The N-Terminal Domain of Human Topoisomerase IIα Is a DNA-Dependent ATPase†

Laurence P. Gardiner,‡ David I. Roper,§ Timothy R. Hammonds,‡ and Anthony Maxwell*,‡

Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH, United Kingdom, and Department of Chemistry, University of York, Heslington, York YO1 5DD, United Kingdom

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ABSTRACT: We have constructed clones encoding N-terminal fragments of human DNA topoisomerase IIα. We show that the N-terminal domain (∼50 kDa) has an intrinsic ATPase activity that can be stimulated by DNA. The enzyme obeys Michaelis−Menten kinetics showing a ∼6-fold increase in k_{cat} in the presence of DNA. Cross-linking studies indicate that the N-terminal domain is a dimer in the absence and presence of nucleotides. Using site-directed mutagenesis, we have identified the catalytic residue for ATP hydrolysis as Glu86. Phosphorylation of the N-terminal domain with protein kinase C does not affect the ATPase activity. The ATPase domain of human topoisomerase IIα shows significant differences from its counterpart in DNA gyrase and we discuss the mechanistic implications of these data.

DNA topoisomerases are enzymes that catalyze topological changes in DNA (*1*). These enzymes have been found in all cell types and are essential for cell viability. Their roles include maintenance of the level of intracellular DNA supercoiling, removal of supercoils that build up ahead of and behind transcription and replication complexes, and decatenation of daughter chromosomes following replication. The topoisomerase reaction involves the breakage of DNA in one or both strands, the formation of protein−DNA covalent bonds, and the passage of another segment of DNA through the enzyme-stabilized break. In the case of type II enzymes this DNA strand-passage reaction generally requires the hydrolysis of ATP.

As a consequence of their essential roles in cells, DNA topoisomerases have become important drug targets. For example, the prokaryotic type II enzyme DNA gyrase is the target of a range of antibacterial agents such as the quinolone and coumarin drugs (*2*), and eukaryotic topoisomerase II is the target of a variety of antitumor drugs that include amsacrine, epipodophyllotoxins, and merbarone (*3*, *4*). Many of these compounds (e.g., quinolones and amsacrine) act by stabilizing a cleavable complex between the topoisomerase and DNA, in which the enzyme is covalently linked to the DNA. Arresting of DNA replication forks by this complex is thought to initiate events that lead to cell death (*3*, *4*). Other topoisomerase-targeted compounds act by different mechanisms; e.g. coumarin drugs (such as novobiocin) act as competitive inhibitors of the DNA gyrase ATPase reaction (*5*, *6*).

On the basis of the alignment of their amino acid sequences, DNA topoisomerases can be grouped into three subfamilies: type IA, type IB, and type II (*7*). All type II enzymes are evolutionarily and structurally related, each possessing two distinct catalytic centers: a DNA cleavage and rejoining site and a site for ATP hydrolysis (*7*−*11*). The enzymes differ in their molecular weights and subunit composition; e.g. DNA gyrase from *Escherichia coli* consists of two subunits, GyrA[1] and GyrB, of molecular masses 97 and 90 kDa, which associate as an A_2B_2 complex. GyrA contains the DNA cleavage activity while GyrB catalyzes ATP hydrolysis. Eukaryotic type II enzymes are homodimers, where each monomer can be regarded as a fusion of a GyrB and GyrA subunit. Homology between eukaryotic and prokaryotic enzymes is closest in the region containing the active site for DNA cleavage and the N-terminal region (corresponding to the N-terminal domain of GyrA and the ATPase domain of GyrB, respectively); the C-termini tend to be divergent. The molecular masses of the eukaryotic enzymes show some diversity; the enzyme from *Saccharomyces cerevisiae* (yeast topoisomerase II) has a monomer molecular mass of 164 kDa (*12*), whereas the two isoforms of the human enzyme, α and *β*, are 170 and 180 kDa, respectively (*13*).

The mechanism of eukaryotic topoisomerase II (topo II) is now understood in some detail as a consequence of a number of structural and mechanistic studies (*14*−*16*). The enzyme binds two segments of DNA. The gate (or G) segment (∼25 bp) is cleaved in both strands with a 4-base stagger between the break sites; this leads to the formation of covalent bonds between the 5′-phosphates at the break site and the active-site tyrosines. The transported (or T) segment is captured by an ATP-operated clamp (comprising the N-terminal domains of each monomer), which presents the T segment to the double-stranded break in the G segment and facilitates the strand-passage reaction. Resealing of the

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* Corresponding author.
‡ University of Leicester.
§ University of York.

[1] Abbreviations: ADPNP, 5′-adenylyl-*β*,*γ*-imidodiphosphate; bp, base pairs; DTT, dithiothreitol; GyrA, DNA gyrase A protein; GyrB, DNA gyrase B protein; topo II, DNA topoisomerase II.

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break in the G segment leads to a change in linking number of the DNA by 2, in the case of intramolecular reactions (e.g., DNA relaxation), or catenation or decatenation in the case of intermolecular strand passage.

The role of ATP hydrolysis in the mechanism of topo II remains to be clarified. ATP hydrolysis is normally required to drive reactions that are energetically unfavorable. Indeed, in the case of the prokaryotic type II topoisomerase, DNA gyrase, the requirement for ATP hydrolysis is clear. Gyrase can introduce negative supercoils into DNA, an energetically unfavorable reaction that is coupled to ATP hydrolysis. In this case there appears to be an approximate correspondence between the free energy available from the hydrolysis of ATP and the energy required to introduce supercoils (*17*−*20*). In the absence of ATP, gyrase can catalyze the relaxation of negative supercoils (an energetically favorable reaction), albeit less efficiently than the introduction of supercoils (*21*, *22*). Eukaryotic topoisomerase II cannot introduce supercoils into DNA but relaxes DNA in an ATP-dependent reaction. Given that this is an energetically favorable reaction, it is unclear why ATP is required. Recent studies on the decatenation, unknotting, and relaxation reactions of type II enzymes have shown that topo II (from a variety of sources) is apparently able to generate nonequilibrium distributions of topoisomers (*23*). This behavior can be rationalized by invoking the free energy of ATP hydrolysis to drive the reactions away from equilibrium. A tracking mechanism has been proposed to account for these observations (*23*).

