard protein; the concentration of subunit B was estimated by comparing the intensity of coomassie blue staining bands on a sodium dodecyl sulfate (SDS)-containing polyacrylamide gel with different levels of subunit A. Amounts of subunit A or B are expressed as dimers of the 110,000 or 95,000 dalton protomers respectively. This is done for convenience since only the A subunit is known to exist as a free dimer, but two copies of both protomers are in active gyrase (9, 10). A sonicated extract of rat liver nuclei rich in the nicking-closing enzyme (17) was a gift of A. Sugino of the National Institute of Environmental Health Sciences. Bacteriophage T4 polynucleotide kinase was purified as described previously (18) and EcoRI restriction endonuclease was a gift of H. Boyer of the University of California at San Francisco. Proteinase K was from Sigma Chemical Company.

Enzyme reactions. Gyrase reaction mixtures contained 50 mM Tris-HCl (pH 7.6), 20 mM KCl, 2 mM dithiothreitol, 10 mM $MgCl_2$, 5mM spermidine-HCl, bovine serum albumin at 50 μg/ml, and the indicated levels of gyrase subunits and ColE1 DNA. E·DNA complexes were formed by incubating enzyme with DNA at 23° for 15-30 min and were converted to E*DNA by incubation with 77 mM oxolinic acid for 30 min. Conversion of E*DNA to E-DNA was effected by adding 0.1% SDS. The efficiency of this conversion is the same over a range of SDS concentrations from 0.01% to 1%.

Relaxed DNA was produced in 250-μl reaction mixtures containing 10 μg of native ColE1 DNA, 40 mM Tris-HCl (pH 7.8), 5 mM EDTA, 200 mM NaCl, 100 μg bovine serum albumin per ml, and 7.5 μg of rat liver nicking-closing enzyme preparation. After 30 min at 30°, the covalently-closed relaxed product was isolated by CsCl equilibrium gradient centrifugation in the presence of ethidium bromide (14). 5'-^{32}P-labeled DNA was prepared by digestion of native ColE1 DNA with EcoRI restriction nuclease followed by exchange of the terminal phosphate with that of [γ-^{32}P]ATP using T4 polynucleotide kinase (18). [γ-^{32}P]ATP was synthesized by the method of Glynn and Chappell (19).

Nitrocellulose filter-binding assays. Nitrocellulose filters were presoaked overnight in binding buffer containing 25 mM Tris-HCl (pH 7.6), 20 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 5% (v/v) dimethylsulfoxide. Gyrase reactions (5-20 μl) were mixed with 1 ml of binding buffer in 1.5-ml plastic Eppendorf centrifuge tubes. Using a 2-ml plastic pipette, the sample was applied dropwise to the center of a nitrocellulose filter with a flow rate of 1 ml per min. The filters were washed with 3 ml of binding buffer, dried, and the radioactivity determined. Unless indicated otherwise, the data were corrected for the 2-5% of radioactive DNA that bound to the filter in control reactions lacking gyrase.

Other materials. Oxolinic acid was a gift of Warner Lambert Research Institute. Nitrocellulose filters (BA 85, 25 mm diameter, 0.45 μ pore size) were from Schleicher and Schuell. $^{32}P_i$ was from New England Nuclear Corp. Bovine serum albumin, agarose (type II), spermidine trihydrochloride, and dithiothreitol were from Sigma Chemical Corporation. Sepharose 4B was from Pharmacia and electrophoresis purity acrylamide and N,N'-methylenebisacrylamide were from BioRad Laboratories.

RESULTS

Requirements for formation of gyrase DNA complex (E·DNA). Duplex DNA passes through a nitrocellulose filter whereas many proteins are retained

(20-23). A convenient assay for formation of a complex of a protein with labeled DNA is thereby the retention of label by the filter. Using this assay, reaction conditions favorable for the binding of gyrase to linear Col E1 DNA were determined (Table I). Mg^{++}, which is required for all previously-characterized reactions of gyrase with DNA, was needed for optimum complexation. Since the binding buffer contained Mg^{++}, the reduction of binding was not a secondary effect on the absorption of gyrase to the filter. Both KCl and spermidine slightly stimulate supercoiling (24), although their omission from a binding mixture leads to a slight enhancement of binding. The sulfhydryl reacting reagent, N-ethylmaleimide, abolished binding, thus explaining its abolition of DNA-dependent ATPase and supercoiling (15). Bovine serum albumin stimulated binding at low gyrase concentrations, such as 0.2 fmol/µl, but had little effect when the gyrase concentration was raised to 5 fmol/µl (not shown); thus albumin probably stabilizes gyrase.

