Volume 8 Number 17 1980 Nucleic Acids Research

DNA gyrase subunit stoichiometry and the covalent attachment of subunit A to DNA during DNA cleavage

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Received 12 May 1980

ABSTRACT

Escherichia coli DNA gyrase contains a 1:1 ratio of protomers coded by the genes *gyrA* and *gyrB*. This along with previous results shows that the enzyme has two copies of each protomer and thus a molecular weight of 400,000. Abortion of the gyrase reaction results in double-strand breakage of the DNA and covalent attachment of both *gyrA* protomers to the 5'-cut ends. We conclude that the *gyrA* protomer contains a critical part of the active site for the concerted breakage and reunion reaction of gyrase, the topoisomerase activity of the enzyme.

INTRODUCTION

DNA gyrase introduces negative supercoils into DNA in the presence of ATP (1). It is an essential enzyme with important roles in DNA replication, recombination, repair, and transcription (reviewed in ref. 2). The enzyme is composed of two subunits, termed A and B (3). For *Escherichia coli* gyrase, subunit A is a homodimer of 105,000 dalton protomers coded by the *gyrA* (formerly *nalA*) gene; subunit B is the product of the *gyrB* (formerly *cou*) gene, whose protomer molecular weight is 95,000 (3-9). A similar structure has been found for the gyrases from *Micrococcus luteus* (10) and *Bacillus subtilis* (A. Sugino and K. Bott, unpublished data). Each subunit has been separately purified (3, 7, 10) but both are needed for all gyrase activities (2).

The role of the gyrase subunits has been revealed primarily by studying enzyme inhibitors. Binding of ATP to gyrase subunit B provides energy to fuel supercoiling; this subunit controls sensitivity to the antibiotics novobiocin and coumermycin A_1 that poison energy transduction by blocking ATP binding (6, 11). The related supercoil relaxing enzyme, topoisomerase II', is composed of gyrase subunit A and a subunit homologous to a portion of subunit B and shows no apparent interaction with ATP or novobiocin (12, 13). Nalidixic and oxolinic acids act

through the A subunit (4, 5), entrapping a stable complex of enzyme and DNA. If strong protein denaturants are added to the trapped complex, site-specific double-strand breaks result with concomitant covalent linkage of gyrase protein to the termini. This abortive reaction illuminates three important aspects of the gyrase mechanism. First, it suggests that gyrase breaks and rejoins internucleotide bonds of DNA without an outside energy source by the mechanism first proposed by Wang for topoisomerase I (14). In this scheme a covalent protein-DNA intermediate results from the breakage reaction and preserves the energy of the phosphodiester linkage for the reunion step. Second, transient double-strand breaks in DNA are an essential feature of the sign inversion mechanism for supercoiling proposed by Brown and Cozzarelli which explains how gyrase changes the linking number of DNA in steps of two (15). Third, breakage of both strands is needed to account for the gyrase-catalyzed reactions of catenation, uncatenation, knotting, and unknotting of closed duplex circular DNA (16, 17).

Sequence analysis of the aborted gyrase product has revealed that cleavage is precise to the nucleotide level and generates 4-nucleotide long 5'-extensions with a protein covalently attached to each 5'-phosphoryl (18). In this report, we show that the attached protein is a single *gyrA* protomer. The active site for the breakage and reunion reaction is therefore in the A subunit and when gyrase acts it contains at least two *gyrA* protomers. We also find a precise 1:1 ratio of the *gyrA* and *gyrB* protomers in active enzyme. Taken with previous data on gyrase structure (2) we can conclude that gyrase is a tetramer containing two each of the *gyrA* and *gyrB* gene products.

EXPERIMENTAL PROCEDURES

Enzymes. Constituted DNA gyrase which contains both subunits (3) and subunits A (3) and B (19) were purified as described. Pancreatic DNase I, staphylococcal nuclease, and proteinase K were purchased from Boehringer Mannheim.