Early studies on topo II prepared from HeLa cells, *Drosophila melanogaster*, and calf thymus showed that the enzyme possessed a DNA-dependent ATPase activity (*24*−*26*). The degree of stimulation by DNA was 3−17-fold, depending on the source of the enzyme. The ATPase reaction of yeast (*S. cerevisiae*) topo II has been analyzed by Lindsley and Wang (*27*). The enzyme has an intrinsic ATPase activity that is stimulated 19-fold by DNA. The DNA-independent reaction follows Michaelis−Menten kinetics with an estimated k_{cat} of 1 s^{-1}. The DNA-dependent reaction shows cooperative binding of ATP to the two monomers in the enzyme dimer. Determination of the coupling between ATP usage and DNA strand passage showed that ~2 ATPs are hydrolyzed per strand-passage event at low ATP concentrations, whereas at saturating ATP concentrations ~7−8 ATPs are hydrolyzed per event.

Recently the ATPase reaction of human DNA topo IIα has been studied in detail (*28*). The ATPase activity is stimulated by DNA and shows apparent Michaelis−Menten kinetics. Although the ATPase activity of human topo IIα is lower than that of yeast, it is more active in decatenation, implying more efficient coupling of the ATPase to DNA strand passage. With plasmid pBR322 as the DNA cofactor, the reaction shows hyperstimulation by DNA at a base pair to enzyme dimer ratio of 100−200:1. When DNA fragments are used as the cofactor, the reaction requires ~100 bp to stimulate the activity, and fragments of ~300 bp show hyperstimulation. This behavior can be rationalized in terms of the enzyme requiring fragments that can bind to both the DNA gate and the ATP-operated clamp in order for the ATPase reaction to be stimulated. Hyperstimulation is a consequence of the saturation of DNA with enzyme, i.e., possibly due to protein−protein interaction between topo II dimers (*28*).

Eukaryotic topoisomerase II is known to be phosphorylated at multiple sites and there is evidence that phosphorylation modulates catalytic activity (*29*). For example, *Drosophila* topo II, when phosphorylated by casein kinase II or protein kinase C, shows increased relaxation activity (*30*) and stimulation of the ATPase activity (*31*, *32*). Phosphorylation of topo II predominantly occurs in the C-terminal domain. In *S. cerevisiae* multiple phosphorylation sites have been mapped to the C-terminal domain and it is proposed that phosphorylation has a regulatory effect on the enzyme (*33*, *34*). In the case of the human enzyme, topo IIα, phosphorylation by three separate kinases, protein kinase C, casein kinase II, and p34cdc2, has been demonstrated (*35*−*37*). Although phosphorylation predominantly occurs in the C-terminal domain, in the case of protein kinase C, phosphorylation at Ser29 in the N-terminal ATPase domain has been found (*37*). The effect of phosphorylation on the ATPase reaction of the human enzyme is presently not clear.

The ATPase reaction of DNA gyrase has also been investigated. In this case, recent work has focused on the ATPase reaction of the 43-kDa N-terminal ATPase domain of GyrB (*38*, *39*). It was found that this domain is a monomer in the absence of nucleotide but dimerizes in the presence of ATP or ADPNP (5′-adenylyl-β,γ-imidodiphosphate). The ATPase activity is distinctly non-Michaelian, and the ATPase and binding data are consistent with the active form of the enzyme being a dimer and dimerization being the rate-limiting step of the ATPase reaction. In conjunction with the X-ray crystal structure of this domain complexed with ADPNP (*40*), these data support the idea that the N-terminal domain of GyrB, and by implication the N-terminal domains of other type II topoisomerases, acts as an ATP-operated clamp in the topoisomerase reaction cycle.

To further our understanding of ATP hydrolysis by DNA topoisomerase II we have studied the ATPase reaction of the N-terminal domain of topo IIα cloned in *E. coli*.

EXPERIMENTAL PROCEDURES

Cloning. A variety of constructs were produced by PCR in order to express portions of the ATPase region of human topo IIα in an *E. coli* T7 (pET) vector system. These constructs were designed on the basis of sequence similarity with the equivalent region of *E. coli* DNA GyrB, for which the crystal structure of the N-terminal 43 kDa domain is known (*40*). Three different oligonucleotides (oligos) were designed to introduce translational stop codons after Lys399, Glu419, and Lys435, along with a 3′ restriction enzyme site. The oligos designed to terminate translation at 399 and 419 contained 3′ *Sac*I restriction sites, whereas the oligo designed to terminate translation at 435 incorporated the naturally occurring *Eco*RI site within the human topo IIα gene. Oligo sequences were as follows: 399 K stop = 5′-TTA GCT GAG CTC TTA GAT AAA TTT TTC ACT CAA-3′; 419 Q stop = 5′-TTA GCT GAG CTC TTA GGC CTT AAA CTT CAC CCA-3′; 435 K stop = 5′-TGG GAA TTC CTT TGA TTC AAT TAT GTT TTA C-3′. These oligos were used in PCR reactions with M13 universal primer and a cDNA clone for human topo IIα (*41*). The PCR fragments produced contained 3′ restriction sites as described above in addition to a naturally occurring 5′ *Nco*I site that straddles the ATG initiation codon in the human topo IIα gene. This allowed

unidirectional cloning of the PCR fragments after restriction digest into suitably prepared pET21d (Novagen). After selection of recombinants, the cloned DNA was sequenced to check for the incorporation of PCR-directed mutations. These constructs were then used to transform *E. coli* BL21(DE3) in expression studies.

A fourth construct was produced incorporating a carboxy-terminal polyhistidine tag to enable the protein to be purified directly from a crude extract by metal chelate affinity chromatography. An oligonucleotide (439 K HisTag = 5′-TTT GCG GAG CTC TTG GGA ATT CCC TTG ATT CT-3′) was synthesized that introduces an in-frame *Sac*I site after the codon for Lys439 and was used in PCR amplification of the ATPase region as described above. Cloning of this fragment into pET21d as an *Nco*I−*Sac*I fragment produces an in-frame fusion with a 17 amino acid carboxy-terminal peptide that includes a 6-histidine repeat sequence. This plasmid was named pTOPSTOP/His.

