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The C-terminal domain of the *Escherichia coli* DNA gyrase A subunit is a DNA-binding protein

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ABSTRACT

We have constructed a clone which over-produces a 33 kDa protein representing the C-terminal portion of the *Escherichia coli* DNA gyrase A subunit. This protein has no enzymic activity of its own, but will form a complex with a 64 kDa protein (representing the N-terminal part of the A subunit) and the gyrase B subunit, that will efficiently catalyse DNA supercoiling. We show that the 33 kDa protein can bind to DNA on its own in a manner which induces positive supercoiling of the DNA. We propose that the 33 kDa protein represents a domain of the gyrase A subunit which is involved in the wrapping of DNA around DNA gyrase.

INTRODUCTION

DNA gyrase (E.C. 5.99.1.3) is the exclusively prokaryotic enzyme which introduces negative supercoils into closed-circular DNA using the free energy derived from ATP hydrolysis (reviewed in 1, 2). The enzyme from *Escherichia coli* consists of two proteins, A and B, of molecular masses 97 and 90 kDa respectively. The active enzyme is an A_2B_2 tetramer (3, 4). The genes for the A and B proteins, *gyr*A and *gyr*B, have been cloned, sequenced and can be over-expressed (5−11). In the absence of ATP, gyrase will catalyse the relaxation of negative supercoils in DNA.

The mechanism of DNA supercoiling by gyrase is thought to involve the ATP-driven translocation of a segment of DNA through a double-stranded DNA break. This break is stabilised in part by transient covalent bonds between GyrA (the DNA gyrase A protein) and the DNA (12). It has been shown that GyrA is involved in the DNA breakage and reunion aspects of the supercoiling reaction, while GyrB (the DNA gyrase B protein) is the site of ATP hydrolysis. Evidence for the roles of the A and B subunits has come, in part, from studies using antibacterial agents. GyrA is thought to be the target of the quinolone drugs (e.g. nalidixic acid, ciprofloxacin) and GyrB is the target of the coumarins (e.g. coumermycin A_1 and novobiocin) (reviewed in 13). The quinolones inhibit DNA supercoiling by interrupting the DNA breakage-reunion activity of gyrase. Incubation of gyrase with DNA in the presence of a quinolone drug leads to the cleavage of DNA in both strands if the reaction is terminated by the addition of a protein denaturant such as SDS (14). Moreover, following cleavage, GyrA is found to be covalently bound to the DNA at the break site *via* a phosphotyrosine linkage between the newly formed 5′-phosphate and Tyr-122 (8). The coumarin drugs inhibit DNA supercoiling by preventing the hydrolysis of ATP by gyrase (15−17).

A number of structural studies have been performed on gyrase and its complexes with DNA. Electron microscopy experiments have suggested that the active enzyme (A_2B_2) has a 'heart-shaped' structure with the A subunits forming the upper lobes of the heart (18). Transient electric dichroism experiments suggested that about 120 bp of DNA are wrapped around a globular protein core in a single positive superhelical turn (19). Small angle neutron scattering and light scattering data support the idea that gyrase is an oblate particle containing cavities or channels between the protein subunits (4). In addition, these scattering data are consistent with the existence of domain structure within the subunits.

Other evidence for domain structure has been provided for both the A and B proteins. GyrB isolated from some bacterial strains contains a significant proportion of a 47 kDa protein comprising the C-terminal portion of GyrB (9, 20, 21). When complexed with the A protein, this fragment will support DNA relaxation but not DNA supercoiling. These data suggest that the C-terminal part of GyrB binds to GyrA and will form a complex capable of catalysing DNA breakage and reunion. The N-terminal portion of GyrB is proposed to contain the site of ATP hydrolysis (9, 20). A clone has recently been constructed which synthesises this N-terminal domain of GyrB as a gene product (22); this protein is found to have novobiocin-sensitive ATPase activity. These data support the idea that GyrB consists of two domains, an N-terminal domain (43 kDa) which contains the ATP binding site and a C-terminal domain (47 kDa) which interacts with GyrA and DNA. The 43 kDa N-terminal domain of GyrB, GyrB(1−393), has been crystallized (22) and a high resolution three-dimensional structure has recently been obtained (D.B. Wigley, personal communication).

Cleavage of GyrA with the proteases trypsin or chymotrypsin generates two stable fragments with molecular masses 64 and

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33 kDa (23, 24). The 64 kDa tryptic fragment, derived from the N-terminus (residues 6−571 of the intact protein), will support DNA supercoiling (albeit with low efficiency) in the presence of GyrB, and has been proposed to comprise the DNA breakage-reunion domain of GyrA (23). When produced as a direct gene product, the N-terminal portion of the A protein (GyrA(1−572)) retains enzymic activity and has been crystallised and shown to diffract X-rays (25), although a high resolution structure has not yet been determined. A number of deletion derivatives of GyrA have been constructed (26), and a 58 kDa protein, GyrA(7−523), was the smallest fragment found to support the DNA breakage and reunion activities. The precise function of the C-terminal portion of the GyrA protein is not clear. The trypsin-generated 33 kDa C-terminal fragment is able to enhance the supercoiling activity of the N-terminal 64 kDa tryptic fragment (23) and was therefore suggested to stabilise the interactions between gyrase and DNA. It is proposed that GyrA consists of two domains, an N-terminal domain (59−64 kDa) which contains the DNA breakage and reunion activities and a C-terminal domain (33 kDa) involved in stabilising the DNA-protein complex.

