Evidence for a conformational change in the DNA gyrase – DNA complex from hydroxyl radical footprinting

George Orphanides and Anthony Maxwell*

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, UK

Received February 15, 1994; Revised and Accepted March 25, 1994

ABSTRACT

We have used the technique of hydroxyl radical footprinting to probe the complex between DNA gyrase and a 198 bp DNA fragment containing the preferred gyrase cleavage site from plasmid pBR322. We find that gyrase protects 128 bp from the hydroxyl radical with the central 13 bp (adjacent to the gyrase cleavage site) being most strongly protected. Flanking the central region are arms showing periodic protection from the reagent suggesting a helical repeat of 10.6 bp, consistent with the DNA being wrapped upon the enzyme surface. The presence of 5'-adenylyl- β , γ -imidodiphosphate or a quinolone drug causes alteration of the protection pattern consistent with a conformational change in the complex involving one arm of the wrapped DNA. The significance of these results for the mechanism of DNA supercoiling by gyrase is discussed.

INTRODUCTION

DNA gyrase is the enzyme from bacteria which can introduce negative supercoils into closed-circular DNA using the free energy of ATP hydrolysis (1). The enzyme from *Escherichia coli* consists of two proteins, GyrA and GyrB, of molecular masses 97 and 90 kDa respectively. The active enzyme is an A₂B₂ complex. The mechanism of DNA supercoiling by gyrase involves the following steps (reviewed in 1, 2): (i) The binding of gyrase to DNA and the wrapping of a segment of DNA (~120 bp) around the A₂B₂ complex. (ii) Cleavage of this wrapped DNA in both strands, involving the formation of covalent bonds between the 5'-phosphates at the break sites and Tyr122 of the GyrA subunits. (iii) Passage of another segment of DNA through this break. (iv) Resealing of the break. Catalytic supercoiling requires the hydrolysis of ATP but in the presence of the non-hydrolysable ATP analogue ADPNP (5'-adenylyl- β , γ -imidodiphosphate) limited supercoiling can be achieved (3), indicating that nucleotide binding will promote one round of supercoiling but that hydrolysis is required for the enzyme to turnover.

The gyrase proteins have been shown to be organised as functional domains (reviewed in 1). The GyrA protein consists

of an N-terminal domain (59–64 kDa) which contains the DNA breakage-reunion site and has interactions with the quinolone drugs (4, 5) and a C-terminal domain (33 kDa) which is thought to be involved in the wrapping of DNA around the protein particle (6). The 33 kDa domain has been shown to wrap DNA on its own in the absence of a divalent metal cation (6). The GyrB protein consists of an N-terminal domain (43 kDa) which hydrolyses ATP and binds the coumarin drugs (7), and a C-terminal domain (47 kDa) which binds to the A protein and DNA (8–10). The structure of the 43 kDa N-terminal domain complexed with ADPNP has been solved to 2.5 Å resolution by X-ray crystallography (11).

Gyrase is the target of a number of antibacterial agents most of which belong to the quinolone and coumarin classes. Quinolone drugs (e.g. oxolinic acid and ciprofloxacin) inhibit DNA supercoiling by interrupting the breakage and reunion of DNA by gyrase (reviewed in 12–14). Incubation of gyrase and DNA in the presence of a quinolone drug and termination of the reaction with SDS leads to double-strand cleavage of DNA and attachment of the GyrA proteins to the 5' phosphates (15, 16). Such cleavage is also observed in the presence of Ca²⁺ (17). Although it is clear that quinolones inhibit supercoiling by disrupting the DNA cleavage reaction, their mode of bactericidal action is less certain. Recent experiments support the idea that the gyrase–quinolone complex on DNA forms a barrier to polymerases and that it is this event which is lethal (18). Coumarin drugs inhibit supercoiling by preventing the hydrolysis of ATP by gyrase (reviewed in 12, 13, 19).

Although the mechanism of DNA supercoiling by gyrase is understood in outline many details are unclear. For example the way in which the DNA is wrapped around the gyrase complex and how this wrap changes during supercoiling are not known. This issue has been examined using a number of methods (reviewed in 1). Early studies by Liu and Wang (20) showed that incubation of gyrase with nicked-circular DNA led to the introduction of positive supercoiling upon ligation of the nick and removal of protein. These and other similar experiments (1) have suggested that approximately one positive superhelical turn is wrapped around gyrase. Digestion of such gyrase–DNA complexes with staphylococcal nuclease leads to the formation of DNA fragments ~140 bp in length (21). Further digestion of these core complexes with DNase I results in a set of fragments

*To whom correspondence should be addressed

differing in length from each other by about 10 nucleotides (21). Taken together these data suggest that ~140 bp of DNA is wrapped around gyrase in a manner reminiscent of the nucleosome, but with opposite handedness.

More detailed analysis of the gyrase–DNA complex has been carried out in several laboratories using DNase I footprinting (22–25). In summary, these studies showed that gyrase protects 100–155 bp of DNA from the nuclease with a central region of 40–50 bp being most strongly protected. This region contains the quinolone-induced gyrase cleavage site and is thought to be where the DNA breakage and reunion reactions take place. The DNA flanking this region is less well protected and has been found to contain sites of enhanced sensitivity to DNase I spaced 10–11 bp apart, a feature suggestive of DNA wrapped on a surface. Very little difference in the DNase I protection pattern has been observed upon the addition of ADPNP or quinolone drugs suggesting that there is no significant change in the DNA wrap in the presence of these ligands.