Protein Purification. To prepare the 52 kDa N-terminal fragment of human topo IIα, 1 L of Nutrient Broth (Oxoid) plus 50 μg/mL ampicillin was inoculated with a 5 mL overnight culture of *E. coli* BL21(DE3)::pLysS::pTOPSTOP/His and incubated at 37 °C until the $A_{600} = \sim 0.5$. Isopropyl β-D-thiogalactoside (50 μM) was then added and growth continued for a further 3 h. Cells were harvested by centrifugation and resuspended in 10 mL of 50 mM Tris·HCl (pH 7.5) and 10% sucrose. Cells were disrupted by sonication and the cell debris was pelleted by centrifugation. (At this stage the vast majority of the 52 kDa protein was in the pellet.) The pellet was washed with 1% Triton X-100 (Sigma), pelleted again, and washed with water. The pellet was then resuspended in 50 mM Tris·HCl (pH 8.0) and 8 M urea and applied to a Talon (Clontech) Co^{2+} affinity column preequilibrated with 50 mM Tris·HCl (pH 8.0), 0.5 M KCl, and 8 M urea. The column was washed with the same buffer and then developed with 50 mM Tris·HCl (pH 8.5), 50 mM imidazole (Sigma), and 8 M urea. The eluted protein was refolded by dialysis overnight at 4 °C against 50 mM Tris·HCl (pH 9.0), 0.1 M KCl, 1 mM EDTA, 1 mM DTT, and 10% (w/v) glycerol. Following dialysis, the protein solution was centrifuged in a microfuge (15 min) to remove any residual insoluble material. The yield from a 1 L culture was 0.5 mg.

To purify proteins from polyacrylamide gels, samples in 125 mM Tris·HCl (pH 6.8), 4% SDS, 20% (w/v) glycerol, 10% (w/v) β-mercaptoethanol, and 0.002% (w/v) bromophenol blue were run on an SDS−12% polyacrylamide gel. A section of the gel was stained and a scalpel blade was used to carefully cut out the band of interest from the remaining (unstained) part of the gel. The gel slice was then crushed and 1 mL of 50 mM Tris·HCl (pH 7.5), 100 mM EDTA, 5 mM DTT, 150 mM NaCl, and 1% SDS was added. The gel matrix was left overnight in the elution solution. After filtration through an Econocolumn (Bio-Rad) to remove gel debris, the protein was precipitated by the addition of an equal volume of acetone (at −20 °C) and this mixture was then kept at −20 °C for 1 h. The precipitated protein was then pelleted in a benchtop centrifuge at 13 000 rpm for 30 min. The resulting pellet was then resuspended in 50 mM Tris·HCl (pH 8.8), 100 mM KCl, and 8 M urea for subsequent refolding (as above).

ATPase Assays. ATPase determinations were carried out by the pyruvate kinase/lactate dehydrogenase assay described previously (*28*, *38*), with the following modifications. Reactions (200 μL) contained 50 mM Tris·HCl (pH 7.5), 80 mM KCl, 2 mM ATP, 2 mM $MgCl_2$, 0.4 mM phosphoenolpyruvate, 0.25 mM NADH, 5 μL pyruvate kinase/lactate dehydrogenase [in 50% (w/v) glycerol, 100 mM KCl, 10 mM HEPES (pH 7.0), and 0.1 mM EDTA; Sigma] and topo II fragment (up to 60 μL). Plasmid pBR322 DNA (supercoiled, relaxed, or linear; gifts of Mrs. A. J. Howells, University of Leicester) was also added where indicated. Assays were carried out at 37 °C in a Perkin-Elmer Lambda 5 spectrophotometer or a Bio-Tek EL340 microplate reader. Novobiocin and amsacrine were purchased from Sigma, ICRF-159 was a gift of Dr. J. R. Jenkins (University of Leicester), and merbarone was a gift of NCI (National Institutes of Health, Bethesda, MD). DNase I was purchased from Boehringer.

Protein Cross-Linking. Protein was dialyzed into 50 mM HEPES (pH 8.5), 100 mM KCl, 4 mM DTT, and 4 mM $MgCl_2$ at 4 °C. Where indicated, samples were incubated for 1 h at 25 °C with ATP, ADPNP, or ADP. Dimethyl suberimidate (40 μg/mL) was added and the incubation was continued for 2 h at 25 °C. Samples were applied to an SDS−polyacrylamide gel and analyzed by Western blotting, which was carried out with a rabbit polyclonal antibody raised against the N-terminal domain of topo IIα (gift of Dr. J. R. Jenkins, University of Leicester) as described previously (*42*).

Site-Directed Mutagenesis. To mutate Glu86 to Ala and Asp, the QuikChange (Stratagene) procedure was employed using the following pairs of complementary oligonucleotides: 5′-TGT TCC TGG TTT ATA CAA AAT CTT TGA **TGC** GAT TCT AGT TAA TGC-3′ and 5′-TGT TCC TGG TTT ATA CAA AAT CTT TGA **TGA** TAT TCT AGT TAA TGC-3′, respectively. To mutate Ser29 to Ala and Asp, the following pairs of complementary oligonucleotides were used: 5′-AAG AAA AAT GAA GAT GCA AAG AAA AGA CTG **GCT** GTT GAA AGA A-3′ and 5′-AAG AAA AAT GAA GAT GCA AAG AAA AGA CTG **GAT** GTT GAA AGA A-3′, respectively. In each case only the top strand is shown and the mutated codon is shown in boldface type. In addition to introducing the desired mutations, restriction enzyme sites (*Bsp*1407I or *Dde*I) were removed during the mutagenesis at the locations indicated by underlining. Colonies were screened by performing plasmid minipreps and restriction enzyme analysis. Mutations were confirmed by DNA sequencing.