In this paper, we describe a detailed analysis of the C-terminal 33 kDa domain of GyrA. The protein has been generated as a direct gene product and purified to homogeneity. A functional analysis of this protein, and its complexes with the 64 kDa N-terminal domain of GyrA and DNA, has been performed. On the basis of these experiments we suggest that the C-terminal portion of GyrA imparts stability to the gyrase-DNA complex by acting as a non-specific DNA-binding domain.

MATERIALS AND METHODS

Enzymes and DNA

The A and B subunits of *E. coli* DNA gyrase were prepared from strains JMtacA and JMtacB as described by Hallett *et al.* (11). The N-terminal 64 kDa fragment of GyrA was prepared from *E. coli* strain JM109 containing plasmid pRJR242 as described previously (26). The N-terminal 43 kDa fragment of GyrB, prepared from *E. coli* strain JM109 containing plasmid pAJ1 (22), was a gift from Alison Grimshaw (this laboratory). All protein samples were dialysed extensively against Enzyme Buffer (50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM DTT, 1 mM EDTA, 10% (w/v) glycerol) before use. Concentrations of the gyrase proteins are expressed in terms of molarities of dimer. Supercoiled plasmid DNA, the relaxed form of plasmid pBR322 and the catenated form of plasmid pRH43-117 were prepared as described previously (23). A 147 bp DNA fragment (which contains the major quinolone-induced gyrase cleavage site in pBR322) was a gift of S.T. Dobbs (this laboratory). Nicked pBR322 DNA was prepared by digestion of the supercoiled form with DNase I in the presence of ethidium bromide (27); 200 μg of supercoiled pBR322 DNA were incubated in a total volume of 4 ml under the following conditions: 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.01% (w/v) gelatin, 150 μg/ml ethidium bromide, 0.003 U/μl DNase I for 30 min at 20°C. The nicked DNA species was subsequently purified by caesium chloride density gradient centrifugation. Oligonucleotides were synthesised using an Applied Biosystems 380B oligonucleotide synthesiser by Debra Langton (University of Leicester). Restriction enzymes, T4 DNA ligase, and *E. coli* DNA ligase were purchased from New England Biolabs and used according to the manufacture's instructions. Mung bean nuclease was from Pharmacia, and Taq DNA polymerase was from Amersham.

Polymerase Chain Reaction

PCR mixtures (50 μl final volume) contained the following: 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 pmol/μl oligonucleotides, 5 Units Taq polymerase. The following steps were repeated for 30 cycles in a Trio-thermoblock thermal incubator (Biometra): Denaturation, 94°C, 1.5 min; annealing, 50°C, 1.5 min; extension, 74°C, 4−10 min. The PCR products were extracted with chloroform:isoamyl alcohol (24:1) and precipitated with ethanol. After digestion with the appropriate restriction enzymes, the amplified DNA was run on a 1% low melting point agarose gel, and the DNA isolated by phenol extraction (28).

Cloning

To construct plasmid pTTQ18*, a 54 bp sequence between *ori* and *tac* in the plasmid pTTQ18 (29) was deleted and replaced with a *Dra* III restriction enzyme site using PCR methodology. We constructed this plasmid because we have found that DNA preparations of pTTQ18 and its derivatives are often contaminated by an approximately 2 kb plasmid which is probably the result of a recombination event between 54 bp homologous sequences within *lac*I^q and residual *lac*I sequence between *ori* and *tac* (M.J.R. Stark, personal communication).

Plasmid pRJR79, encoding the 33 kDa C-terminal domain of GyrA, GyrA(572−875), was constructed in the following manner. Using appropriate oligonucleotides and PCR technology, an *Ssp* I restriction site was generated at nucleotide 1713 in the *gyr*A gene of plasmid pPH3 (11). Digestion of the PCR product with *Ssp* I and *Sph* I yielded a 1.8 kb DNA fragment encoding the C-terminal end of the gyrase A protein (GyrA(572−875)). Plasmid pTTQ18* was digested with *Eco* RI and subsequently treated with mung bean nuclease. The blunted DNA was then cut with *Sph* I, and the large fragment (~4.5 kb) was gel purified and ligated to the 1.8 kb *Ssp* I + *Sph*-I-cut pPH3 PCR fragment described above. The resulting plasmid (pRJR79) was transformed into competent *E. coli* JM109 cells. DNA sequence analysis of about 350 nucleotides (from approximately 200 bp downstream of the gene start through the ribosome binding site and the promoter) showed no anomalies (data not shown).