Labeled DNA. To prepare uniformly ^{32}P-labeled Colicin E1 (ColE1) DNA, *E. coli* strain JC411, kindly provided by Dr. D. Helinski, was grown at 37° in 100 ml of low TPA-medium (20) containing 75 μM KH_2PO_4; at a density of 5 x 10^8 cells/ml, 10 mCi of $^{32}P_i$ was added. After 60 min at 37°, chloramphenicol (Sigma) was added to 0.15 mg/ml and incubation continued for 20 h. ColE1 DNA was isolated as described (21) except CsCl

equilibrium density gradient centrifugation was used instead of CsCl-ethidium bromide equilibrium density gradient centrifugation. The final specific activity was 5.1 x 10^5 cpm/μg of DNA; 90% of the radioactivity was in supertwisted ColE1 DNA and the remainder in nicked ColE1 DNA. ^{3}H-labeled ColE1 DNA was prepared similarly. 5'-^{32}P-labeled DNA was prepared by end labeling *Eco*RI restriction enzyme cleaved ColE1 DNA with [γ-^{32}P]ATP and phage T4 polynucleotide kinase.

Labeling of gyrase protomer covalently attached to DNA. A covalent complex of DNA gyrase and labeled DNA was formed in a 4-ml reaction mixture containing 35 mM Tris-HCl (pH 7.8), 10 mM potassium phosphate, 6 mM $MgCl_2$, 5 mM spermidine-HCl, 5 mM dithiothreitol, 180 μg tRNA, 100 μg of ^{32}P-labeled ColE1 DNA linearized by *Eco*RI nuclease digestion (5.1 x 10^7 cpm), 0.2 mg of freshly dissolved oxolinic acid, and 10^4 units (20 μg) of DNA gyrase either reconstituted with subunits A and B or the constituted form purified as such from cells. After 60 min at 30°, 0.5% sodium dodecyl sulfate ($NaDodSO_4$) was added. About 50% of the radioactive DNA was attached to gyrase as judged by retention by nitrocellulose filters. The DNA-protein complex was concentrated by ethanol precipitation and dialyzed for two days at room temperature against 50 mM Tris-HCl (pH 7.5). The DNA was digested in a 1.0-ml reaction mixture containing 0.5 ml of the concentrated, dialyzed DNA-protein complex, 50 mM Tris-HCl (pH 7.8), 2 mM $CaCl_2$, and 1000 units of staphylococcal nuclease. After 30 min at 37°, an additional 500 units of the nuclease was added and incubation was continued for 30 min. 99% of total radioactivity was rendered acid soluble. The digest was chromatographed on a 1.0 x 20 cm Sephacryl S-200 column equilibrated with 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, and 50 mM KCl. Nearly excluded radioactive material (5 x 10^4 cpm) was pooled, retreated with the nuclease as above, and precipitated with 5% trichloroacetic acid. After resuspension in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, two extractions with ether, and bubbling with N_2, the sample was subjected to electrophoresis through a 10% polyacrylamide gel containing 1% $NaDodSO_4$ (22).

Gyrase protomers covalently and non-covalently attached to DNA. Gyrase was bound to DNA in 250-μl reaction mixtures containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 20 mM KCl, 5 mM dithiothreitol, 5 mM spermidine-HCl, 50 μg of bovine serum albumin per ml, 5'-^{32}P-labeled *Eco*RI restricted ColE1 DNA, and subunits A and B in a ratio of 0.38, 1.3, or 2.4. After 45 min at 23°, the sample was filtered through a 28 x 0.68 cm

Sepharose-4B column equilibrated with 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 20 mM KCl, and 5 mM 2-mercaptoethanol, and 0.34 ml fractions were collected. The complex of gyrase with DNA was in fractions 8-10, well separated from the free gyrase subunits in fractions 22-25. Oxolinic acid (100 µg/ml) was added to one-half of the complex; after 40 min at 23°, 0.5 mM ATP was included to enhance covalent complex formation (23). After 5 min, 50 µg/ml albumin was added as a carrier and then covalent attachment to DNA was induced by addition of 0.1% $NaDodSO_4$. The samples were electrophoresed through a 6-15% gradient polyacrylamide gel (24) along with untreated complex from the Sepharose column. The gel was stained with Coomassie brilliant blue and scanned at 650 nm using a Gilson spectrophotometer and a linear gel transport. The peaks corresponding to *gyrA* and *gyrB* were cut out and weighed to determine the protomer ratio.