The gyrase–DNA complex has also been probed with exonuclease III (24). These data were broadly in agreement with the DNase I studies, however the presence of ADPNP led to changes in the protection pattern consistent with an altered conformation of this complex. Experiments with dimethyl sulphate showed that the entire length of DNA on the gyrase–DNA complex could be modified by this agent suggesting that the wrapped DNA is solvent-accessible (23).

A range of biophysical methods has been applied to the study of the gyrase–DNA complex. High resolution electron microscopy suggested that the A_2B_2 complex is 'heart-shaped' and that the length of the wrapped segment is consistent with that determined by nuclease studies (26). Analysis of gyrase complexed with a range of short DNA fragments by transient electric dichroism suggested that ~120 bp of DNA is wrapped around the enzyme with the entry and exit points being close together with an angle of ~120° between the emerging tails (25). The addition of ADPNP caused changes in dichroism consistent with the tails being wrapped upon the protein core. This technique thus suggested a fairly dramatic conformational change in the gyrase–DNA complex upon the addition of ADPNP which has been interpreted as representing an intermediate stage in the supercoiling cycle. Analysis of gyrase and its complexes with DNA by small-angle neutron scattering and dynamic light scattering suggest that the particle is a flattened sphere with the DNA wrapped such that it does not significantly change the radius of gyration (27).

A picture of the complex between DNA gyrase and DNA is thus now emerging, but there are still many uncertainties concerning the manner in which the DNA is wrapped and the changes in the complex which occur during DNA supercoiling. One technique which has not yet been applied to the study of the gyrase–DNA complex is hydroxyl radical footprinting. This involves the generation of hydroxyl radicals *in situ* from the reaction between iron–EDTA and hydrogen peroxide. The highly reactive hydroxyl radical can cleave DNA by attacking the deoxyribose sugar (28). The hydroxyl radical has a number of advantages over DNase I as a footprinting reagent: it is small, highly reactive and cleaves at every nucleotide with almost equal efficiency, and therefore produces data of a much higher resolution. In this paper we have used hydroxyl radical footprinting to probe the structure of the DNA gyrase–DNA complex.

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 previously (29). Plasmid DNA was prepared by alkaline lysis followed by two rounds of fractionation in CsCl gradients, so that mostly supercoiled DNA was isolated. DNA was stored as a precipitate in ethanol or as a frozen solution in TE buffer [10 mM Tris.HCl (pH 8), 1 mM EDTA]. To allow flexibility in the length of DNA fragment footprinted, and to enable either strand to be labelled, the major gyrase cleavage site from plasmid pBR322 was inserted into the *EcoRI* site in the polylinker region of plasmid pBluescript II (Stratagene) using standard protocols (30) to produce plasmid pBS147. A 198 bp uniquely end-labelled DNA fragment was obtained by digesting plasmid pBS147 with *XhoI* (Boehringer Mannheim), labelling at the 3' end with α -[32 P]dCTP and the Klenow fragment of DNA polymerase I (Gibco-BRL), and digesting with *XbaI* (Boehringer Mannheim). The labelled fragment was then purified from a 5% polyacrylamide non-denaturing gel. DNA labelled in the other strand was obtained by labelling at the *XbaI* site before digesting with *XhoI*.

Footprinting

Gyrase–DNA complexes (28 μ l) were formed by incubating 1 pmole of end-labelled DNA fragment with 3–10 pmole of pre-formed A_2B_2 complex in 50 mM HEPES (pH 7.5), 55 mM KCl, 4 mM $MgCl_2$, and 2 mM DTT, for 1 hour at 25°C. When included, ADPNP or ciprofloxacin were at 1 mM and 27 μ M respectively. Cleavage of DNA with the hydroxyl radical was initiated by the addition of 4 μ l each of freshly prepared solutions of 1 mM ferrous ammonium sulphate (Aldrich) containing 2 mM EDTA (Aldrich), 10 mM ascorbic acid (Aldrich) and 0.3% H_2O_2 (Sigma). Reactions were quenched after 2 min by the addition of glycerol to 5% and gyrase–DNA complexes were separated from any free DNA in the sample on a 5% polyacrylamide (acrylamide:bis, 37.5:1) native gel containing 90 mM Tris/borate and 5 mM $MgCl_2$. In order to prevent cleavage of bound DNA in samples containing ciprofloxacin, excised gel slices were treated at 80°C for 10 min (15). Complexes were eluted from the gel by the crush and soak method (30) and the samples deproteinised by extracting twice with phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous layers were then extracted with ether and the DNA recovered by ethanol precipitation. Samples were resuspended in TE buffer, the DNA recovery quantified by scintillation counting, and the samples dried.

For footprinting with DNase I, gyrase–DNA complexes were prepared as described for hydroxyl radical reactions. Digestions were initiated by adding 0.1–0.5 units DNase I (Boehringer Mannheim) and allowed to proceed for 1 min at 25°C before being quenched by the addition of EDTA to 15 mM. Again, where ciprofloxacin was present, samples were treated at 80°C for 10 min after footprinting. DNA was recovered as described above. Maxam–Gilbert G markers were prepared by the standard protocol (30).