Protein purification

The 33 kDa protein was purified from *E. coli* strain JM109 containing plasmid pRJR79. Cells were grown at 37°C in 2 L of 2×YT broth (10 g/L Bacto-tryptone (Difco), 10 g/L Bacto-yeast extract (Difco) and 5 g/L NaCl) containing 100 μg/ml ampicillin and induced (at an optical density of 0.5 at 595 nm) by the addition of isopropyl-β-D-thiogalactopyranoside (to 50 μM). Cells were harvested after 4 hr and resuspended in 25 ml of 50 mM Tris-HCl (pH 7.5), 10% sucrose, and the following were added: DTT to 2 mM, EDTA to 20 mM, and KCl to 100 mM. The cells were disrupted using a French press at a pressure of 8,000−12,000 psi; at this stage the 33 kDa protein, GyrA(572−875), represented approximately 20−30% of the total cell protein. The resulting extract was spun at 130,000 *g* in a Beckman Ti50 rotor for 15 min at 4°C. The pellet was the resuspended in Enzyme Buffer containing 6 M guanidine hydrochloride. After 20 min on ice, this suspension was again

spun at 130,000 *g* for 15 min. The supernatant (20 ml) was dialysed against Enzyme Buffer (in four 250 ml aliquots) overnight at 4°C. The protein was then precipitated using 30–70% (w/v) ammonium sulphate fractionation. The pellet was collected by centrifugation at 10,000 *g* for 15 min at 4°C, and resuspended in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, 5 mM DTT. The protein was then purified by chromatography using FPLC mono Q and phenyl superose columns (Pharmacia). Peak fractions were pooled and dialysed into Enzyme Buffer overnight, frozen in liquid nitrogen and stored at −70°C. The 33 kDa protein was estimated to be >98% pure as judged by SDS-polyacrylamide gel electrophoresis.

Other methods

DNA supercoiling, relaxation, decatenation and cleavage assays were carried out as described previously (23). Gel-retardation assays were performed as follows. Samples of the gyrase proteins were incubated with linear DNA fragments (ranging from 24 to 147 bp in length) in 50 mM Tris-HCl (pH 7.5), 55 mM KCl, 4 mM $MgCl_2$, 5 mM dithiothreitol, 5% (w/v) glycerol for 1 hr and electrophoresed through 5 or 10% polyacrylamide gels (acrylamide:bis, 29:1) in 90 mM Tris-borate (pH 7.0), 10 mM $MgCl_2$. The DNA was visualised after staining in ethidium bromide. The sequences of the oligonucleotides used in gel-retardation assays are shown below:

52 bp:

5′ AGCTTAGCGGGTCATTTCACGCGCGCGACGCGCCGCTTCACGGGCACGGGCAGCAT 3′

3′ ATCGCCCAGTAAAGTGCGCGCGCTGCGCGGCGAAGTGCCCGTGCCCGTCGTAGC 5′

35 bp:

5′ ACCCGCCGTAAAGGTGCGCTCGACTTAGCGGGCCTGCC 3′

3′ TGGGCGGCATTTCCACGCGAGCTGAATCGCCCGGA 5′

24 bp:

5′ GGTGACGTAATCGGTAAATACCATCCC 3′

3′ CCACTGCATTAGCCATTTATGGTA 5′

Each single-stranded oligonucleotide was precipitated with ethanol and resuspended in sterile distilled water. The complementary pairs were mixed in equimolar amounts and incubated at 65°C for 10 min, before cooling on ice. The concentration of each pair was determined from their absorbance at 260 nm.

The extent to which the gyrase proteins are able to wrap DNA was determined by incubation with nicked pBR322 DNA. Reaction mixtures (30 μl) containing 50 mM Tris-HCl (pH 7.5), 55 mM KCl, 2 mM $MgCl_2$, 5 mM dithiothreitol, 5% (w/v) glycerol, 13.3 μg/ml nicked pBR322 and protein were incubated at 25°C for 30 min. Nicotine adenine dinucleotide and *E. coli* DNA ligase were then added to final concentrations of 30 μM and 0.13 U/μl respectively. Incubations were continued for 30 min at 25°C, after which the DNA was extracted with chloroform and subjected to agarose gel electrophoresis. In order to distinguish between negatively and positively supercoiled DNA, samples were analyzed by electrophoresis through agarose gels containing chloroquine (2–6 μg/ml) (30).

DNA sequencing was carried out by the dideoxynucleotide chain termination procedure (31) using Sequenase version 2.0 (United States Biochemical Corporation), directly on plasmid DNA (32). N-terminal sequencing of proteins was carried out by Kathryn Lilley (University of Leicester) as described by Matsudaira (33).