Assay of complexes of gyrase and DNA by binding to nitrocellulose filters (23). The 10-µl samples containing the complex of gyrase and labeled DNA were diluted with 1 ml of binding buffer [20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 5% dimethylsulfoxide] and filtered through 25 mm Schleicher and Schuell nitrocellulose filters. The filters were washed twice with 5 ml of binding buffer, dried, and counted.

CsCl equilibrium density gradient centrifugation. A covalent complex of uniformly ^{32}P-labeled ColE1 DNA (10^5 cpm) and gyrase was diluted to 3 ml with 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1% Triton X-100, mixed with 4 g CsCl and a ^3H-labeled ColE1 DNA reference, and centrifuged at 36,000 rpm for 48 h at 15° in a Spinco SW 50.1 rotor; the polyallomer tubes were prewashed with 10 µg/ml of albumin to enhance recovery of label. 50-µl fractions were collected from the bottom of the tube directly into scintillation vials. The density gradient was calculated from the refractive index of selected fractions.

RESULTS

Number of gyrase protomers linked to cleaved DNA. Successive addition of oxolinic acid and $NaDodSO_4$ to a gyrase reaction causes double-strand breaks in the DNA substrate terminated by covalently attached gyrase protomers (4, 5, 18, 25). The bound protein reduces the buoyant density of the DNA (Fig. 1). The complex with ^{32}P-labeled ColE1 DNA had a density of 1.685 gm/cm^3 and was resolved from unreacted substrate and an internal ^3H-labeled ColE1 DNA reference at 1.705 g/cm^3 (Fig. 1b). The density shift