Protein Phosphorylation. Protein phosphorylation experiments were carried out on the 52 kDa fragment with protein kinase C from rat brain (Boehringer Mannheim) under the following conditions: 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 5 μg/mL dicaproin (Sigma), 100 mM ATP, and 10 μCi of [γ-^{32}P]ATP (Amersham), where indicated. Reactions (20 μL) were incubated at 30 °C for 15 min. Radiolabeled samples were applied to an SDS−12% polyacrylamide gel, which was then dried in a vacuum dryer, and the gels were exposed to Fuji X-ray film.

Other Methods. Protein concentrations were determined by the method of Bradford (*43*). Novobiocin affinity chromatography was carried out as described previously (*44*).

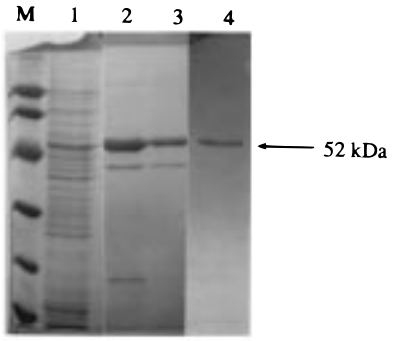

FIGURE 1: SDS−polyacrylamide gel showing purified 52 kDa protein. Lane 1, cell extract from overproducing strain; lanes 2 and 3, fractions from a Talon column eluted with imidazole; lane 4, purified 52 kDa protein after refolding. Molecular mass markers (M) are 94, 67, 43, 30, 20, and 14.4 kDa.

RESULTS

Cloning and Purification of the N-Terminal Domain. Three *E. coli* clones containing pET-based plasmids encoding N-terminal fragments of human topo IIα were constructed, producing proteins of 45.8, 47.9, and 49.8 kDa. Of these the largest protein, ~50 kDa (residues 1−435), showed the best expression; its C-terminus is close to the putative domain boundary defined by homology with GyrB (the N-terminal ATPase domain ends at Arg393 (*38*, *45*), which corresponds to Lys425 in human topo IIα). This protein was made both with and without a C-terminal His_6 tag (50 and 52 kDa). In the case of the His-tagged protein, four further C-terminal amino acids were included (436−439), plus 11 extra amino acids and 6 His residues introduced during cloning.

Both the native and His-tagged versions of the N-terminal domain were found to be in the insoluble fraction following cell lysis but the proteins could be resolubilized following treatment with urea and subsequent dialysis into nondenaturing buffers (see Experimental Procedures). The purification of the 52 kDa (His-tagged) protein is summarized in Figure 1.

ATPase Activity. Using the pyruvate kinase/lactate dehydrogenase-linked assay, the purified 52 kDa protein was found to have an ATPase activity. Typically 0.1 μM protein was found to have an activity of ~1 nM s^{-1}. That this activity was intrinsic to the purified 52 kDa protein was indicated by the following experiments. Purified 52 kDa protein was applied to an SDS−polyacrylamide gel and the protein band was excised and refolded as described in Experimental Procedures. The ATPase activity of this gel-purified protein was essentially unchanged. The non-His-tagged version of the N-terminal fragment (50 kDa) was also purified from an SDS gel and found to possess a similar ATPase activity to the 52 kDa protein (data not shown).

The intrinsic ATPase activity of the 52 kDa protein was investigated as a function of enzyme concentration and found to exhibit a linear dependence of initial rate on enzyme concentration in the range 75−450 nM (data not shown). This behavior is in marked contrast to that of the 43 kDa N-terminal domain of GyrB, which exhibits a nonlinear dependence of ATPase rate on enzyme concentration (*38*). As a function of substrate (ATP) concentration, the rate of ATP hydrolysis by the 52 kDa protein shows a hyperbolic dependence (Figure 2), indicative of Michaelis−Menten kinetics, again in contrast to the 43 kDa fragment of GyrB

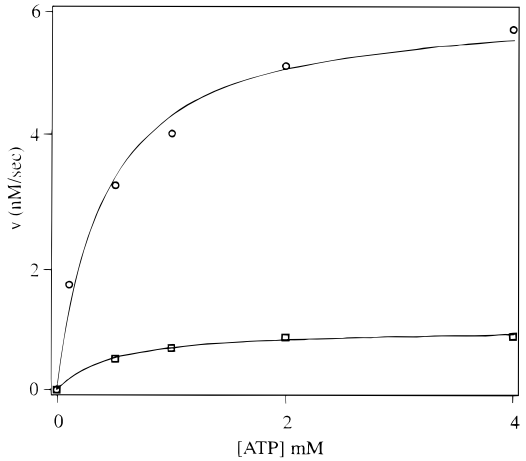

FIGURE 2: Dependence of the ATPase rate of the 52 kDa protein on ATP concentration. Rates (v) are initial velocities in the absence (□) and presence (○) of 80 μg/mL (0.12 mM bp) supercoiled pBR322 DNA.

(*38*). Values of K_M and k_{cat} (0.47 mM and 0.018 s^{-1}) were determined from these data (Figure 2).

DNA Dependence of ATPase Activity. Unexpectedly we found that the ATPase activity of the 52 kDa protein was stimulated in the presence of DNA (Figures 2 and 3). We found that supercoiled, relaxed, and linear pBR322 stimulated the activity to a similar extent (data not shown). Using a range of buffer conditions and with several separate enzyme preparations, we found that the presence of DNA to increase the ATPase rates 5−10-fold (e.g., Figure 2). This is in marked contrast to the 43 kDa domain of GyrB, whose ATPase activity is independent of DNA (*38*), but is similar to the DNA dependence observed with full-length human topo IIα (*28*).

To further investigate the DNA dependence of the ATPase activity, the following experiment was performed. An ATPase reaction containing the 52 kDa protein was initiated by the addition of ATP, and the ATPase rate was monitored. Supercoiled pBR322 DNA was then added and a 9-fold increase in rate was observed. DNase I was then added and a sharp drop in the rate of hydrolysis was observed, the final rate approaching the DNA-independent rate (data not shown). These data support the idea that DNA directly stimulates the ATPase activity and that this stimulation is reversible. It also confirms that the stimulation is due to DNA and not another component in the mixture.