Neither gyrase subunit alone bound DNA (Table I), and this simple assay does not distinguish between effects on subunit assembly and effects on a gyrase tetramer binding to DNA. Therefore, the complementary nature of the

TABLE I: Requirements for gyrase binding to DNA as determined by filter retention assay.

Additions or Omissions	Binding (% of complete reaction)
Complete	100
-$MgCl_2$	39
-$MgCl_2$+4 mM EDTA	24
-KCl	133
-Spermidine	118
-Dithiothreitol	88
-Dithiothreitol +2 mM N-ethylmaleimide	5
-Albumin	17
-Subunit A	<2
-Subunit B	<2

Gyrase (5 fmol) reconstituted at 50 pmol/ml with a 50% excess of subunit A was incubated 40 min at 23° with 12 fmol of 5'-^{32}P-labeled linear Col E1 DNA. The 10-µl complete reaction mixture contained 50 mM Tris-HCL (pH 7.6), 10 mM $MgCl_2$, 2 mM dithiothreitol, 20 mM KCl, 5 mM spermidine, and 50-µg of bovine serum albumin per ml. Reaction mixtures were filtered through nitrocellulose membranes and the amount of retained DNA determined. The data are corrected for DNA (2%) that adhered to the filters in the absence of gyrase; the complete reaction mixture resulted in 2.4 fmol of bound DNA.

binding reaction was investigated (Fig. 1). With addition of increasing amounts of one subunit to a fixed amount of the complementary subunit, binding activity increased to a plateau level where the limiting subunit was saturated with its complement. Thus a 2-fold increase in the limiting component lead to a doubling of the plateau value. The implied ratio of subunit B to subunit A in gyrase that binds to DNA is 1.3; this is very close to the value of 1.0 expected from the structure of the enzyme (9,10). The plateau value also indicates that only 1.25-1.5 gyrase molecules were required to attach a DNA molecule to the filter. Therefore, the formation of gyrase from its subunits, the binding of the enzyme to the DNA, and the adsorption of the enzyme DNA complex to the filter are all efficient processes and most of the gyrase subunits were active.

The shapes of the complementation curves are different. As seen in similar experiments with supercoiling (4), there was a lag and slower rise in the curve with constant subunit A and increasing subunit B (Fig. 1B). This could indicate a cooperativity in the stepwise assembly of individual B protomers into gyrase whereas the A protomers exist as a stable dimer in the absence of the B subunit (6). Alternatively, the stability or association of the more thermolabile B protomers could be increased by high levels of subunit A.

The assembly of gyrase subunits into a filter binding complex occurs very rapidly at 23° (Fig. 2). However, both the rate of complex formation and the plateau value were increased by preincubation of the gyrase subunits at high concentration prior to their dilution into the reaction mixture (Fig. 2).

Stability of the E DNA complex. Once formed the E·DNA ensemble is quite stable (Fig. 3). Complexes formed at 23° and then diluted 67-fold in the

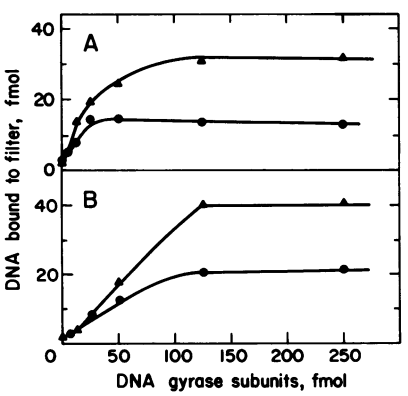

Fig. 1. Dependence of DNA binding on gyrase subunits A and B. The 20-µl reaction mixtures containing 80 fmol of ^{3}H-labeled native Co1E1 DNA were incubated with subunits A and B for 45 min at 23° and then diluted and filtered. In panel A, 25 (●) or 50 (▲) fmol subunit B were mixed with the indicated amounts of subunit A. In panel B, 25 (●) or 50 (▲) fmol subunit A were mixed with the indicated amounts of subunit B.