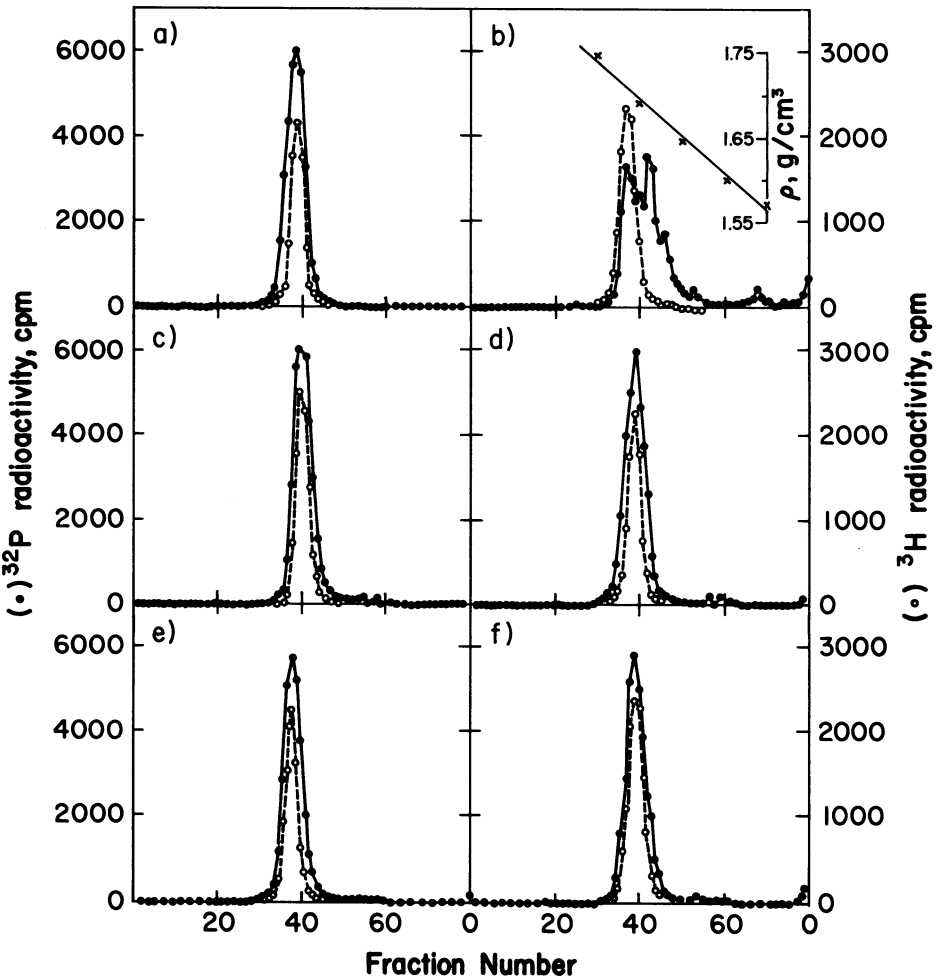

Fig. 1. Reduction in buoyant density of DNA by covalently bound gyrase. The complete 17-µl reaction mixtures contained 0.1 µg of native ^{32}P-labeled ColE1 DNA, 5 units each of subunits A and B, and 50 µg/ml of oxolinic acid. After 60 min at 30°, 0.5% NaDodSO4 was added. The samples were mixed with 20 µg of ^{3}H-labeled ColE1 DNA and analyzed by CsCl equilibrium density gradient centrifugation. No gyrase (a); complete (b); no subunit B (c); no subunit A (d); no oxolinic acid (e); complete but then treated with 50 µg/ml of proteinase K at 37° for 30 min (f).

required both gyrase subunits (Fig. 1, a-d) and oxolinic acid (Fig. 1e) and was abolished by treatment with proteinase K (Fig. 1f). The reduction in density of about 45% of the DNA molecules is in good agreement with the 52% of the DNA which was bound to gyrase as judged by retention by a nitrocellulose filter; moreover, agarose gel electrophoresis showed that a similar proportion of the DNA was converted to full length linear molecules. The small shoulder on the light side of the bound DNA peak may result from double cutting by gyrase. The molecular weight of the protein attached to the DNA was calculated using the equation (26):

$$m_p = \frac{m_D \rho_p (\rho_D - \rho_c)}{\rho_D (\rho_c - \rho_p)}$$

where m_p and m_D stand for the mass of the protein and DNA in the complex, and ρ_p, ρ_D, and ρ_c refer to the buoyant density of the protein, DNA, and the complex, respectively. If the molecular weight of the Cs^+ salt of ColE1 DNA is 5.6×10^6 and the buoyant density of the Cs^+ salt of the protein is 1.29 (27), then the molecular weight of the protein in the complex is about 210,000. The molecular weight of the attached *gyrA* protomer (see below) is 105,000. Thus, there are two protomers attached to each ColE1 DNA molecule and since both 5'-cut ends are blocked by protein (18, 25) the protomers are solely on the cut ends.

Stoichiometry of gyrase subunits bonded to DNA. The buoyant density shift does not identify the protomers attached to cleaved DNA since the *gyrA* and *gyrB* gene products have similar molecular weights. The covalently attached polypeptide was identified in two ways.

The first method also demonstrated the stoichiometry of the protomers in enzymatically active gyrase, a number that heretofore had been elusive. Although neither gyrase subunit alone interacts detectably with nucleic acids, together they bind ColE1 DNA in a very stable complex with a half-life of about 60 h at 23° (28). This stable complex has been shown to be fully functional using several tests (23, 28). The ratio of A and B protomers in functional enzyme was determined by mixing with DNA subunits A and B in different ratios and purifying the active complexes from unassociated subunits and any inactive enzyme by Sepharose-4B chromatography. The complex was subjected to gel electrophoresis in the presence of $NaDodSO_4$ and the subunit ratio determined by scanning the Coomassie blue-stained bands and assuming that the protomers stain equally (29). An example of the scans is shown in Fig. 2a and Fig. 2b.