RESULTS

Over-production and purification of the 33 kDa C-terminal domain of GyrA

The 33 kDa protein, GyrA(572–875), was over-produced from *E. coli* as described in 'Materials and Methods'. After disruption of the cells by French press, the 33 kDa protein was found to be associated with the insoluble fraction (data not shown). However, the protein could be solubilised by suspension of the pellet in 6 M guanidine hydrochloride. Relatively slow dialysis into Enzyme Buffer (see Materials and Methods) retained the solubility of the 33 kDa protein, whereas rapid dialysis into a large volume of buffer led to significant protein precipitation. It is possible that the 33 kDa protein is over-produced in a misfolded form which cannot assume its correct conformation until it has been completely denatured and allowed to refold slowly. It has previously been shown that both the N- and C-terminal tryptic fragments of GyrA can be recovered from polyacrylamide gels and successfully renatured from guanidine hydrochloride-containing solutions, although in very low yields (23). Following the procedure described here, we were able to purify 15–20 mg of 33 kDa protein per litre of bacterial culture. N-terminal amino acid sequencing of the purified 33 kDa protein revealed the sequence MIKEE, indicating that the N-terminal methionine residue is still present. Hence, GyrA(572–875) contains an extra methionine residue at the N-terminus when compared to the 33 kDa tryptic fragment of GyrA (23).

Enzymic properties of the 33 kDa protein

DNA Supercoiling and Relaxation. DNA supercoiling and relaxation by DNA gyrase will only occur in the presence of both the A and B proteins (12). The 33 kDa protein, GyrA(572–875), assayed either on its own or in the presence of GyrB, showed no DNA supercoiling or relaxation activities (data not shown). The N-terminal 64 kDa fragment of the gyrase A protein, GyrA(1–572), when complexed with GyrB, has previously been shown to have a considerably reduced supercoiling activity compared to that of the intact A protein; GyrA(1–572) has a specific activity of 10^3 U/mg compared with 10^6 U/mg for

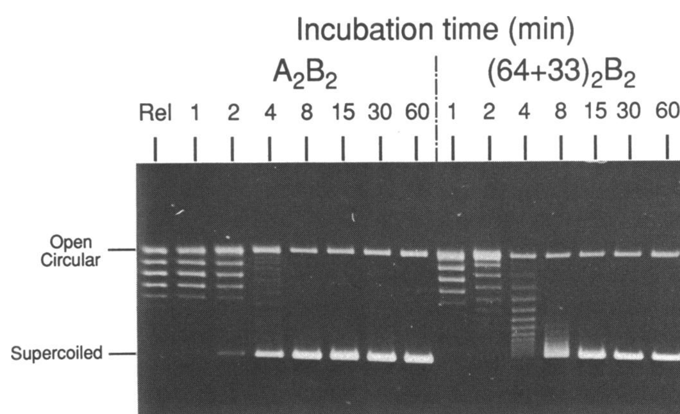

Fig. 1. Supercoiling time course of gyrase containing either GyrA or GyrA(1-572) and GyrA(572-875). Samples of GyrA (15 nM) or the 64 kDa and 33 kDa fragments (15 nM each), complexed with GyrB (15 nM) were incubated in 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 1.8 mM spermidine, 0.36 mg/ml bovine serum albumin, 18 μg/ml tRNA, 6.5% (w/v) glycerol, 5 mM dithiothreitol, 1.4 mM ATP, 10 μg/ml relaxed pBR322 in a total volume of 90 μl. At the times indicated, 10 μl samples were removed and the reaction terminated by chloroform extraction. Samples were electrophoresed through a 1% agarose gel.

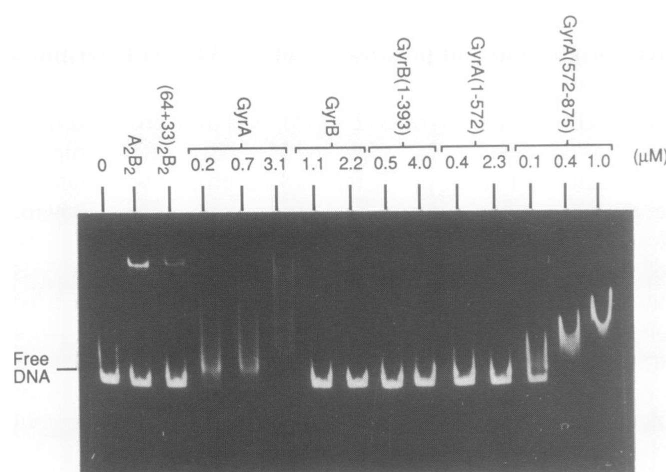

Fig. 2. Gel Retardation assay using various gyrase proteins and their fragments. Protein concentrations (expressed as μM dimer in each case) were incubated with a 147 bp DNA fragment (84 nM) as described in 'Materials and Methods'. A_2B_2 and $(64+33)_2B_2$ were at a concentration of 50 nM.