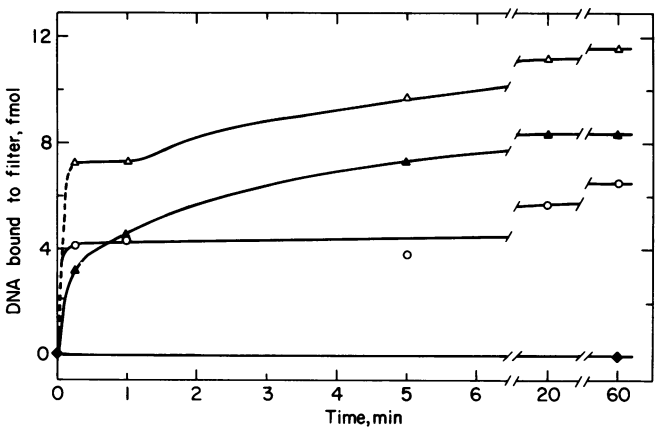

Fig. 2. Time course of gyrase binding to DNA. 110-µl DNA binding mixtures contained 120 fmol of 5'-^{32}P-labeled EcoRI cleaved Co1E1 DNA and 50 fmol (○) or 100 fmol (△) of gyrase reconstituted at a concentration of 50 pmol/ml with 50% excess of subunit A, or 150 fmol subunit A and 100 fmol subunit B added separately to the reaction mixture (▲). The control lacked enzyme (◆). After the indicated times at 23°, 20-µl samples were diluted into 1 ml of binding buffer, filtered, and counted.

presence of a 3-4 fold excess of competitor DNA decayed with a half-life of 60-70 h. There appeared to be two rate components in the dissociation at 37° of complexes formed at 23°. One third of the complexes decayed more rapidly over a 4 h period, whereas the rest decayed at a rate similar to that at 23° (Fig. 3A). The rate of dissociation was also influenced by the concentration of competitor DNA. If the competitor was in the same 3-fold excess but in a 50-fold higher concentration, two populations of complexes were apparent at 23°. One-third of the total complexes decayed in the first 3 h, but the remaining had the high stability of the complexes in the presence of lower competitor concentrations (Fig. 3B). These differences probably reflect the difference in stability of gyrase bound to different DNA sites (25,26). With both a high concentration of competitor and incubation at 37°, again about one third of the complexes dissociated rapidly (not shown). Thus the molecules decaying rapidly at high temperature are probably the same ones competed off by high DNA concentrations. The fact that concentration and not the excess of competitor is the important parameter implies that gyrase may be exchanged directly between different DNA molecules.

The apparent dissociation constant for E·DNA complexes formed and held at 23° averaged 2 x 10^{-10} M as determined by Klotz plots (27) such as shown in

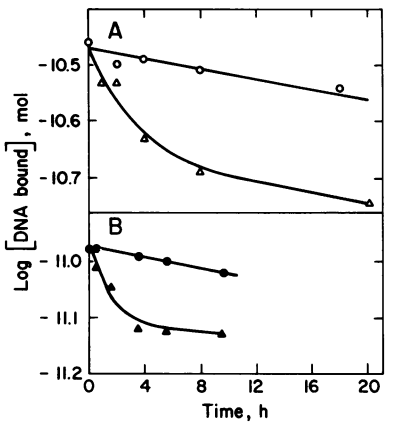

Fig. 3. Stability of gyrase DNA complexes. In (A) the 0.4-ml reaction mixtures containing 1.3 pmol gyrase reconstituted with a 4-fold excess of subunit B and 1.5 pmol of Col E1 [^{3}H] DNA were incubated for 40 min at 23°. 15-µl samples were then diluted with 1 ml of binding buffer containing a 4-fold excess of unlabeled competitor Col E1 DNA and incubation was continued at 23° (o) or 37° (△). At the indicated times, the amount of DNA retained by the filter was determined. In (B) 500 fmol of gyrase reconstituted with a 50% excess of subunit A was incubated for 30 min at 23° in 0.6-ml reaction mixtures containing 740 fmol of ^{3}H-labeled relaxed Col E1 DNA. 20-µl samples were either diluted into 1 ml of binding buffer containing a 3-fold excess of unlabeled relaxed competitor Col E1 DNA (●), or 3-fold competitor excess was added to 20-µl aliquots without dilution (▲) and incubation continued at 23°. At the indicated times, samples were either diluted into 1 ml of binding buffer and filtered (▲) or filtered directly (●). The slopes were 4.5 x 10^{-3}(○) and 5.0 x 10^{-3}(●) giving first-order dissociation rate constants of 1.0 x $10^{-2}h^{-1}$ and 1.2 x $10^{-2}h^{-1}$, respectively. Initial slopes for the faster dissociating species (△,▲) were 6 x 10^{-2}.

Fig. 4. This experiment was carried out four times with enzyme reconstituted with either subunit in excess and values ranged between 1 and 3 x 10^{-10} M.