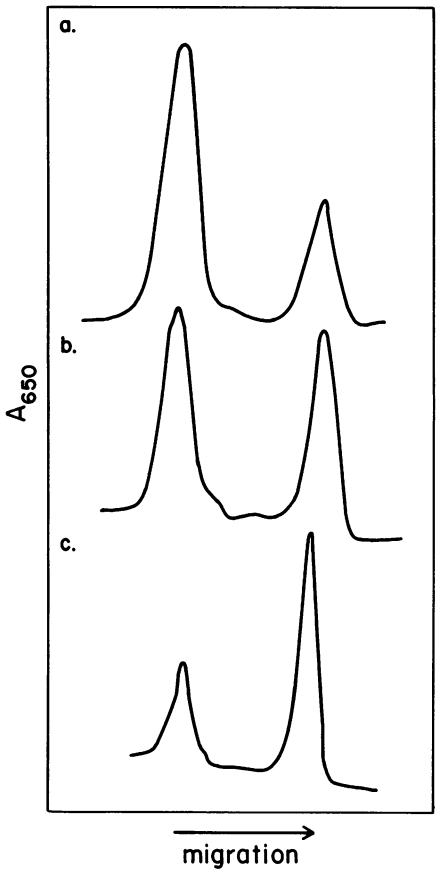

Fig. 2. Amount of *gyrA* and *gyrB* in non covalent and covalent complexes with DNA. 5'-^{32}P-labeled linear ColE1 DNA (30 µg) was mixed with 20 µg of subunit A and 8.3 µg of subunit B and the DNA-bound enzyme was purified by Sepharose-4B gel filtration. One-half of the complex was directly subjected to gel electrophoresis in the presence of NaDodSO$_4$ (lane b) and the other half was treated with oxolinic acid before electrophoresis (lane c). Lane a shows the subunits prior to Sepharose-4B chromatography. The gels were stained with Coomassie blue and scanned at 650 nm; the two peaks shown correspond to *gyrA* and *gyrB* polypeptides in order of increasing electrophoretic mobility.

Final A to B ratios of 1.01, 1.03, and 0.97 were measured for subunits initially mixed at ratios of 0.38, 1.3, and 2.4, respectively. Thus, active gyrase has a 1:1 protomer ratio. The addition of oxolinic acid to the gyrase DNA complex resulted in the disappearance of most of the *gyrA* protomer electrophoretic band but essentially none of the *gyrB* protomer band; in the experiment in Fig. 2c about three times as much *gyrB* as *gyrA* protomer remained. It is likely that in the aborted reaction the *gyrA* protomer becomes covalently attached to the DNA and therefore does not enter the polyacrylamide gel. Some free *gyrA* protomer was detected in all the experiments probably because not all bound enzyme cleaved (23) or some enzyme released from the DNA prior to cleavage.

A direct demonstration of attachment of the *gyrA* protomer to DNA employed the procedure used by Rekosh et al. to determine the protein

attached to the ends of adenovirus DNA (30). A covalent complex of gyrase with highly ^{32}P-labeled ColE1 DNA was induced by treatment with oxolinic acid and $NaDodSO_4$ as above, and the DNA was exhaustively digested with staphylococcal nuclease. The covalently bonded protomer was then identified by the attached ^{32}P-labeled fragment. Display of this material by polyacrylamide gel electrophoresis showed that the predominant labeled band migrated at the position of the *gyrA* protomer (Fig. 3b). The average chain length of the covalently attached oligonucleotide was about five nucleotides which did not change the mobility of the polypeptide. Both