GyrA (26). Fig. 1 shows a comparison of the supercoiling reaction performed by DNA gyrase and a complex comprising equimolar amounts of GyrA(1−572), GyrA(572−875) and GyrB. We suggest that this complex will contain two copies each of the 64 kDa and 33 kDa proteins, by analogy with intact GyrA. This suggestion is supported by the mobility of this complex when bound to DNA in gel-retardation assays (see below). It was observed that there is an approximate factor of two difference in the supercoiling abilities of gyrase containing the intact A protein (A_2B_2) compared to the enzyme containing the two fragments that have been produced in isolation and reconstituted ($(64+33)_2B_2$) (see Fig. 4). Hence it would appear that the GyrA N- and C-terminal domains can be produced separately and, when added together, virtually reproduce the activity of intact GyrA. This result also suggests that the denaturation step in the purification of the 33 kDa fragment, GyrA(572−875), has little effect on the activity of the protein. Gyrase is generally found to supercoil DNA in a processive manner (12), with fully supercoiled products appearing before all the relaxed substrate has been utilised (see Fig. 1 at 2 min for A_2B_2). However, gyrase containing the reconstituted 64 kDa and 33 kDa domains shows a more distributive reaction, similar to that previously observed for the trypsin-treated A protein (GyrA′) (23). In other experiments, the ability of the two complexes to catalyse the relaxation of negatively supercoiled DNA was determined (data not shown). An approximate two-fold difference in activity between the native and reconstituted complexes was again found. It therefore appears that the reformed complex is proficient at both supercoiling and relaxation reactions, but with activities reduced by about a factor of two when compared with gyrase containing the intact A protein. Although there are difficulties in accurately quantitating the DNA supercoiling and relaxation reactions, these differences have consistently been observed in several experiments.

Decatenation and DNA cleavage. DNA gyrase can decatenate duplex DNA in the presence of ATP; this activity being considerably less efficient than the supercoiling reaction (34). The decatenation reaction cannot be detected using the 64 kDa protein, GyrA(1-572), in the presence of GyrB (26); this result is not surprising given the low supercoiling activity of the 64 kDa protein. In a similar reaction, the 33 kDa protein, GyrA(572-875), was also found to have no detectable decatenation activity (data not shown). However, if GyrA(1-572) and GyrA(572-875) are mixed in equimolar amounts, then, in the presence of GyrB, decatenation is found to proceed with equal efficiency as with intact gyrase. Since neither GyrA fragment has the ability to promote the reaction on its own, it must be assumed that the two fragments are interacting in some way to perform this reaction. This is consistent with the observation that the trypsin-treated gyrase A protein (GyrA′), in the presence of GyrB, can also support the decatenation reaction (23).

An essential step in the DNA supercoiling pathway is the cleavage of DNA in both strands. Quinolone drugs (e.g. oxolinic acid, ciprofloxacin) are thought to inhibit the supercoiling of gyrase by interrupting the cleavage and rejoining of the DNA strands (14). The addition of SDS to reactions containing gyrase, DNA and a quinolone drug, and subsequent digestion of the protein, leads to the appearance of DNA cleaved in both strands (14). It has previously been found that the 64 kDa fragment, GyrA(1-572), and a 58 kDa fragment, GyrA(7-523), will complement GyrB and perform the quinolone-induced cleavage reaction with an efficiency equal to that of intact GyrA (26). When the 33 kDa protein, GyrA(572-875), was used in a similar reaction, no cleaved DNA products could be observed (data not shown). It was also found, as expected, that a mixture of GyrA(1-572) and GyrA(572-875), complexed with GyrB, had a cleavage efficiency equal to that of the native gyrase complex. Therefore it is likely that the 33 kDa domain of GyrA plays no significant role in the DNA breakage and reunion reaction by DNA gyrase.

Table 1. The binding of GyrA and GyrA(572-875) to linear DNA fragments.

DNA length (bp)	Molar excess of protein required to leave no free DNA	
	GyrA	GyrA(572-875)
147	4	3
52	N.B.	4
35	N.B.	45
24	N.B.	N.B.

N.B. indicates that no binding was detected with at least a 30-fold molar excess of protein over DNA.

DNA binding. DNA gyrase forms an organised complex with DNA in which about 120 bp of DNA are wrapped around the protein in a single turn with a positive superhelical sense (19). The formation of such a complex can be detected by a number of techniques, including gel retardation assays (12). Complexes between gyrase and short DNA fragments have been found to be stable during electrophoresis on 5% polyacrylamide gels in the presence of Mg^{2+}; the complex forms a discrete band which can be resolved from free DNA (35). In addition, complexes between the A protein alone and DNA have been observed by electron microscopy (36).