Enzymatic activity of stable E·DNA. The E·DNA complexes with very long half-lives are not inactive forms of the enzyme. To demonstrate this, enzyme DNA complexes were filtered through a Sepharose-4B column to remove unbound enzyme (Fig. 5). Chromatography was carried out slowly - the DNA peak eluted after 3 h - so that only very stable complexes remained. 63% of the E·DNA complexes could still be converted to E-DNA compared to 72% before chromatography. Moreover, upon addition of ATP the relaxed substrate was supercoiled. Thus all gyrase molecules which had been bound to a DNA molecule for 3 hours retained DNA cleavage and supercoiling activity. A kinetic experiment showed that stably bound gyrase supercoiled DNA at the normal rate (Fig. 6). Relaxed Col E1 DNA was incubated at 30°C for 15 min

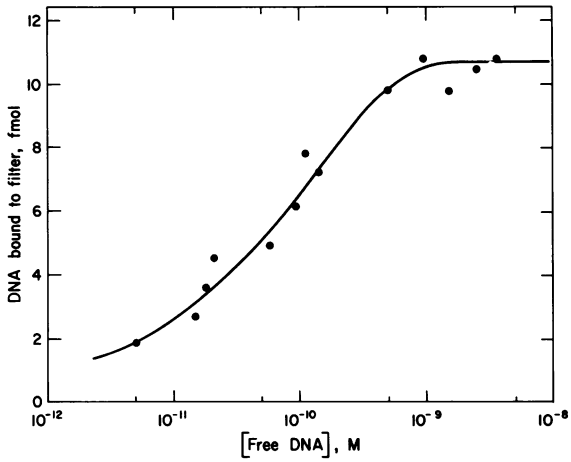

Fig. 4. Measurement of the dissociation constant for gyrase and linear duplex DNA. Gyrase (15 fmol) reconstituted at a concentration of 50 pmol/ml with a 4-fold excess of subunit B was added to 20-µl reaction mixtures containing from 2 to 80 fmol ^{32}P-labeled EcoR1 cleaved ColE1 DNA. After 45 min at 23°, the amount of DNA bound to gyrase was measured by retention to filters. The data is plotted by the method of Klotz (27) in which the apparent dissociation constant is the midpoint of the curve.

with gyrase in the absence of ATP. Part of the reaction was provided with ATP and the rate of supercoiling measured. The remainder of the reaction was incubated for 25 min with excess phage λ DNA to compete out unstable gyrase DNA complexes before ATP addition. In this case, only processive supercoiling of ColE1 substrates was measured because gyrase released from ColE1 binds to the more abundant λ sequences. Supercoiling in the latter reaction with competitor occurred at 75% the initial rate of the control reaction in its absence (Fig. 6). Since the competitor removes approximately 30% of the enzyme molecules from the ColE1 DNA, the 25% diminution in rate is anticipated and thus stable E·DNA complexes are fully active for supercoiling.

Influence of superhelicity on binding. To determine the effect of superhelicity on binding, reactions were carried out with an equimolar mixture of ^{3}H-labeled native ColE1 DNA (containing 50 negative supercoils/molecule) and ^{32}P-labeled linear ColE1 DNA (Fig. 7A). A preferential binding to the linear molecules in the mixture was observed at sub-saturating enzyme levels; after correction for contaminating nicked circles in the native substrate the selectivity is approximatly tenfold. At enzyme concentrations in excess of the DNA concentration the curves are nearly parallel. ^{32}P-

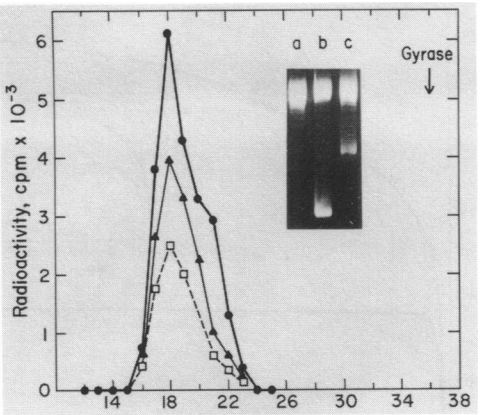

Fig. 5. Stable gyrase-DNA complexes are active for both supercoiling and cleavage. The 60-µl reaction mixtures contained 700 fmol of relaxed Co1E1 DNA, 700 fmol of ^{3}H labeled nicked Co1E1 DNA and 1 pmol gyrase. After 40 min at 30°, the mixture was applied to a 5.2-ml Sepharose-4B column equilibrated with 50 mM Tris-HCl (pH 7.5), 10 mM MgCl , 0.1 mM EDTA, 5 mM 2-mercaptoethanol, and 20 mM KCl. The column was developed at 23° with the equilibration buffer and 110-µl fractions were collected. The DNA peak, which emerged after 3 h, was identified by counting (●) and the position of gyrase in a control column in the absence of DNA is indicated by an arrow. The amount of DNA which was enzyme bound (▲) was determined by the retention to nitrocellulose membranes. Oxolinic acid (200 µg/ml) was added to samples and after incubation at 30° for 30 min, 0.1% SDS was added and the amount of DNA with covalently bound protein determined by filter retention (□). A portion of the peak fraction was incubated for 30 min at 30° with either no addition (lane A), 500 µM ATP (lane B), or 200 µg/ml oxolinic acid (lane C) and displayed by agarose gel electrophoresis. The relative mobility in ascending order is nicked or relaxed DNA, linear duplex DNA, and supercoiled DNA.

labeled linear DNA is an appropriate model for covalently closed relaxed DNA, since binding was equally efficient to both of these forms (Fig. 7B).