The optimal conditions for the ATPase reaction were explored. Over a range of pH values (7.0−9.0) we found the optimum pH to be 7.5. At high ionic strength (>200 mM KCl) the ATPase activity was abolished; optimal activity occurred at ~100 mM. This mirrors the salt dependency of the DNA-dependent ATPase of the full-length enzyme. We found that the Mg^{2+} dependency showed an optimum that was slightly higher than the ATP concentration (e.g., 2−3 mM at 1.25 mM ATP). At higher $MgCl_2$ concentrations (e.g., >5 mM) there was a significant fall in ATPase activity that could not be accounted for by ionic strength effects; in subsequent experiments the Mg^{2+} concentration used was equimolar with the ATP concentration. The following ATPase reaction conditions were therefore used in the experiments described in this paper: 50 mM Tris·HCl (pH

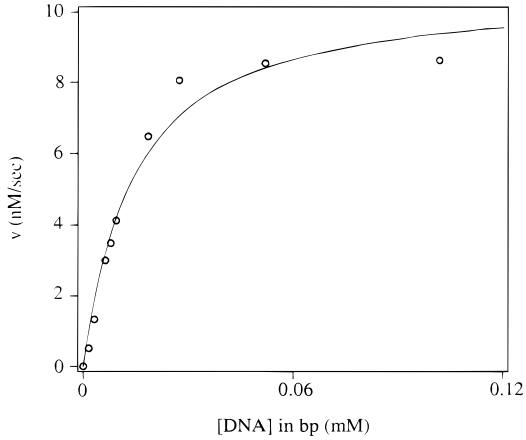

FIGURE 3: Dependence of the ATPase rate of the 52 kDa protein on DNA concentration. Rates (v) are initial velocities at 2 mM ATP; DNA was supercoiled pBR322.

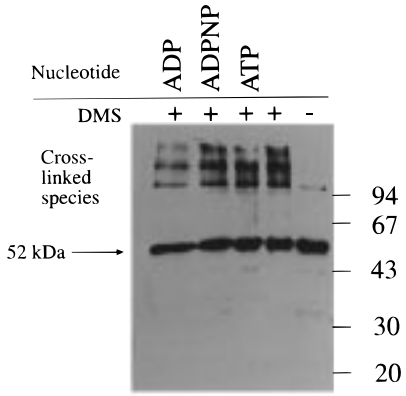

FIGURE 4: Cross-linking of the 52 kDa protein with dimethyl suberimidate. Shown is a Western blot (with antibody specific to the N-terminal domain of topo IIα) of an SDS−polyacrylamide gel showing cross-linking of the 52 kDa protein by dimethyl suberimidate in the absence and presence of 2 mM nucleotides, as indicated. Sizes of molecular mass markers in kilodaltons are shown.

7.5), 80 mM KCl, 2 mM ATP, and 2 mM $MgCl_2$, except where indicated.

The His-tagged protein was generally prepared with a Co^{2+} column. When this protein was prepared from a Ni^{2+} column it was found to be largely inactive in ATPase assays. We found that the ATPase activity of protein prepared with a Co^{2+} column could be inhibited by Ni^{2+}, and we conclude that Ni^{2+} is an inhibitor of the ATPase activity of the N-terminal domain of topo II. Experiments with full-length topo II showed that Ni^{2+} could also inhibit the full-length enzyme (data not shown). We also tested a range of topo II inhibitors (merbarone, amsacrine, and ICRF-159) and found no evidence of significant inhibition of the DNA-dependent ATPase of the 52 kDa fragment (data not shown).

Novobiocin and other coumarins have been suggested as being inhibitors of the ATPase reaction of *Drosophila* topo II (*25*, *46*), although recent experiments with human topo IIα showed little effect of novobiocin on the ATPase reaction (*28*). We found that the ATPase reaction of the N-terminal 52 kDa fragment of topo IIα was insensitive to novobiocin and that the protein did not bind to a novobiocin affinity column (data not shown). Taken together, these results suggest that novobiocin is not an effective inhibitor of the ATPase reaction of human topo IIα.

At a range of enzyme concentrations (0.9−10.5 nM) we found that the DNA-dependent ATPase was linearly dependent on enzyme concentration (data not shown). Figure 2 shows the dependence of ATPase rate on ATP concentration in the presence of DNA. The calculated values of K_M and k_{cat} (0.40 mM and 0.11 s^{-1}) suggest that in the presence of DNA the K_M is essentially the same as in its absence but that the k_{cat} increases by ∼6-fold.

We have investigated the stimulation of the ATPase of the 52 kDa protein at a range of DNA concentrations (Figure 3). These data suggest that a considerable excess of DNA over protein is required to fully stimulate the ATPase activity. These data can be treated as a binding isotherm (see Discussion) and an approximate value for the equilibrium dissociation constant for the binding of the 52 kDa to DNA was calculated to be ∼14 μM.

Dimerization. A salient feature of the ATPase activity of the N-terminal domain of GyrB is its nonlinear dependence on enzyme concentration, an observation that, when coupled with binding and structural data, strongly supports the idea that this domain dimerizes in the presence of nucleoside triphosphate (*38*−*40*). The observation of a linear dependence of ATPase rate on enzyme concentration (both with and without DNA) for the 52 kDa protein raises the possibility that dimerization is either not occurring with this fragment or is not the rate-limiting step of the ATPase reaction. To address this issue we have performed cross-linking experiments with this fragment. Figure 4 shows that dimethyl suberimidate cross-links higher molecular weight forms of this protein in both the absence and presence of ADPNP, ATP, and ADP. Although there are several cross-linked bands in these lanes, the two principal species have sizes consistent with dimeric forms. In experiments with the N-terminal domain of DNA gyrase we have previously noted that multiple cross-linked forms are apparent in the presence of dimethyl suberimidate (*6*, *38*); these species are thought to be dimers cross-linked in different ways. (We found also that the untreated 52 kDa protein, lane 1, contains a small amount of dimer. It is possible that treatment with SDS does not completely dissociate the dimeric form.) From these data we suggest that the dimer form of the N-terminal domain of topo IIα is stable in both the absence and presence of nucleotides.