Gel electrophoresis

Samples containing equal amounts of radioactivity were resuspended in 3 μ l of 98% (w/v) formamide containing 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol and

heated at 90°C for 3 min. Electrophoresis was performed on denaturing 6% or 8% polyacrylamide gels, depending on the size range of DNA fragments to be resolved. Gels were transferred to Whatman 3MM paper, dried under vacuum at 80°C and exposed to either preflashed X-ray film or to PhosphorImager screens (Molecular Dynamics).

Data quantitation and analysis

Gel images (autoradiographs or PhosphorImages) were quantitated on a Molecular Dynamics PhosphorImager (funded by the Wellcome Trust) using Imagequant software. Band integrals were determined by performing area integration across the whole width of a lane. Integrals of bands from the naked DNA sample were subtracted from the integrals of the corresponding bands in the gyrase–DNA complex samples. Data from three independent experiments were averaged to produce values which represent the relative amount of cleavage at each nucleotide in the gyrase–DNA complex.

RESULTS

Selection of DNA fragment for analysis

The 198 bp DNA fragment used in these studies contains the major quinolone-induced gyrase cleavage site from plasmid pBR322, centred at position 990 (22). Control experiments show that cleavage occurs at the same position in the 198 bp fragment (data not shown). The interaction between gyrase and a fragment containing this site has previously been studied by DNase I footprinting and its determinants for site-specific cleavage by gyrase have been examined (22, 31). A plasmid containing 22 copies of a 147 bp fragment containing this site has been constructed and it has been shown that the fragment is bound efficiently by gyrase and is cleaved uniquely in the presence of quinolone drugs (17). This fragment has also been used in experiments investigating the binding of quinolone drugs to the gyrase–DNA complex (32) and in transcriptional footprinting studies (18).

Hydroxyl radical footprinting of the gyrase–DNA complex

Earlier footprinting studies used the nuclease DNase I to investigate the accessibility of DNA in the gyrase–DNA complex (22–25). We decided to exploit the small size and lack of sequence specificity of the hydroxyl radical to generate a higher resolution footprint. Initial experiments produced a weak footprint, barely visible by eye (data not shown). The appearance of the footprint was improved markedly when footprinting was followed by separation of gyrase–DNA complexes from any uncomplexed DNA by native gel electrophoresis. This treatment produced the footprints displayed in figures 1A and 2A (lanes 3 and 4) which represent the bottom and top strand of the DNA respectively. Quantitative analysis of the cleavage patterns reveals that the region of DNA over which modulation of cleavage occurs extends from position –54 to +74 on the top strand and –57 to +70 on the bottom strand (where positions –2 and +2 are the bases 3' to the bonds cleaved by gyrase; see Fig. 3C), i.e. a footprint of 128 bp. This region is represented by the black bar in figures 1A and 2A. Strongest protection against hydroxyl radical attack occurs over an area of about 13 bp, situated asymmetrically, adjacent to the position of double-stranded DNA cleavage. DNA between the staggered gyrase cleavage sites is cleaved by the hydroxyl radical, implying that this region is

Figure 1. Hydroxyl radical footprints of the gyrase–DNA complex (bottom strand). (A). The 198 bp DNA fragment, containing the major gyrase cleavage site from plasmid pBR322, was radiolabelled in the bottom strand. Samples contained 1 pmole DNA and either no gyrase (lane 2), 3 pmole gyrase (lanes 3, 5 and 7) or 10 pmole gyrase (lanes 4, 6 and 8). Where indicated, ciprofloxacin (CFX) and ADPNP were present at 27 μ M and 1 mM respectively. Hydroxyl radical cleavage was allowed to proceed for 2 mins and was quenched by the addition of glycerol. Lane 1 contains G markers (M) generated by chemical cleavage of the 198 bp fragment. (B). Densitometer scans of the hydroxyl radical footprints. Cleavage patterns were scanned across the whole width of each lane. The vertical lines are drawn through the cleavage maxima of the complex with gyrase alone and serve to illustrate the ADPNP- and CFX-induced conformational change. The black bars indicate the extent of hydroxyl radical cleavage modulation in the presence of gyrase alone and the hatched bars that seen with the addition of ADPNP and CFX. The centre of gyrase cleavage is indicated (i.e. the bond between positions –1 and +1) and the base numbering is relative to this point.

Figure 2. Hydroxyl radical footprints of the gyrase-DNA complex (top strand). (A). The 198 bp DNA fragment, containing the major gyrase cleavage site from plasmid pBR322, was radiolabelled in the top strand. Samples contained 1 pmole DNA and either no gyrase (lane 2), 3 pmole gyrase (lanes 3, 5 and 6) or 10 pmole gyrase (lane 4). Where indicated, ciprofloxacin (CFX) and ADPNP were present at 27 μ M and 1 mM respectively. Samples were treated as described in the legend to figure 1A. Lane 1 contains G markers (M) generated by chemical cleavage of the 198 bp fragment. (B). Densitometer scans of the hydroxyl radical footprints (as described in the legend to figure 1B). (C) The same samples as in figure 2A were electrophoresed on a denaturing 6% acrylamide gel to resolve the larger fragments. (D). Densitometer scans of the cleavage patterns shown in figure 2D.

accessible to solvent (see Discussion). Flanking this highly protected region are two areas of approximately 60 bp, over which cleavage is modulated with a period of 10–11 bp. The positions of cleavage maxima on the two strands are shifted by

on average 3 bp (see Fig. 3), characteristic of the cleavage chemistry of the hydroxyl radical which is centred in the minor groove.