Production of derivative gyrase DNA complexes. The initial ensemble of gyrase and DNA (E·DNA) can be destroyed by heating to 70° or the addition of SDS or high salt concentrations (ref. 28). However, addition of oxolinic acid to E·DNA complexes changes the complex so that incubation with SDS results irreversibly in E-DNA formation, i.e., breakage of the DNA and covalent attachment of subunit A protomers (9). This new complex trapped by oxolinic acid that we designate E*DNA is stabilized by Mg^{++}, because addition of EDTA prior to the denaturing agent prevents the covalent attachment of protein and rupture of DNA backbone bonds (Table II). The destabilization of E*DNA is rapid at 30° but slow at 0°. E*DNA then can decay in one of two ways. It can dissociate in a reaction stimulated by EDTA or it can result in

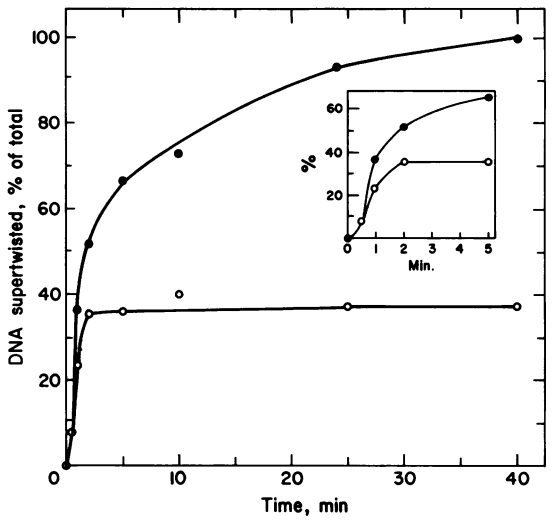

Figure 6: Supercoiling of DNA by stably-bound gyrase. The 0.21-ml reaction mixture contained 600 fmol relaxed Col E1 DNA and 100 fmol DNA gyrase. After 15 min at 30°C a 90-µl portion was mixed with 22 µg phage λ DNA in a volume of 45 µl; 1 mM ATP was added to the control reaction lacking λ DNA at 20 min (●) and to the reaction with λ DNA at 40 min (○). The product DNA was displayed by agarose gel electrophoresis and the fully supertwisted band was quantitated by densitometry.

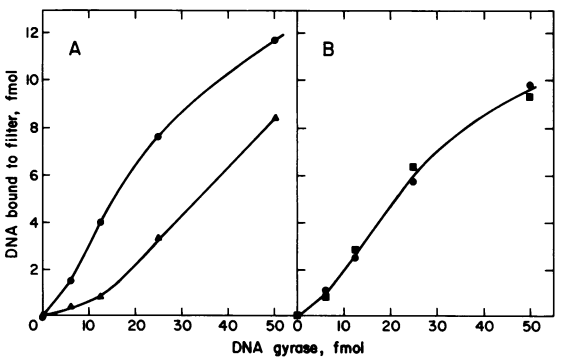

Fig. 7. Selective binding of gyrase to DNA without negative supercoils. The indicated amounts of gyrase reconstituted at 50 pmol/ml with a 50% excess of subunit A were incubated for 30 min at 30° in 20 µl reaction mixtures containing 14 fmol of ^{32}P-labeled EcoRI cleaved Col E1 DNA (●) plus either 14 fmol of ^{3}H-labeled native supercoiled Col E1 DNA having 18% nicked forms (▲) in (A) or ^{3}H-labeled relaxed Col E1 DNA (■) in (B). The label retained by nitrocellulose filters was measured.