Figure 2 shows the ability of DNA gyrase, and a number of fragments of the gyrase proteins, to bind to a 147 bp linear DNA fragment. This fragment is derived from the plasmid pBR322 and contains the preferred gyrase cleavage site in this plasmid, at nucleotide 990 (37). Both native gyrase (A_2B_2) and a complex of GyrA(1-572) and GyrA(572-875) with GyrB ($(64+33)_2B_2$) form a discrete DNA-protein complex. The position of this

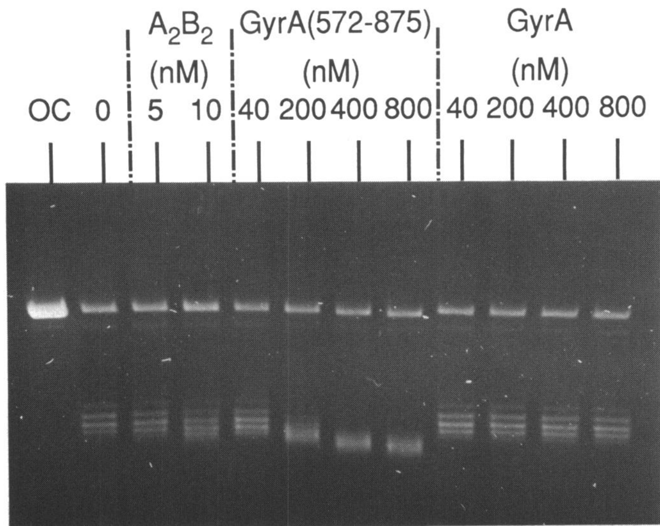

Fig. 3. The wrapping of DNA around gyrase and GyrA(572-875). Protein samples, at the concentrations indicated, were incubated with nicked pBR322 DNA as described in 'Materials and Methods'. After the nicks had been sealed with DNA ligase, the reactions were terminated by chloroform extraction, and the samples run on a 1% agarose gel containing 4 μg/ml chloroquine. Track '0' is a control containing no added gyrase and serves as a marker for the position of relaxed DNA under these conditions.

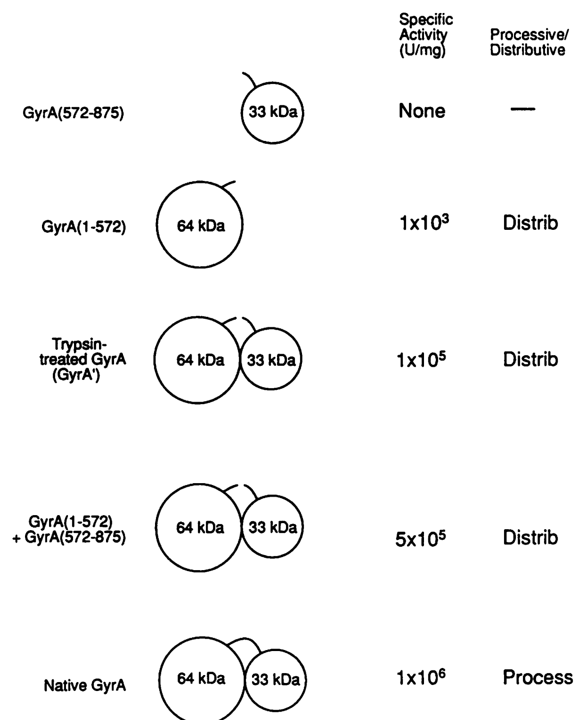

Fig. 4. Diagrammatic representation of the stabilising effect of GyrA(572-875), the C-terminal 33 kDa fragment of the gyrase A protein, on the supercoiling reaction by GyrA(1-572), the N-terminal 64 kDa fragment. The two protein fragments can be prepared separately and reconstituted. The trypsin-treated A' protein consists of the N- and C-terminal fragments which have not been separated (23). Whether the supercoiling reaction was found to be largely processive or distributive is also indicated. All supercoiling reactions were performed in the presence of GyrB.

complex on the gel is similar in both cases. Therefore we suggest that the protein content of each is similar, and that the reconstituted fragments do indeed form a $(64+33)_2B_2$ complex. At similar protein concentrations, there is less DNA-protein complex formed by $(64+33)_2B_2$ than with A_2B_2 by approximately a factor of two. This apparently weaker interaction with DNA could explain the differences in the supercoiling and relaxation activities observed between A_2B_2 and $(64+33)_2B_2$ (see Fig. 1). In addition, we have found no evidence for a protein-DNA complex between GyrA(1-572), GyrB, and DNA as judged by the gel-retardation assay (data not shown).