Conformational changes in the gyrase-DNA complex

The DNA supercoiling cycle catalysed by gyrase implicitly requires conformational changes in the gyrase-DNA complex (1). Presently, it is unclear whether this involves alterations in the binding of the wrapped DNA, although this has been implied by transient electric dichroism studies of gyrase-DNA complexes (25). Early DNase I footprinting experiments failed to detect alterations in DNA binding in the presence of ATP or its non-hydrolysable analogue ADPNP (22–25). However, minor changes to the footprint upon the addition of the quinolone drugs have been reported (22, 24).

We decided to probe for conformational changes in the gyrase-DNA complex by hydroxyl radical footprinting, anticipating that the increased resolution and sensitivity of the reagent might reveal changes in DNA binding which are too subtle to be detected by DNase I. Figure 1A shows the hydroxyl radical footprints of the bottom strand in the presence of ADPNP (lanes 7 and 8) and the quinolone drug ciprofloxacin (CFX) (lanes 5 and 6). Both ADPNP and CFX induce the same changes in the cleavage pattern. The most immediately obvious alteration is a shift of about 3 bp in the phase of the cleavage maxima on one side of the cleavage site, in the region between –12 and –54. This is accompanied by the appearance of further modulation of cleavage with an additional cleavage maximum at position –67 (see Fig. 1B), suggesting that additional DNA is being wrapped onto the surface of the enzyme. This results in an extended footprint of 142 bp in the presence of ADPNP and CFX. These changes can be seen more clearly in the densitometric scans of the footprints (Fig. 1B). Here, vertical lines have been drawn through the cleavage maxima seen in the complex with gyrase alone, which helps to illustrate the shifted maxima in the +ADPNP and +CFX scans. The additional wrapping is illustrated by the hatched bars in figures 1A and 1B. Figure 3A is a difference cleavage plot and shows the variation in the modulation of cleavage along the DNA fragment for each complex. This figure more clearly depicts the phase shift and the additional interaction detected. Each point in this plot represents data averaged from three independent experiments. It is interesting to note that no such shift in cleavage maxima occurs in the wrapped DNA on the other side of the centrally protected region. Quantitative analysis of the footprints reveals an additional alteration of the footprint in the vicinity of the site of gyrase-catalysed double-stranded cleavage. Figure 3C shows the degree of hydroxyl radical cleavage in the central 40 bp of the footprint. The asymmetry of protection in the complex formed with gyrase alone is evident. However, in the presence of ADPNP or CFX an additional cleavage maximum appears at position +13 and this reduces the asymmetry of the interaction around this central region.

So far, in describing the changes to the footprint, we have concentrated on the bottom strand only. However, the changes were also seen in footprints of the top strand. Figure 2A shows footprints of the top strand obtained in the presence of ADPNP (lane 6) and CFX (lane 5). The location of the phase shift and additional cleavage maximum at the top of the gel makes it difficult to see any changes by eye. Therefore, to resolve the larger fragments, the samples were run on a gel containing a lower concentration of polyacrylamide for a longer time. This

Figure 3. Plots of relative hydroxyl radical cleavage frequency at each nucleotide in the gyrase-DNA complex. (A). Bottom strand. (B). Top strand. The area of each band was determined by integration. Integrals of bands in the naked DNA sample were subtracted from integrals of the corresponding bands in the +ADPNP and +CFX samples. Each point represents data averaged from three independent experiments. For each experiment two gels were run, in order to resolve bands along the entire length of the footprint, and the data sets joined at position +23. The small arrows mark the positions of hydroxyl radical cleavage maxima along with the corresponding nucleotide positions. The plots have been stacked along the Y-axis to aid comparison of the three data sets. (C). Plots of hydroxyl radical cleavage frequency in the central 40 bp surrounding the gyrase cleavage-site. ●; gyrase-DNA complex, ○; ADPNP and □; CFX. The plots for the bottom strand have been reversed to allow comparison with plots for the top strand. The DNA sequence of this region is shown and the arrows indicate the positions of double-stranded cleavage by gyrase.

gel is shown in figure 2C and the corresponding densitometric scans in figure 2D, where the phase shift can now be seen. The corresponding appearance of an additional cleavage maximum in the central 40 bp of the footprint is evident in figures 3B and 3C. The implications of these rearrangements in DNA binding for the supercoiling mechanism of gyrase is discussed below.

Experiments carried out in the presence of ATP resulted in

a poorly defined footprint that resembled that obtained with ADPNP (data not shown). Footprints obtained in the presence of the quinolone drugs oxolinic acid and norfloxacin produced the same results as ciprofloxacin (data not shown). No change in the protection pattern was seen in the footprints obtained in the presence of the coumarin drugs novobiocin and coumermycin A₁, or when Mg²⁺ was replaced by Ca²⁺ (data not shown).

Figure 4. DNase I footprints of the gyrase-DNA complex. (A). The 198 bp DNA fragment was radiolabelled in the top strand. Samples contained 1 pmole DNA and either no gyrase (lane 2), 1 pmole gyrase, (lane 3), 3 pmole gyrase (lanes 4, 6 and 8) or 10 pmole gyrase (lanes 5, 7 and 9). Samples were digested with 0.1 units of DNase I and the DNA electrophoresed on an 8% denaturing acrylamide gel. (B). The same samples were electrophoresed on a 6% denaturing acrylamide gel in order to resolve the larger fragments.