TABLE II: Effect of EDTA on formation of covalent gyrase-DNA complexes

Addition	Relative Efficiency of Complex Formation
0.2% SDS	100 3%
30 mM EDTA plus 0.2% SDS	108 5%
30 mM EDTA for 6 min at 23°, then 0.2% SDS	16 6%
30 mM EDTA for 6 min at 0°, then 0.2% SDS	84 4%

Reaction mixtures (20 -µl) containing 6 fmol of gyrase and 12 fmol of ^{32}P-labeled EcoRI cleaved Co1E1 DNA were incubated at 23° for 30 min; 100 µg/ml oxolinic acid was added and incubation at 23° continued for 10 min. Duplicate mixtures were treated as indicated, diluted with 1 ml binding buffer, filtered, and counted. Addition of SDS led to formation of 1.6 fmol of covalent complex.

strand rupture and concomitant attachment of gyrase to the DNA. Direct evidence for such an oxolinic acid-trapped intermediate is shown in Fig. 8. E·DNA complexes are dissociated by increased ionic strength; only 25% remains at 0.16 M KCl. However, addition of oxolinic acid produces a complex which is considerably more salt stable and that, upon addition of SDS, results in cleavage and formation of E-DNA. The formation of E-DNA was measured both by binding of labeled DNA to a filter in the presence of SDS (Fig. 8A) and by cleavage of the substrate (Fig. 8B). Most cleavages occured at 2 positions which generate the 4 major bands in the gel. The 2 middle bands come from site a and the two outer bands from site b, which were footprinted by Morrison and Cozzarelli (26). The a site was most resistant to ionic strength (lane 5). If E·DNA is dissociated by addition of 0.2 M KCl prior to oxolinic acid addition, cleavage is prevented.

DISCUSSION

Each gyrase subunit plays a role in productive DNA binding. Subunit A must bind to DNA since it contains the active site for the DNA breakage-and-reunion component of gyrase (9,29). However, subunit B must also have a DNA contact or provide for proper enzyme folding since three independent measures of DNA binding - adsorption to nitrocellulose filters (this paper), protection of positive supercoils (28), and footprinting (A. Morrison, unpublished observations) each require the complete enzyme. Only about half of subunit B in the form of a protein designated ν is needed for DNA binding since subunit A plus ν has topoisomerase activity and protects the same DNA region from nuclease as does gyrase (26).

Only 2-3 A and B protomers are needed to bind Co1E1 DNA to a

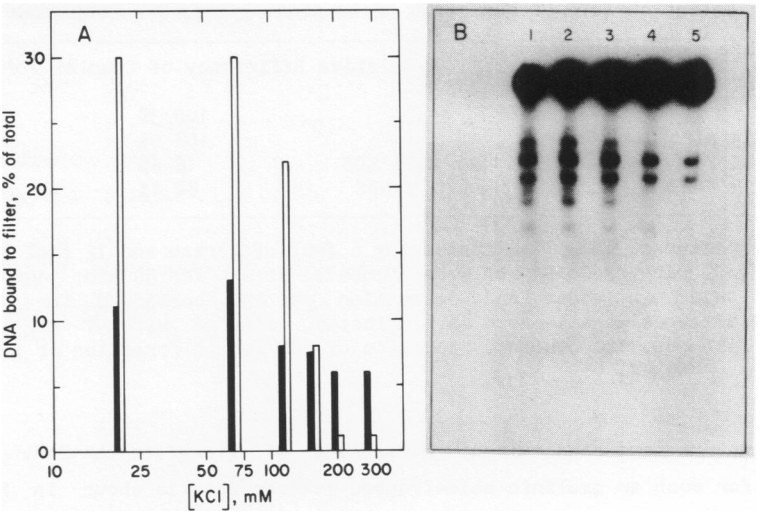

Fig. 8. Differential salt stability of gyrase E*DNA and E·DNA complexes. The 20-µl reaction mixtures containing 20 fmol of ^{32}P-labeled EcoRI cleaved Co1E1 DNA, 20 fmol subunit B, and 30 fmol subunit A were incubated at 23 for 30 min in the presence or absence of 100 µg/ml oxolinic acid. The indicated KCl concentrations were added and incubation was continued for 10 min at 23°. Reaction mixtures lacking oxolinic acid were diluted into 1 ml of binding buffer, filtered, and the amount of DNA retained was measured (open bars in panel A). Reactions containing oxolinic acid were stopped by the addition of 0.1% SDS and either filtered to measure the covalent gyrase DNA complex formed (solid bars in panel A) or digested with proteinase K (50 µg/ml) and the cleavage pattern displayed by agarose gel electrophoresis (Panel B). The KCl concentration was 20 mM, lane 1; 68 mM, lane 2, 115 mM, lane 3, 160 mM, lane 4, or 280 mM, lane 5.

nitrocellulose filter (Fig. 1). Since gyrase has the form $\alpha_2\beta_2$, a single gyrase molecule suffices for binding both to DNA and to the filter. Even though the assembly of E·DNA is rapid at 23°, preincubation of the A and B subunits prior to addition of DNA enhances the rate as well as the extent of complex formation (Fig. 2). If B protomers are not a dimer, as are A protomers, then a complex order of formation could exist, such as cooperative binding of a second B protomer to an $\alpha_2\beta_1$ complex. This form has been identified by Klevan and Wang (10). The sigmoidal complementation curve (Fig. 1B) is consistent with this possibility.