ATPase Active Site. Previous work with the 43 kDa N-terminal domain of GyrB showed that Glu42 was very likely to be the catalytic residue in the ATPase reaction (*47*). As the sequences of type II topoisomerases are conserved in this part of the protein, it was possible to suggest that the corresponding catalytic base in human topo IIα is Glu86 (*47*). To verify this proposition we have mutated this residue in the N-terminal domain of human topo IIα to Ala and Asp. The purified 52 kDa proteins bearing these mutations were found to have very low ATPase activities that showed only low-level stimulation in the presence of DNA (Figure 5). In control experiments we found that cell extracts containing no 52 kDa protein and purified in the same way as the 52 kDa protein also showed these low-level ATPase activities; i.e., we conclude that this activity is a consequence of contaminating proteins and that the two mutations at Glu86 render the protein essentially inactive.

Phosphorylation. Phosphorylation has been proposed to affect the catalytic activity of topo II (*29*). Although phosphorylation predominantly occurs in the C-terminal

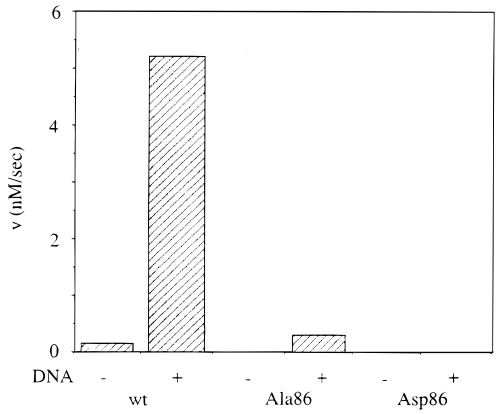

FIGURE 5: ATPase activities of wild-type 52 kDa protein and proteins bearing mutations at Glu86. Rates (v) are initial velocities at 2 mM ATP of ~0.2 μM of each protein in the absence and presence of 80 μg/mL supercoiled pBR322 DNA.

domain, a site for phosphorylation by protein kinase C has been mapped to Ser29 in the N-terminal ATPase domain of topo IIα (*37*). Treatment of the 52 kDa fragment with protein kinase C and radiolabeled ATP resulted in labeling of the protein (data not shown). Following phosphorylation, we found the phosphoprotein to have ATPase activity identical to that of the untreated protein. To probe further the potential effects of phosphorylation, we engineered two mutant 52 kDa fragments bearing either Ala or Asp at residue 29. The Ala mutation generates a protein that cannot be phosphorylated at this position (and hence controls for the possibility of phosphorylation in vivo in *E. coli*) while the Asp mutant potentially mimics a phosphorylated enzyme. Again we found that both mutant proteins had ATPase activities that were similar to that of wild type, suggesting that phosphorylation at Ser29 does not appreciably affect the ATPase activity of this fragment and that Ser29 is not an essential residue in the ATPase reaction.

DISCUSSION

We have constructed a number of *E. coli* clones that express N-terminal fragments of human DNA topoisomerase IIα. We found that the proteins expressed by these clones were largely insoluble. The best expression was found with a ~50 kDa protein (amino acids 1−435), which was also made with a C-terminal His tag. It is not known where the domain boundary for the ATPase function of topo IIα lies. A proteolysis site occurs between Glu410 and Asn411 in yeast topo II (*48*), which corresponds to the C-terminal side of Gln420 in human topo IIα. In *E. coli* GyrB a proteolysis site occurs between Arg393 and Arg394, corresponding to the C-terminal side of Lys425 in human topo IIα. As the sequence similarity in this stretch of amino acids is not high (*7*), it is not entirely clear where the boundary for the ATPase domain of human topo IIα will occur, but we might expect, on the basis of the above information, that the 50 kDa N-terminal fragment (*1*−*435*) should encompass this domain. However, the fact that this protein and those made by our other constructs are insoluble may suggest that the C-termini of these fragments do not coincide with a domain boundary and may lie within a region of secondary structure. Alternatively, the human ATPase domain may be intrinsically less soluble or fold less readily than its prokaryotic counterpart (gyrase). Clones expressing alternative-sized fragments are currently under construction.

We found that both the 50 kDa fragment and the His-tagged construct (52 kDa) showed ATPase activity. Although the rates were low, they were comparable between the two proteins and showed an approximate linear dependence on enzyme concentration. This is in marked contrast to the N-terminal ATPase domain of GyrB (43 kDa protein), which shows a distinctly nonlinear dependence of ATPase rate on enzyme concentration (*38*). This difference is likely to correlate with the observation that the human protein appears to be a dimer under these conditions (see below).

Perhaps the most striking difference between the ATPase activities of the N-terminal domains of the human and *E. coli* enzymes is that the human enzyme shows DNA stimulation (Figures 2 and 3). No direct evidence for the interaction of the ATPase domain of GyrB with DNA exists, although this is strongly suggested by X-ray crystallography and site-directed mutagenesis studies (*40*, *49*). Such an interaction is in keeping with the role of this domain as an ATP-operated clamp (*14*). The level of stimulation of the ATPase activity by DNA is 5−10-fold (~6-fold comparing k_{cat} values). With full-length topo IIα the level of stimulation by DNA was ~10-fold (*28*), depending on the enzyme to DNA ratio, and has been shown to be 3−17-fold for other type II enzymes. The K_M values in the absence and presence of DNA were found to be essentially the same (0.4−0.47 mM), whereas the k_{cat} values were 0.018 and 0.11 s^{-1} in the absence and presence of DNA, respectively. It is interesting to compare these values with those found for full-length topo IIα: k_{cat} and K_M values of 2.17 s^{-1} and 0.78 mM, respectively, at low DNA to enzyme ratios and 0.59 s^{-1} and 0.56 mM, respectively, at high enzyme to DNA ratios (*28*). Thus the K_M value for the ATPase domain of topo IIα is in keeping with the values for the full-length enzyme and also with those for other type II topoisomerases such as DNA gyrase [0.21−0.45 mM (*50*)]. The k_{cat} values for the ATPase domain are somewhat less than that for the full-length enzyme, suggesting that binding of DNA at the DNA "gate" may be required for full DNA stimulation of the ATPase activity.