To rule out the possibility that the conformational change observed is a peculiarity of the complex formed between gyrase and this particular DNA fragment, experiments were repeated with a second fragment. The fragment chosen was 207 bp in length, derived from the sea urchin 5S rRNA gene, and was previously used in DNase I footprinting experiments (25). The results obtained are in agreement with those presented above, with ~124 bp of DNA being protected and a similar change in the footprint upon the addition of ADPNP or CFX (data not shown).

DNase I footprinting

The hydroxyl radical footprinting data prompted us to repeat the above experiments using DNase I as the footprinting reagent under the same conditions. Figure 4A shows the DNase I footprints of the top strand obtained with gyrase alone (lanes 3, 4 and 5), in the presence of ADPNP (lanes 8 and 9) and CFX (lanes 6 and 7). The footprint with gyrase alone is similar to that obtained in other studies, where enhanced cleavage occurs every 10–11 bp and a centrally located region of about 40 bp is most highly protected (22–25). Examination of the cleavage pattern at the top of the gel (Fig. 4B) reveals subtle differences between the gyrase-alone footprint and the +ADPNP and +CFX footprints (compare for example tracks 5, 7 and 9 around positions -30 to -35, where a shift in cleavage maxima can be seen). The additional wrapping of DNA seen in the hydroxyl radical footprints is also detected by DNase I footprinting, as indicated by the hatched bar.

DISCUSSION

The structure of the gyrase-DNA complex

In this study we have examined the wrapping of DNA around the gyrase-DNA complex by hydroxyl radical footprinting. We used a fragment containing the major quinolone-induced gyrase

Figure 5. Summary of hydroxyl radical cleavage of the gyrase-DNA complex. (A). Gyrase-DNA complex alone. (B). Gyrase-DNA complex in the presence of ADPNP/CFX. Sites of hypersensitivity to and protection from hydroxyl radical cleavage are marked on a cylindrical projection of a schematic double helix on which the base pairs are drawn across the minor groove. For illustrative purposes the helical repeat of the DNA is drawn as 10.5 bp/turn. Base numbering is as described in the legend to Fig 1B. Sites of hypersensitivity are indicated by arrows and sites of protection by filled circles. The size of the arrow or circle indicates the relative degree of hypersensitivity and protection respectively. The filled triangles mark the positions of double-stranded gyrase cleavage.

cleavage site from plasmid pBR322, which has previously been used in a variety of studies both in this laboratory and in others (see Results). The results obtained are consistent with the widely-accepted model of the gyrase–DNA complex in which the DNA is wrapped around the enzyme in approximately one complete turn. The small size of the hydroxyl radical allows us to determine the length of DNA wrapped by gyrase with a higher degree of accuracy than in previous footprinting studies. This was found to be 128 bp, with 54 bp being protected on one side of the gyrase cleavage site and 74 bp on the other. An interesting feature of the footprint is a region of about 13 bp which is protected to a higher degree than the surrounding DNA. This area is adjacent to the site of gyrase-catalysed double-stranded cleavage and it is therefore likely that DNA in this region is buried in an active site in which the cleavage reaction occurs. In corresponding DNase I experiments this highly protected region was found to be ~40 bp (Figs. 4A and 4B); this difference is likely to reflect the relative sizes of the two footprinting reagents.

Two further observations regarding this central region deserve comment. Firstly, the bases between the staggered gyrase cleavage sites are available to the hydroxyl radical (see Fig. 3C), suggesting that this region is accessible to solvent. In addition, the bases directly adjacent to the gyrase cleavage sites, on either side, are protected from hydroxyl radical attack. The significance of these observations becomes apparent when the architecture of the DNA double-helix is considered. Figure 5A is a cylindrical projection of a schematic DNA double helix on which sites of cleavage hypersensitivity and protection are indicated by arrows and filled circles respectively. The two filled triangles indicate the bonds that are cleaved by gyrase. It can now be seen that the hydroxyl radical cleavage maxima between the gyrase cleavage sites lie across the minor groove from each other. In contrast, the sites protected from hydroxyl radical cleavage at this position lie across the major groove on the opposite side of the DNA. These data suggest that in order to cleave DNA the enzyme interacts in the major groove. Indeed, this conclusion is supported by the fact that the two bonds attacked by gyrase lie almost directly opposite each other across the major groove.

The second striking feature of the cleavage pattern around the active site of DNA cleavage is the asymmetric nature of the protection from hydroxyl radical attack. The 13 bp region of highest protection is centred at position +11 on the top strand and +8 on the bottom strand (see Figs. 3C and 5A), although positions –5 to –2 on the top strand and –9 to –6 on the bottom strand are also heavily protected. The implication here is that the site at which DNA cleavage occurs is not in the centre but adjacent to the buried region.