Optimal DNA binding requires a divalent metal ion such as Mg^{++} and an N-ethylmaleimide-sensitive group, presumably cysteine. Since all other gyrase reactions require DNA, they should be, and are, EDTA and N-ethylmaleimide sensitive. Electrostatic forces are clearly important in maintaining the

structure of E·DNA because 0.2 M KCl in the presence of 10 mM Mg^{++} destroys the ensemble (Fig. 8).

Most of our detailed information on enzyme binding comes from stable sites. Gyrase is aligned precisely, to the nucleotide level, over these positions on DNA (26,29). Fine structure mapping of gyrase molecules has shown that the enzyme interacts with 100-140 base pairs of DNA with the cleavage site located near the center (26,30,31). Enzymes bound to stable sites are active. Even after a three-hour purification by gel filtration, gyrase-Co1E1 DNA complexes are fully functional for supercoiling and oxolinic acid induced cleavage (Fig. 5).

Comparison of the DNA binding reactions of gyrase to the similarly sized RNA polymerase molecule is revealing (23,32). RNA polymerase holoenzyme binds promoter sites in DNA rapidly and stably. The dissociation constant for bacteriophage T7 promoters is 10^{-12} to 10^{-14}M; the half time for association is about 20 sec, and for dissociation it is 20-60 h. Core polymerase forms filter binding complexes with DNA at more sites, and much more rapidly (within 5 sec); the half time for dissociation though is only 20 min. Since there are many more weak binding sites that promoters, Hinkle and Chamberlin (32) suggested that the rate limiting step in promoter binding was binding and release from non-promoter sequences. Sigma aids the release of polymerase from weak sites. Gyrase also ultimately becomes located over a number of stable sites in DNA. The reaction appears intermediate between core and holoenzyme. Like the core enzyme, formation of a filterable gyrase complex with DNA occurs within the first few seconds of reaction (Fig. 2). However, after 40 min of binding, gyrase is distributed over a number of sites on Co1E1 DNA having varying stabilities (Fig. 4). The average dissociation constant for these sites is 10^{-10}M and the dissociation rate constant is $3 \times 10^{-6} sec^{-1}$ (Fig. 3). Therefore, the expected rate constant for binding to the stable sequences is $3 \times 10^{4} M^{-1} sec^{-1}$. This is equivalent to a half time of association of 8-9 min instead of the observed seconds. Thus initial binding is probably to weak sites and the enzyme moves slowly to the more stable sites.

Gyrase is unusual in its selective binding to DNA molecules lacking negative supercoils (Fig. 7). The mechanism for this is very likely the positive supercoiling of DNA around gyrase (33). This causes a counterposing negative supercoil in other regions of the DNA and the energy needed to put in an additional supercoil increases with the square of the supercoil density (34).

Finally, what do our results indicate about the mechanism of inhibition by oxolinic acid? This drug stabilizes a complex, designated E*DNA, which resists salt concentrations that destroy the less stable complex E·DNA (Fig. 8). Footprinting experiments also show that oxolinic acid strengthens the binding of DNA around several cleavage sites (26,30,31). The changes in the nature and tightness of gyrase binding in the presence of oxolinic acid suggests a significant alteration in enzyme conformation although there is no gross change in the footprint. There are two extreme models for the structure of E*DNA. Subunit A could be attached via an ester bond to phophates in intact circular DNA or gyrase could link linearized DNA into a circle with covalent bonding of subunit A protomers to the 5'-phosphoryl ends of the double strand break and noncovalent bonding to the adjacent 3'-hydroxyls. The prevention of conversions of E*DNA to E-DNA by EDTA (Table II) is compatable with both alternatives. If the latter model is correct, then Mg^{++} is probably required for DNA reunion but not breakage so that EDTA dissociates E*DNA back to E·DNA even in the presence of oxolinic acid. In the former model, the same reaction is postulated but the Mg^{++} requiring step is the formation of the triester bond. Whichever model is correct, E*DNA, trapped in vivo over important DNA sequences, could represent a significant impediment to normal replication and transcription. This argument has been invoked to explain the inhibition of replication of T7 DNA by oxolinic acid, a process that does not require functional gyrase (35).

Acknowledgments. This work was supported by grants GM-21397, PCM 8004689, and CA-19265. We are indebted to A. Morrison for aid in preparing the manuscript and the execution of some of the early experiments.

† Footnote. The three complexes between gyrase and DNA are designated by E·DNA, gyrase bound to DNA under standard reactions conditions; E*DNA, gyrase stably bound to DNA in the presence of oxolinic acid; and E-DNA, a derivative of E*DNA created by protein denaturation which contains subunit A covalently linked to cleaved DNA.