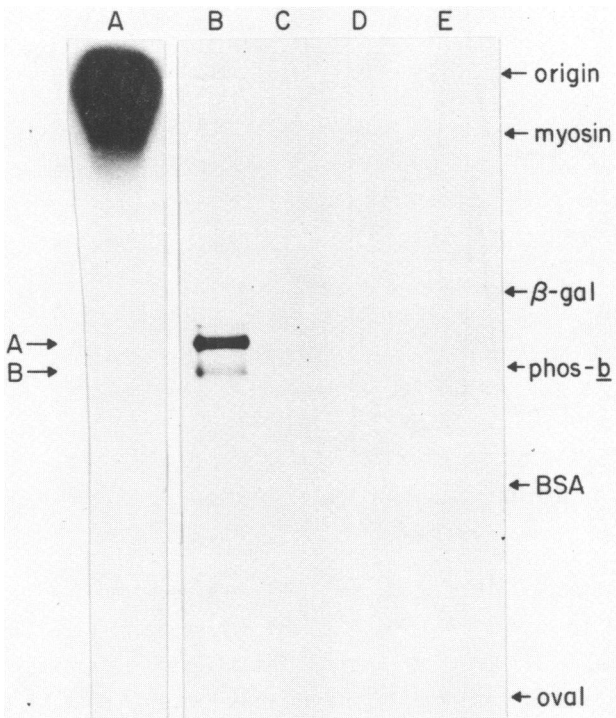

Fig. 3. Gyrase subunit A is covalently attached to cleaved DNA. The covalent complex formed between ^{32}P-labeled ColE1 DNA and 10^4 units of DNA gyrase was applied to a $NaDodSO_4$-polyacrylamide gel either directly (A) or after staphylococcal nuclease digestion (B-E). Gyrase was replaced by 10^4 units of subunit A in lane C and 10^4 units of subunit B in lane D. The nuclease digested sample was treated with proteinase K before addition to lane E. After electrophoresis the gel was stained with Coomassie blue and the positions of gyrase subunits A and B, myosin, β-galactosidase (β-gal), phosphorylase-b (phos-b), bovine serum albumin (BSA), and ovalbumin (oval) are shown. The gel was autoradiographed: lane a was exposed for 5 h and the other lanes for three days.

gyrase subunits were required for *gyrA* protomer labeling (Fig. 3c and d) and no labeled band was seen after proteinase K treatment (Fig. 3e). In the absence of nuclease digestion, the label was at the top of the gel (Fig. 3a). There was 3000 cpm in the *gyrA* band and 200 cpm in a band that moved a little slower than the *gyrB* standard; the latter could be a breakdown product of *gyrA* or be derived from *gyrB*. The preferential labeling of subunit A was also found with constituted DNA gyrase as well as enzyme reconstituted in vitro (data not shown).

DISCUSSION

Gyrase reactions involve concerted backbone breakage and reunion, the hallmark of topoisomerases, and coupled energy transduction that allows catalysis of endergonic topoisomerizations such as supercoiling. Although all gyrase reactions require both subunits A and B, substantial division of labor between the subunits exists. A key portion of the active site of the breakage and reunion component is now demonstrated to be in subunit A, since interruption of this activity leads to covalent attachment of A protomers to the ends of the DNA. This was adumbrated by the finding that subunit A is the target for oxolinic acid, a drug that greatly accentuates $NaDodSO_4$-induced cleavage (2). Subunit B, on the other hand, is the critical subunit for energy transduction (11). It contains the ATP binding site (6), is the target for drugs that specifically block energy requiring gyrase reactions (11), and is replaced in topoisomerase II' which is limited to exergonic reactions (12, 13).

It is clear now that there are two copies of each gyrase gene product in the active enzyme. Gyrase that binds to DNA contains an equal number of protomers (Fig. 2). The 2:2 ratio comes from the finding that during cleavage, one *gyrA* protomer is attached to each of the two revealed 5' ends (Figs. 1 and 3, ref. 18). Y.-C. Tse, K. Kirkegaard, and J. C. Wang (personal communication) have shown that subunit A protomers of *M. luteus* gyrase are specifically attached to DNA via phosphotyrosine bonds after cleavage induced by alkali. The $\alpha_2\beta_2$ structure for gyrase is consistent with all other data. First, while the estimates for the native molecular weight of gyrase have varied, the best data gave a value of about 400,000 (L. L. Liu and J. C. Wang, personal communication). Second, a 1:1 molar ratio of subunits is needed to reconstitute all of the gyrase activities (2, 3). Third, subunit A, at least, is clearly a dimer of protomers (3, 5). Fourth, about two copies of each protomer are needed to form a complex competent to adhere DNA to a nitrocellulose filter (28).

ACKNOWLEDGMENTS

This work was supported by grants from NIH GM-21397 and CA-19265.

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