On either side of the heavily protected central region are two segments of DNA where cleavage by the hydroxyl radical is modulated with a period of 10–11 bp (see Figs. 3A and 3B). This type of cleavage pattern is characteristic of DNA lying on a surface and is reminiscent of the footprint of DNA in the nucleosome (33). Hydroxyl radical cleavage maxima on the two DNA strands are shifted on average by 3 bp, reflecting the minor groove-centred cleavage chemistry of the hydroxyl radical. Overall, the periodically-modulated cleavage pattern in these two flanking arms is consistent with the DNA being wrapped onto the surface of the enzyme, as in the current model of the gyrase–DNA complex (1).

A feature of the gyrase–DNA complex that has previously been commented on is the helical periodicity of the bound DNA. This value has been put at 10–11 bp in two DNase I footprinting

studies (22, 23) and at 9.9 bp in a third (24). Recently, Fourier analysis of hydroxyl radical footprinting data has been used to accurately determine the helical repeat of DNA in the nucleosome (33, 34). A simple count of bases between cleavage maxima reveals that the helical repeat in the two flanking arms is 10.6 bp (53 bp separating 5 cleavage maxima in both flanking arms), implying that it does not change significantly upon being bound by gyrase. It must be stressed however that in making this statement we are assuming that the helical repeat of the DNA in solution is 10.5–10.6 bp, that of average B-DNA (35–38), which may not be the case. In addition, our value for the helical repeat of DNA in the gyrase–DNA complex is for one particular DNA sequence only. In order to accurately evaluate the average helical periodicity of DNA in the gyrase–DNA complex by this method one must obtain a cleavage pattern for random sequence DNA, as has been performed for the nucleosome (34).

Examination of the positions of cleavage maxima in the two flanking arms of the wrapped DNA reveals that the two sets of maxima are not separated by integral multiples of 10.5–10.6 bp (Figs. 3A and 3B), they are in fact out of phase by about half a turn of the helix. This observation is consistent with earlier studies by Liu and Wang (21). Two possible scenarios can account for this observation. In the first case the two arms are wrapped independently by two DNA-binding surfaces (e.g. the GyrA subunits) such that they bind to opposite faces of the helix. Alternatively, the DNA between the two arms could be significantly under- or over-wound so that, although being separated by non-integral multiples of 10.5–10.6 bp, the exposed surfaces would lie on the same side of the helix. In this case, the helical repeat of this region in figure 5 would need to be adjusted to accommodate this change.

Conformational changes in the gyrase–DNA complex

In view of the high resolution information afforded by hydroxyl radical footprinting, we decided to use the technique to examine the effect of nucleotide binding to the gyrase–DNA complex. It is well known that binding of the non-hydrolysable ATP analogue ADPNP is sufficient for gyrase to catalyse one round of supercoiling (3). In addition, electric dichroism experiments suggested that this analogue promotes a conformational change in the gyrase–DNA complex (25).

Hydroxyl radical footprinting reveals that binding of ADPNP induces a conformational change which involves an alteration in the wrapping of DNA in one flanking arm accompanied by a change in protection at the active site of DNA cleavage. The change in wrapping in one arm is displayed in figures 3A and 3B, where numbered arrows indicate the positions of cleavage maxima. A shift in the phase of the cleavage maxima is seen between the regions –9 and –51 on the top strand and –12 and –54 on the bottom strand. The shift is by 3 bp and is in the 5' direction on the top strand and the 3' direction on the bottom strand (see Figs. 3A and 3B). This alteration in the protection pattern is consistent with a rearrangement in protein–DNA contacts over this region so that a different face of the DNA is bound by the enzyme (see Fig. 5B). In addition, a further cleavage maximum appears at position –64 on the top strand and –67 on the bottom strand, suggesting that more DNA is being wrapped. In contrast, the protection pattern along the other flanking arm remains unchanged. The asymmetric nature of this conformational change may be an intrinsic part of the supercoiling mechanism and is discussed below.

The second feature of this nucleotide-induced conformational change involves a loss of protection at the active site. Figure 3C shows the modulation of hydroxyl radical cleavage in the central 40 bp surrounding the gyrase cleavage site. Upon the addition of ADPNP a new cleavage maximum appears at position +16 on the top strand and +13 on the bottom strand where DNA was previously protected. The implication is that the conformational change causes DNA which was previously protected to become more exposed and accessible to hydroxyl radical attack. Taken together, these changes to the footprint suggest that ADPNP binding induces a conformational change in the enzyme which alters the way in which the DNA is bound and wrapped. Experiments performed in the presence of ATP produced a footprint resembling that with ADPNP, albeit poorly defined (data not shown). The poor definition of this footprint is likely to be due to the hydrolysis of ATP which reverses the conformational change. Hence, the resulting footprint represents a state somewhere between the two conformations.

As a confirmatory measure the experiments were repeated using DNase I as the footprinting reagent. Although less striking, a similar phase shift and additional wrapping is seen, but no loss of protection is seen at the active site. The implication here is that the conformational change at the active site is sufficient to make this region of DNA accessible to the hydroxyl radical but not to DNase I. This is probably a direct reflection of the relative sizes of the two DNA cleavage reagents and suggests that the DNA that becomes accessible to the hydroxyl radical is still buried to a certain extent and so cannot be cut by DNase I.

The appearance of a conformational change in our footprinting studies led us to question whether this effect was indeed a manifestation of part of the supercoiling cycle or was due to a peculiar feature of the DNA fragment used. To resolve this question, identical experiments were performed with an unrelated DNA sequence, and these served to confirm the results discussed above (data not shown). It is therefore likely that the observed conformational change is a general feature of the gyrase supercoiling reaction.