References

1. Wang, J.C. and Liu, L.F. (1979) in Molecular Genetics, Taylor, J.M., Ed., Part 3, pp. 65-88. Academic Press, New York
2. Cozzarelli, N.R. (1980) Science 207, 953-960
3. Gellert, M. (1981) Ann. Rev. Biochem. 50, 879-910
4. Higgins, N.P., Peebles, C.L., Sugino, A. and Cozzarelli, N.R. (1978) Proc. Natl. Acad. Sci. USA 75, 1773-1777
5. Liu, L.F. and Wang, J.C. (1978) Proc. Natl. Acad. Sci. USA 75, 2098-2102

6. Sugino, A., Peebles, C.L., Kreuzer, K.N. and Cozzarelli, N.R. (1977) Proc. Natl. Acad. Sci. USA 74, 4767-4771
7. Gellert, M., Mizuuchi, K., O'Dea, M.H., Itoh, T. and Tomizawa, J. (1977) Proc. Natl. Acad. Sci. USA 74, 4772-4776
8. Gellert, M., O'Dea, M.H., Itoh, T. and Tomizawa, J. (1976) Proc. Natl. Acad. Sci. USA 73, 4474-4478
9. Sugino, A., Higgins, N.P. and Cozzarelli, N.R. (1980) Nuc. Acids Res. 8, 3865-3874
10. Klevan, L. and Wang, J.C. (1980) Biochemistry 19, 5229-5234
11. Brown, P.O. and Cozzarelli, N.R. (1979) Science 206, 1081-1083
12. Sugino, A., Higgins, N.P., Brown, P.O., Peebles, C.L. and Cozzarelli, N.R. (1978) Proc. Natl. Acad. Sci. USA 75, 4838-4842
13. Mizuuchi, K., O'Dea, M. and Gellert, M. (1978) Proc. Natl. Acad. Sci. USA 75, 5960-5963
14. Staudenbaner, W.L. (1970) Molec. Gen. Genet. 145, 3175-3186
15. Sugino, A. and Cozzarelli, N.R. (1980) J. Biol. Chem. 255, 6299-6306
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
17. Champoux, J.J. and McConaughy, B.L. (1976) Biochemistry 15, 4638-4642
18. Van de Sande, J.H., Kleppe, K., and Khorana, H.G. (1973) Biochemistry 12, 5050-5055
19. Glynn, I.M. and Chappell, J.B. (1964) Biochem. J. 90, 147-149
20. Jones, O.W. and Berg, P. (1966) J. Mol. Biol. 22, 199-209
21. Yarus, M. and Berg, P. (1970) Analyt. Biochem. 35, 450-465
22. Riggs, A.D., Suzuki, H. and Bourgeois, S. (1970) J. Mol. Biol. 48, 67-83
23. Hinkle, D.C. and Chamberlin, M.J. (1972) J. Mol. Biol. 70, 157-185
24. Gellert, M., Mizuuchi, K., O'Dea, M.H. and Nash, H.A. (1976) Proc. Natl. Acad. Sci. USA 73, 3872-3876
25. Morrison, A., Higgins, N.P. and Cozzarelli, N.R. (1980) J. Biol. Chem. 255, 2211-2219
26. Morrison, A. and Cozzarelli, N.R. (1981) Proc. Natl. Acad. Sci. USA 78, 1416-1420
27. Klotz, I.M. (1974) Accounts Chem. Res. 7, 162-168
28. Peebles, C.L., Higgins, N.P., Kreuzer, K.N., Morrison, A., Brown, P.O., Sugino, A. and Cozzarelli, N.R. (1978) Cold Spring Harbor Symp. Quant. Biol. 43, 41-52
29. Morrison, A. and Cozzarelli, N.R. (1979) Cell, 17, 175-184
30. Kirkegaard, K. and Wang, J.C. (1981) Cell, 23, 721-730
31. Fisher, L.M., Mizuuchi, K., O'Dea, M.H., Ohmori, H. and Gellert, M. (1981) Proc. Natl. Acad. Sci. 78, 4165-4169
32. Hinkle, D.C. and Chamberlin, M.J. (1972) J. Mol. Biol. 70, 187-195
33. Liu, L. and Wang, J.C. (1978) Cell 15, 979-984
34. Depew, R.E. and Wang, J.C. (1975) Proc. Natl. Acad. Sci. USA 72, 4275-4279
35. Kreuzer, K.N., McEntee, K., Geballe, A.P. and Cozzarelli, N.R. (1978) Mol. Gen. Genet. 167, 129-137