Figure 3 shows the stimulation of the ATPase activity of the 52 kDa protein as a function of DNA concentration. If we assume that the reaction velocity is directly proportional to the concentration of DNA-bound enzyme molecules then these data can be treated as a binding isotherm. This yields an equilibrium dissociation constant (K_d) of 14.3 (±2.5) μM. Although this binding constant is weak, it is based on a DNA concentration in base pairs as we do not know the binding-site size for the ATP-operated clamp. If we were to assume that a 10 bp segment of DNA binds in the clamp, i.e., the DNA-binding site accommodates 10 bp [such a value would be consistent with the crystal structure of the N-terminal domain of GyrB (*40*)], then the K_d would be ~1.4 μM. However, further experiments are required to establish the DNA-binding characteristics of this domain. In recent work we have constructed two fusion clones of the N-terminal domain of human topo IIα (which are expressed as soluble protein), which both show DNA-dependent ATPase activities very similar to that of the 52 kDa protein described in this paper (S. Campbell and A.M., unpublished data), further endorsing the DNA dependence described here.

A further difference between the ATPase domain of gyrase and human topo IIα is that, at least as judged by cross-linking experiments, the human fragment is a dimer, whereas dimerization of the gyrase domain only occurs in the presence of nucleoside triphosphate (*38*, *39*). This observation is supported by structural data in the case of gyrase (*40*); although structural data for the human domain does not currently exist, the existence of this protein as a dimer is consistent with the linear dependence of its rate on enzyme concentration (i.e., no dimerization step is detectable in the kinetics of ATP hydrolysis) and its ability to bind DNA (see below). It is possible that the cross-linking results shown in Figure 4 are a consequence of aggregation of the 52 kDa protein. However, we favor the interpretation given above for a number of reasons. First, the size of the cross-linked products is suggestive of dimers and the existence of multiple cross-linked forms is similar to what has been observed previously with the corresponding gyrase domain in the presence of ADPNP and coumermycin (*6*, *39*). Second, we do not see evidence of high molecular weight species that fail to enter the gel, an expected feature of nonspecific aggregation. Third, fusion constructs of the N-terminal domain of human topo IIα (which produce souble protein) do not show the greater than first-order dependence of rate on enzyme concentration found with the 43 kDa domain of GyrB (S. Campbell and A.M., unpublished data); i.e., dimerization is not a rate-limiting step with these proteins, supporting the idea that this domain is a stable dimer.

Site-directed mutagenesis of the gyrase B protein identified Glu42 as the catalytic residue; the corresponding residue in human topo IIα is Glu86 (*47*). Therefore we used site-directed mutagenesis to engineer 52 kDa proteins bearing Ala or Asp at this position. Neither of these proteins had appreciable ATPase activity in the absence or presence of DNA, supporting the proposed role of Glu86 as the catalytic base in the ATP hydrolysis reaction. Using a combination of site-directed mutagenesis and phosphorylation experiments, we showed that phosphorylation of the 52 kDa protein by protein kinase C has an insignificant effect on the ATPase activity. It is possible that phosphorylation exerts a subtle effect or that a more profound effect on ATPase activity would be found in the context of the full-length enzyme.

Mechanistically, the proposed role for the N-terminal domain of human topo IIα and other type II topoisomerases is as an ATP-operated clamp that captures a segment of DNA (the T segment) to be transported through a double-stranded break in the G segment, which is bound at the DNA gate (*14*). Therefore we would expect this domain to bind and hydrolyze ATP and to bind DNA. We have found that the 52 kDa protein is capable of both activities, consistent with its proposed role. In the context of the intact topo II molecule the capture of DNA by the ATP-operated clamp ought to be coordinated with the binding and cleavage of DNA at the DNA gate (the G segment). Studies on the ATPase activity of full-length human topo IIα have shown that >100 bp of DNA is required to stimulate the ATPase activity, consistent with a requirement for DNA to be bound both at the DNA gate and at the ATP-operated clamp (*28*). The stimulation of the ATPase activity of topo II by DNA is ~10-fold, depending on the reaction conditions. The fact that we find a similar level of stimulation of the ATPase activity of the N-terminal domain by DNA suggests that a substantial element of the DNA stimulation of the ATPase of topo II derives from the interaction of DNA with the ATP-operated clamp.

Taken together, the structural and ATPase data on the N-terminal domains of gyrase and topo II support their proposed role as ATP-operated DNA-binding clamps. The origin of the observed differences between the prokaryotic and eukaryotic enzymes is presently unclear. It is possible that the eukaryotic domain forms a more stable dimer, through interactions at its C-terminus, such that it does not dissociate during the ATP binding and hydrolysis cycles that result in clamp closing and opening. As a consequence, the N-terminal fragment behaves as a permanent dimer and shows ATPase kinetics that are not dependent upon a dimerization step. Its prokaryotic counterpart has been shown to be a monomer in the absence of nucleotide and a dimer in the presence of nucleotide triphosphate (*38*, *39*). Alternatively it is possible that the longer C-terminus in the case of the eukaryotic N-terminal fragment permits protein−protein interactions that cannot occur in the shorter prokaryotic protein; i.e., the differences in the ATPase, oligomeric state, and DNA-binding properties that we have observed are merely a consequence of the C-terminal sequences in the two constructs. This would imply that important dimerization interactions occur just beyond the boundary of the N-terminal domains, within a relatively short stretch of amino acids. Further N-terminal clones of topo IIα are currently under construction in order to resolve this question.