Mechanistic implications

The supercoiling mechanism of all type II DNA topoisomerases involves the passage of a DNA double-helix through a transient double-stranded break stabilised by the enzyme (2). For DNA gyrase it is known that the double-stranded break (or DNA 'gate') is located roughly in the centre of the wrapped DNA. What is less well understood is the location of the DNA segment which is passed through this break (the 'passage helix') or, more precisely, whether this region is part of the wrapped DNA or from a completely separate region of the same molecule. Two lines of experimental evidence suggest that the passage helix is part of, or close to, the wrapped DNA. Firstly, DNA supercoiling by gyrase is directional: the enzyme must be able to detect the relative orientations of the DNA gate and the passage helix. Failure to do this would result in a higher tendency to knot rather than supercoil DNA. The second piece of evidence comes from work which looked at the supercoiling of small DNA circles by gyrase (39), where it was found that gyrase could supercoil DNA rings as small as 174 bp. Considering that the length of DNA wrapped in the gyrase-DNA complex is about 130 bp, the passage helix in these small circles must be part of, or at least very close to, the wrapped DNA. However, it must be stressed that this does not necessarily mean that the passage helix must always be part of the wrapped DNA. Moreover, in some reactions

catalysed by DNA gyrase (e.g. catenation) the gate and passage helices must come from different DNA molecules.

If the passage helix is part of or close to the wrapped DNA, how then is strand passage achieved? In this study we have detected a conformational change in the wrapped DNA which includes the further wrapping of DNA on one side of the complex accompanied by a rearrangement of enzyme-DNA contacts in the adjacent region. Hence we propose that this conformational change is a manifestation of the manipulation of one arm of the wrapped DNA to facilitate strand passage. This interpretation is partially in agreement with the electric dichroism studies of Rau and colleagues who proposed that upon ADPNP binding the 'tails' adjacent to the bound DNA were wrapped onto the surface of the enzyme (25). However, the dichroism data was interpreted as suggesting a conformational change in which both tails were bound onto the protein core whereas hydroxyl radical footprinting shows a conformational change in only one arm of the wrapped DNA. The reason for this difference is not clear but it is important to point out that the model suggested by the dichroism data is not unambiguous. In addition, the dichroism studies were carried out in the presence of glycerol (at 25–50%) which may stabilise the wrapping of both tails (D.C.Rau, personal communication). Intuitively it seems more likely that only one tail is wrapped onto the enzyme as only one duplex is passed through the DNA gate. If this were the case, it seems likely that the choice of which flanking arm acts as the passage helix would be an energetic one, perhaps influenced by the relative deformabilities of the two DNA arms.

Recently, the crystal structure of a 43 kDa N-terminal domain of the GyrB protein has been solved to high resolution (11). This suggested the presence of a hole in the B subunits, roughly the diameter of B-DNA. Subsequent biochemical studies indicated that the 43 kDa protein is a dimer only in the presence of ADPNP (7). This leads to the idea of a nucleotide-operated hole that can trap a segment of double-helical DNA upon ATP binding. Roca and Wang have since proposed a model for the mechanism of yeast topoisomerase II in which a passage helix is trapped by an ATP-operated clamp before passing through the DNA gate (40). In either case it is possible that the conformational change detected in this study is associated with the movement of DNA into a hole or clamp within the enzyme.

The change in protection pattern at the active site of DNA cleavage can be understood if we consider two conformations of the gyrase-DNA complex: namely where the DNA gate is closed and where it is open. In the absence of nucleotide an equilibrium exists between the gate-open and gate-closed states, with the gate-closed state favoured. Binding of nucleotide to the gyrase-DNA complex stabilises the gate-open state, a conformation where strand passage can occur. This scenario is supported by the findings of Morrison and Cozzarelli who reported that, upon the binding of ADPNP, new barriers to exonuclease III were seen (24). Whereas in the absence of nucleotide the exonuclease was able to digest DNA to approximately 40–80 bp from the gyrase cleavage site, in the presence of ADPNP digestion occurred right through the cleavage site. We infer from this that the exonuclease (which degrades DNA from the 3' termini) can attack the 3' ends at the DNA gate being stabilised by ADPNP. Therefore, the differences in hydroxyl radical cleavage at the active site probably represents a switch from a situation where the DNA is bound but not cleaved, to one where the DNA has been cleaved and the newly-formed ends are held apart. It seems surprising that the footprint

at the active site does not change more dramatically upon the transition to the gate-open state, and hence it is possible that the complex may be in a conformation where the DNA is cut in both strands but the ends are not held apart. From the data presented here it appears that in the gate-closed state the DNA at the active site is in more intimate contact with the enzyme than in the gate-open state.

Hydroxyl radical footprinting performed in the presence of the quinolone drugs produced the same footprint as with ADPNP. These drugs have been shown to bind to the gyrase-DNA complex and are likely to prevent DNA resealing (32, 41). The data presented here suggest that these drugs act by trapping a conformational intermediate in the gyrase supercoiling cycle.

In summary, we interpret the changes in the footprint in terms of a nucleotide-induced conformational change in which the DNA gate is held open and one flanking arm is manipulated to undergo strand-passage. The details of the conformational change in terms of inter-subunit rearrangements and alterations in protein-DNA interactions is unclear and will require further biochemical and ultimately structural investigation.