Figure 2 also shows the ability of GyrA, GyrB, GyrB(1-393), GyrA(1-572) and GyrA(572-875) to bind a 147 bp DNA fragment. Both GyrA and the 33 kDa protein, GyrA(572-875), form complexes with the DNA that differ in mobility from that between A_2B_2 gyrase and DNA. Native gyrase (A_2B_2) forms a discrete DNA-protein complex that increases in intensity on addition of more protein until there is no free DNA. By contrast, GyrA and GyrA(572-875) produce a smeared DNA-protein band (Fig. 2). The effect of adding increasing amounts of either protein is to deplete the free DNA band and further retard the DNA-protein complex band. This suggests that multiple protein molecules are binding to each 147 bp DNA molecule in the case of GyrA and GyrA(572-875). GyrA(572-875) was found to bind the 147 bp linear DNA with an affinity similar to that of GyrA, as judged by the concentration of protein required to retard all of the free DNA from its original position on the gel (see Table 1). In each case an approximately 3–4 fold molar excess of protein over DNA was required to achieve this. In similar experiments, a 1.5 molar excess of A_2B_2 over DNA resulted in no free DNA remaining. We have found no evidence of protein-DNA complex formation by the 64 kDa protein (GyrA(1-572)), GyrB or the 43 kDa fragment of GyrB (GyrB(1-393)) by gel retardation assays.

The ability of GyrA and the 33 kDa protein, GyrA(572-875), to bind to small, double-stranded, oligonucleotides was also measured (see Table 1). GyrA would not bind any DNA fragment of 52 bp or below in length, whereas GyrA(572-875) bound to the 52 bp fragment with a similar efficiency as to the 147 bp fragment. GyrA(572-875) also retarded a 35 bp fragment (albeit weakly), but no binding to a 24 bp fragment could be detected. It was previously observed that DNA gyrase (A_2B_2) bound a 117 bp DNA fragment efficiently, but a 55 bp fragment was bound only weakly (35). Thus the DNA binding properties of gyrase resemble those of GyrA but not the 33 kDa protein (GyrA(572-875)).

The Mg^{2+}-dependence of the binding of DNA gyrase (A_2B_2), GyrA, and the 33 kDa fragment (GyrA(572-875)) to DNA was investigated. It is known that complexes between DNA gyrase and DNA require Mg^{2+} for their formation (12, 19), and are only stable during electrophoresis on polyacrylamide gels when there is Mg^{2+} in the running buffer. We have confirmed this result for DNA gyrase and shown, in experiments similar to Fig. 2, that when $MgCl_2$ is replaced by EDTA in the electrophoresis buffer, only complexes between GyrA(572-875) and DNA could be observed (data not shown). Also, if $MgCl_2$ is omitted from the binding reaction mixtures, GyrA(572-875) is again able to form complexes with DNA. It seems therefore, that complexes between DNA gyrase (A_2B_2) or GyrA and DNA are only stable when Mg^{2+} is present both in the binding reaction and during electrophoresis, but that the 33 kDa fragment forms stable DNA-protein complexes in the absence of Mg^{2+}.

The binding of gyrase to DNA can be shown to involve the positive wrapping of DNA (12). This has been demonstrated with *M. luteus* gyrase by binding the enzyme to nicked-circular DNA

and, in the absence of ATP, sealing the nicks with DNA ligase (38). The resulting closed-circular DNA molecules are found to be positively supercoiled with a linking difference roughly equal to the number of gyrase molecules present. Fig. 3 shows the result of a similar experiment using *E. coli* gyrase (A_2B_2), GyrA, and the 33 kDa protein (GyrA(572-875)). In this experiment, we confirm that gyrase introduces positive supercoils into the DNA template with an approximate stoichiometry of one turn per gyrase tetramer. GyrA alone appears to have no effect on the topoisomer distribution, whilst the C-terminal fragment, GyrA(572-875), clearly promotes supercoiling of the DNA. This supercoiling was shown to be positive (rather than negative) by analysing the DNA by electrophoresis through agarose containing varying amounts of chloroquine (data not shown). We found that with increasing concentrations of GyrA(572-875), a maximum linking difference of 10–11 can be introduced per pBR322 molecule. Under these conditions there are about 400 protein molecules per DNA molecule. Assuming that all the protein is bound to DNA, then there is approximately one molecule of the 33 kDa protein for every 10 bp, and each of them contributes about 1/40 of a positive turn.