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REFERENCES

1. Wang, J. C. (1996) *Annu. Rev. Biochem. 65*, 635−692.
2. Maxwell, A. (1997) *Trends Microbiol. 5*, 102−109.
3. Chen, A. Y., and Liu, L. F. (1994) *Annu. Rev. Pharmacol. Toxicol. 34*, 191−218.
4. Froelich-Ammon, S. J., and Osheroff, N. (1995) *J. Biol. Chem. 270*, 21429−21432.
5. Lewis, R. J., Singh, O. M. P., Smith, C. V., Skarynski, T., Maxwell, A., Wonacott, A. J., and Wigley, D. B. (1996) *EMBO J. 15*, 1412−1420.
6. Gormley, N. A., Orphanides, G., Meyer, A., Cullis, P. M., and Maxwell, A. (1996) *Biochemistry 35*, 5083−5092.
7. Caron, P. R., and Wang, J. C. (1994) *Adv. Pharmacol. 29B*, 271−297.
8. Lynn, R., Giaever, G., Swanberg, S., and Wang, J. C. (1986) *Science 233*, 647−648.
9. Uemura, T., Morikawa, K., and Yanagida, M. (1986) *EMBO J. 5*, 2355−2361.
10. Wyckoff, E., Natalie, D., Nolan, J. M., Lee, M., and Hsieh, T. (1989) *J. Mol. Biol. 205*, 1−13.
11. Huang, W. M. (1990) Virus-encoded DNA topoisomerases, in *DNA topology and its biological effects* (Cozzarelli, N. R., and Wang, J. C., Eds.) pp 265−284, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
12. Giaever, G., Lynn, R., Goto, T., and Wang, J. C. (1986) *J. Biol. Chem. 261*, 12448−12454.
13. Drake, F. H., Zimmerman, J. P., McCabe, F. L., Bartus, H. F., Per, S. R., Sullivan, D. M., Ross, W. E., Mattern, M. R., Johnson, R. K., Crooke, S. T., and Mirabelli, C. K. (1987) *J. Biol. Chem. 262*, 16739−16747.
14. Roca, J., and Wang, J. C. (1992) *Cell 71*, 833−840.
15. Roca, J., and Wang, J. C. (1994) *Cell 77*, 609−616.
16. Berger, J. M., Gamblin, S. J., Harrison, S. C., and Wang, J. C. (1996) *Nature 379*, 225−232.

17. Maxwell, A., and Gellert, M. (1986) *Adv. Protein Chem. 38*, 69−107.
18. Westerhoff, H. V., O'Dea, M. H., Maxwell, A., and Gellert, M. (1988) *Cell Biophys. 12*, 157−181.
19. Bates, A. D., and Maxwell, A. (1989) *EMBO J. 8*, 1861−1866.
20. Cullis, P. M., Maxwell, A., and Weiner, D. P. (1992) *Biochemistry 31*, 9642−9646.
21. Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T., and Tomizawa, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4772−4776.
22. Sugino, A., Peebles, C. L., Kruezer, K. N., and Cozzarelli, N. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4767−4771.
23. Rybenkov, V. V., Ullsperger, C., Vologodskii, A. V., and Cozzarelli, N. R. (1997) *Science 277*, 690−693.
24. Miller, K. G., Liu, L. F., and Englund, P. T. (1981) *J. Biol. Chem. 256*, 9334−9339.
25. Osheroff, N., Shelton, E. R., and Brutlag, D. L. (1983) *J. Biol. Chem. 258*, 9536−9543.
26. Halligan, B. D., Edwards, K. A., and Liu, L. F. (1985) *J. Biol. Chem. 260*, 2475−2482.
27. Lindsley, J. E., and Wang, J. C. (1993) *J. Biol. Chem. 268*, 8096−8104.
28. Hammonds, T. R., and Maxwell, A. (1997) *J. Biol. Chem. 272*, 32696−32703.
29. Watt, P. M., and Hickson, I. D. (1994) *Biochem. J. 303*, 681−695.
30. DeVore, R. F., Corbett, A. H., and Osheroff, N. (1992) *Cancer Res. 52*, 2156−2161.
31. Corbett, A. H., DeVore, R. F., and Osheroff, N. (1992) *J. Biol. Chem. 267*, 20513−20518.
32. Corbett, A. H., Fernald, A. W., and Osheroff, N. (1993) *Biochemistry 32*, 2090−2097.
33. Cardenas, M. E., Dang, Q., Glover, C. V. C., and Gasser, S. M. (1992) *EMBO J. 11*, 1785−1796.
34. Alghisi, G.-C., Roberts, E., Cardenas, M. E., and Gasser, S. M. (1994) *Cell. Mol. Biol. Res. 40*, 563−571.
35. Wells, N. J., Addison, C. M., Fry, A. M., Ganapathi, R., and Hickson, I. D. (1994) *J. Biol. Chem. 269*, 29746−29751.
36. Wells, N. J., and Hickson, I. D. (1995) *Eur. J. Biochem. 231*, 491−497.
37. Wells, N. J., Fry, A. M., Guano, F., Norbury, C., and Hickson, I. D. (1995) *J. Biol. Chem. 270*, 28357−28363.
38. Ali, J. A., Jackson, A. P., Howells, A. J., and Maxwell, A. (1993) *Biochemistry 32*, 2717−2724.
39. Ali, J. A., Orphanides, G., and Maxwell, A. (1995) *Biochemistry 34*, 9801−9808.
40. Wigley, D. B., Davies, G. J., Dodson, E. J., Maxwell, A., and Dodson, G. (1991) *Nature 351*, 624−629.
41. Tsai-Pflugelder, M., Liu, L. F., Liu, A. A., Tewey, K. M., Whang-Peng, J., Knutsen, T., Huebner, K., Croce, C. M., and Wang, J. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7177−7181.
42. Harlow, E., and Lane, D. (1988) *Antibodies: A laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
43. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
44. Gilbert, E. J., and Maxwell, A. (1994) *Mol. Microbiol. 12*, 365−373.
45. 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.
46. Hsieh, T., and Brutlag, D. (1980) *Cell 21*, 115−125.
47. Jackson, A. P., and Maxwell, A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11232−11236.
48. Lindsley, J. E., and Wang, J. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10485−10489.
49. Tingey, A. P., and Maxwell, A. (1996) *Nucleic Acids Res. 24*, 4868−4873.
50. Maxwell, A., and Gellert, M. (1984) *J. Biol. Chem. 259*, 14472−14480.

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