ACKNOWLEDGEMENTS

We thank Andrew Bates and Paul Cullis for critical reading of the manuscript, and the SERC for financial support. GO was supported by an SERC-CASE studentship funded by SERC and Zeneca Pharmaceuticals. AM is a Lister Institute Jenner Fellow.

REFERENCES

1. Reece, R.J. and Maxwell, A. (1991) *CRC Crit. Rev. Biochem. Mol. Biol.*, **26**, 335–375.
2. Maxwell, A. and Gellert, M. (1986) *Adv. Prot. Chem.*, **38**, 69–107.
3. Sugino, A., Higgins, N.P., Brown, P.O., Peebles, C.L. and Cozzarelli, N.R. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4838–4842.
4. Reece, R.J. and Maxwell, A. (1989) *J. Biol. Chem.*, **264**, 19648–19653.
5. Reece, R.J. and Maxwell, A. (1991) *J. Biol. Chem.*, **266**, 3540–3546.
6. Reece, R.J. and Maxwell, A. (1991) *Nucleic Acids Res.*, **19**, 1399–1405.
7. Ali, J.A., Jackson, A.P., Howells, A.J. and Maxwell, A. (1993) *Biochemistry*, **32**, 2717–2724.
8. Brown, P.O., Peebles, C.L. and Cozzarelli, N.R. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 6110–6114.
9. Gellert, M., Fisher, L.M. and O'Dea, M.H. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 6289–6293.
10. Adachi, T., Mizuuchi, M., Robinson, E.A., Appella, E., O'Dea, M.H., Gellert, M. and Mizuuchi, K. (1987) *Nucleic Acids Res.*, **15**, 771–784.
11. Wigley, D.B., Davies, G.J., Dodson, E. J., Maxwell, A. and Dodson, G. (1991) *Nature*, **351**, 624–629.
12. Drlica, K. and Coughlin, S. (1989) *Pharmac. Ther.*, **44**, 107–121.
13. Rádl, S. (1990) *Pharmac. Ther.*, **48**, 1–17.
14. Maxwell, A. (1992) *J. Antimicrob. Chemotherap.*, **30**, 409–416.
15. Gellert, M., Mizuuchi, K., O'Dea, M.H., Itoh, T. and Tomizawa, J. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4772–4776.
16. Sugino, A., Peebles, C.L., Krueger, K.N. and Cozzarelli, N.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4767–4771.
17. Dobbs, S.T., Cullis, P.M. and Maxwell, A. (1992) *Nucleic Acids Res.*, **20**, 3567–3573.
18. Willmott, C. J. R., Critchlow, S. E., Eperon, I. C. and Maxwell, A. (1994) *Submitted to EMBO J.*
19. Maxwell, A. (1993) *Molec. Microbiol.*, **9**, 681–686.
20. Liu, L. and Wang, J. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2098–2102.
21. Liu, L.F. and Wang, J.C. (1978) *Cell*, **15**, 979–984.
22. Fisher, L.M., Mizuuchi, K., O'Dea, M.H., Ohmori, H. and Gellert, M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4165–4169.
23. Kirkegaard, K. and Wang, J. (1981) *Cell*, **23**, 721–729.
24. Morrison, A. and Cozzarelli, N.R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1416–1420.
25. Rau, D.C., Gellert, M., Thoma, F. and Maxwell, A. (1987) *J. Mol. Biol.*, **193**, 555–569.
26. Kirchhausen, T., Wang, J.C. and Harrison, S.C. (1985) *Cell*, **41**, 933–943.
27. Krueger, K., Zaccai, G., Wlodawer, A., Langowski, J., O'Dea, M., Maxwell, A. and Gellert, M. (1990) *J. Mol. Biol.*, **211**, 211–220.
28. Tullius, T. D. (1987) *Trends Biochem. Sci.*, **12**, 297–300.
29. Hallett, P., Grimshaw, A. J., Wigley, D. B. and Maxwell, A. (1990) *Gene*, **93**, 139–142.
30. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual 2nd edition*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
31. Fisher, L.M., Barot, H.A. and Cullen, M.E. (1986) *EMBO J.*, **5**, 1411–1418.
32. Willmott, C.J.R. and Maxwell, A. (1993) *Antimicrob. Agents Chemother.*, **37**, 126–127.
33. Hayes, J. J., Tullius, T. D. and Wolffe, A. P. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7405–7409.
34. Hayes, J.J., Clark, D.J. and Wolffe, A.P. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 6829–6833.
35. Rhodes, D. and Klug, A. (1981) *Nature*, **292**, 378–380.
36. Peck, L. and Wang, J. (1981) *Nature*, **292**, 375–378.
37. Shore, D. and Baldwin, R.L. (1983) *J. Mol. Biol.*, **170**, 983–1007.
38. Horowitz, D.S. and Wang, J.C. (1984) *J. Mol. Biol.*, **173**, 75–91.
39. Bates, A.D. and Maxwell, A. (1989) *EMBO J.*, **8**, 1861–1866.
40. Roca, J. and Wang, J.C. (1992) *Cell*, **71**, 833–840.
41. Yoshida, H., Nakamura, M., Bogaki, M., Ito, H., Kojima, T., Hattori, H. and Nakamura, S. (1993) *Antimicrob. Agents Chemotherap.*, **37**, 839–845.