DISCUSSION

We have constructed a plasmid that allows the production of a 33 kDa protein which represents the C-terminal third of the *E. coli* DNA gyrase A protein (GyrA(572-875)). This protein is over-produced at a high level, but in an insoluble form that can be solubilised by treatment with guanidine hydrochloride. Once in solution, the protein can be easily purified to homogeneity. An enzymic analysis of this protein has been performed. Previous attempts to characterize the C-terminal domain of GyrA by analysis of the 33 kDa tryptic fragment (23) were limited by the small amounts of material that could be isolated from a polyacrylamide gel. We have found that GyrA(572-875) possesses no enzymic activity of its own, but will complement the 64 kDa fragment (GyrA(1-572)) and GyrB to form a complex which can both supercoil and relax DNA. When compared with DNA gyrase (A_2B_2), this complex was found to have a reduced activity with respect to DNA supercoiling and relaxation by about a factor of two. In addition, the supercoiling reaction of the complex involving GyrA(1-572), GyrA(572-875) and GyrB supercoils DNA in a predominantly distributive manner, by contrast with native gyrase (A_2B_2) which demonstrates a largely processive supercoiling reaction (Fig. 1). We found that, in binding experiments (Fig. 2), the gyrase complex containing the GyrA fragments formed less DNA-protein complex compared with native gyrase by about a factor of two. We suggest that the differences in the supercoiling reactions between these two complexes can be attributed to their relative efficacies in forming DNA-protein complexes. Thus, the complex between the 64 kDa protein, the 33 kDa protein, GyrB and DNA is less stable than that between native gyrase (A_2B_2) and DNA. This leads to a distributive rather than processive supercoiling reaction which may account for the observed decrease in the supercoiling activity, i.e. under distributive conditions the rate limiting step may be DNA binding rather than catalysis.

The supercoiling activities of the various fragments of GyrA are represented diagrammatically in Fig. 4. Processivity in the supercoiling reaction can only be observed with intact GyrA (see Fig. 1). The trypsin-treated gyrase A protein (GyrA′) shown a five-fold lower supercoiling activity compared with GyrA(1-572) + GyrA(572-875), despite these species containing the same fragments. It is possible that this difference is attributable to inactivation of GyrA′ caused by further tryptic digestion (23). The 64 kDa protein, GyrA(1-572), has a low supercoiling activity which can be greatly increased by the addition of the 33 kDa protein, GyrA(572-875), although this protein has no activity in itself. Therefore it is likely that the 33 kDa protein provides a stabilising function which promotes more efficient DNA supercoiling.

Although the 64 kDa protein, GyrA(1-572), can obviously interact with both GyrB and DNA (judged by its ability to perform DNA supercoiling, relaxation and cleavage) no complex between the protein and the DNA could be detected by a gel retardation assay. However, complexes could be detected between DNA and the 33 kDa protein (GyrA(572-875)). These complexes could be observed with DNA fragments as small as 35 bp in length (Table 1). Such complexes with small DNA fragments could not be observed with intact GyrA, although GyrA will bind DNA of larger sizes (see Fig. 2 and Table 1). Both GyrA and GyrA(572-875) form complexes with DNA which involve the binding of multiple protein molecules to each DNA, as judged by increasing retardation of the DNA-protein complex band with increasing amounts of protein. In addition, GyrA(572-875), but not GyrA, will form complexes with DNA in the absence of $MgCl_2$. It is possible that the interactions between GyrA and DNA that require Mg^{2+} occur between residues in the 64 kDa N-terminal domain and DNA, while the interactions between the 33 kDa domain and DNA are Mg^{2+}-independent. Clearly, these Mg^{2+}-independent interactions are not sufficient to stabilise a complex between intact GyrA and DNA. Alternatively, it is possible that the DNA-binding portion of the 33 kDa domain may be inaccessible in GyrA, and the binding site is only exposed after a conformational change which occurs when GyrA associates with GyrB.

The binding of the 33 kDa protein, GyrA(572-875), to DNA induces positive supercoiling (Fig. 3). This effect is not observed with native GyrA. Using pBR322 as a substrate, no further increase in positive supercoiling occurs when there is one 33 kDa protein molecule for every 10 bp of DNA. This level of bound protein results in the generation of approximately 1/40 of a positive turn for each protein molecule.

One possibility which accounts for this effect is that the binding of the 33 kDa protein leads to an increase in the twist of the DNA which, following resealing of the backbone and deproteinization, leads to the introduction of positive supercoiling. However, given that the handedness of the supercoiling introduced by the 33 kDa protein is the same as that for DNA gyrase (A_2B_2), we suggest that it is more likely that the 33 kDa protein provides part of the protein surface around which the DNA is wrapped in the gyrase-DNA complex. This could be achieved in two ways. Firstly, the axis of the DNA bound to the 33 kDa protein could follow a helical path, or secondly, the 33 kDa protein could induce a planar bend in the DNA which, for multiple protein molecules bound in a phased fashion, will lead to DNA supercoiling. It should also be pointed out that the binding of gyrase (A_2B_2) to DNA has been shown to stabilise approximately one positive writhe in DNA (24, 37) and that our experiments indicate that the two 33 kDa domains within the A_2B_2 complex would contribute only a small fraction of that writhe. Clearly other parts of the A_2B_2 complex may also be involved in the wrapping process. In addition, we found no induction of supercoiling following the binding of GyrA to DNA.

Given the behaviour of the 33 kDa domain, it is not clear why intact GyrA does not appear to wrap DNA.

We have shown in previous work (23, 26) that the N-terminal domain of GyrA contains the DNA breakage-reunion functions of the enzyme. We now propose that the C-terminal domain has a DNA-binding function that is involved in the wrapping of DNA around the gyrase complex.

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