Hindering the Strand Passage Reaction of Human Topoisomerase IIα without Disturbing DNA Cleavage, ATP Hydrolysis, or the Operation of the N-terminal Clamp*

Vibe H. Oestergaard, Laura Giangiacomo‡, Lotte Bjergbaek§, Birgitta R. Knudsen, and Anni H. Andersen¶

From the Department of Molecular Biology, University of Aarhus, C. F. Møllers Allé, Building 130, 8000 Århus C, Denmark

DNA topoisomerase II is an essential enzyme that releases a topological strain in DNA by introduction of transient breaks in one DNA helix through which another helix is passed. While changing DNA topology, ATP is required to drive the enzyme through a series of conformational changes dependent on interdomain communication. We have characterized a human topoisomerase IIα enzyme with a two-amino acid insertion at position 351 in the transducer domain. The mutation specifically abolishes the DNA strand passage event of the enzyme, probably because of a sterical hindrance of T-segment transport. Thus, the enzyme fails to decatenate and relax DNA, even though it is fully capable of ATP hydrolysis, closure of the N-terminal clamp, and DNA cleavage. The cleavage activity is increased, suggesting that the transducer domain has a role in regulating DNA cleavage. Furthermore, the enzyme has retained a tendency to increase DNA cleavage upon nucleotide binding and also responds to DNA with elevated ATP hydrolysis. However, the DNA-mediated increase in ATP hydrolysis is lower than that obtained with the wild-type enzyme but similar to that of a cleavage-deficient topoisomerase II enzyme. Our results strongly suggest that the strand passage event is required for efficient DNA stimulation of topoisomerase II-mediated ATP hydrolysis, whereas the stimulation occurs independent of the DNA cleavage reaction per se. A comparison of the strand passage deficient-enzyme described here and the cleavage-deficient enzyme may have applications in other studies where a clear distinction between strand passage and topoisomerase II-mediated DNA cleavage is desirable.

The homodimeric eukaryotic topoisomerase II enzyme contains two highly conserved catalytic entities, which also share homology with the bacterial DNA topoisomerase II counterpart, DNA gyrase. The ATPase activity is found in the N-terminal region, whereas the DNA cleavage and ligation activities are held by the central region (6). The extreme C termini of the type II topoisomerases are divergent and dispensable for catalytic activity in vitro (7–9).

The structural data reveal that the N-terminal region of topoisomerase II forms an ATP-operated clamp, which closes upon ATP binding (10, 11). The dimeric N-terminal clamp contains two domains in each protomer (10). The most N-terminal domain is responsible for dimer interactions during clamp closure and also holds the ATP-binding site. The second domain, called the transducer domain, forms the walls of the clamp and connects it to the enzyme core. Furthermore, communication between the ATP-binding domain and the central domain of the enzyme, responsible for DNA cleavage/ligation, has been suggested to go through the transducer domain (4, 12–15). Upon clamp closure, the transducer domain of each protomer approaches one another, creating a very tight cavity that is actually too small to hold a T-segment (10). The surprisingly small hole of the eukaryotic topoisomerase II has been suggested to enable the enzyme to couple T-segment interactions to G-segment cleavage and opening (10).

The core region of topoisomerase II that is responsible for DNA cleavage and ligation is comprised of two domains, A’ and B’, showing homology to the subunits of DNA gyrase, gyrase A and gyrase B, respectively (16). The A’ domain contains both the active site tyrosine covalently attached to the 5’-end of the DNA during cleavage and the primary dimerization region (6). The B’ domain constitutes the interface between the transducer domain and the A’ domain (16) and is essential for DNA cleavage (16, 17).

At low ATP concentrations, the ATP consumption and the DNA strand passage activity of DNA topoisomerase II are coupled tightly (18). The catalytic actions of both the cleavage/ligation site and the ATPase site are governed by extensive interdomain communication, which probably facilitates an appropriate temporal order of conformational changes in the complex catalytic cycle of topoisomerase II and thereby reduces the energy usage. Thus, the binding of ATP to the N-terminal domain stimulates the cleavage of the G-segment bound by the core region (19), and the presence of DNA increases the turnover rate of ATP hydrolysis (2). Studies of a cleavage-deficient topoisomerase II enzyme have shown that an inability to cleave DNA severely reduces the stimulatory effect of DNA on ATP hydrolysis (20). In this enzyme, both DNA cleavage and strand passage were abolished. Therefore, it was suggested that the normal increase in ATP turnover either depends on the ability...
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to cleave the G-segment or is mediated through T-segment binding/transfer (20). Thus, in contrast to ATP-mediated stimulation of DNA cleavage, which only requires ATP binding, a full increase in ATP hydrolysis seems to depend on a catalytic action on DNA rather than just DNA binding.

We have characterized a human topoisomerase IIα enzyme with two amino acids inserted at position 351 in the transducer domain (351l). The insertion abolishes the strand passage reaction of the enzyme but leaves both catalytic domains active. Based on the newly published structure of the N-terminal fragment of yeast topoisomerase II (10), we find it very likely that the insertion impairs the strand passage reaction via sterical hindrance. Our results show that the insertion at position 351 slightly increases topoisomerase II-mediated DNA cleavage complex formation, consistent with a role of the transducer domain in controlling DNA cleavage (15). The biochemical characterization of 351l has revealed further that the enzyme retains DNA-stimulated ATP hydrolysis, although at a lower level compared with that of the wild-type enzyme. The stimulatory effect of DNA in 351l, which lacks strand passage activity, is equal to that obtained with a cleavage-deficient topoisomerase IIα enzyme. Our results demonstrate that DNA-mediated stimulation of ATP hydrolysis is independent of DNA cleavage and rather requires the strand passage reaction per se.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The Saccharomyces cerevisiae strains BJ201 (MATa ura3 trpl pep4 HSL1 prb1 can1 trpl2 HRP1) and JEL1top1 (kindly provided by J. C. Wang) were used for complementation analysis. BJ201 (10) was performed in TBE (100 m M Tris borate, pH 8.3, 2 m M EDTA), and was visualized by UV light.

Topoisomerase II-mediated DNA Ligation—Hybridization and labelling of the synthetic oligonucleotides were done according to the procedures described by Andersen et al. (22). The oligonucleotides used for suicide DNA cleavage and ligation were as described previously (15).

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Polynucleotides—DNA polynucleotides were obtained from DNA Technology Corporation and purified by preparative polyacrylamide gel electrophoresis as described by Andersen et al. (22). The polynucleotides used for suicide DNA cleavage and ligation were as described previously (15).

Clamp-closing Assay—For clamp-closing experiments, 10 nm topoisomerase II was preincubated with 6.5 nm of negatively supercoiled pUC19 DNA for 5 min in a total volume of 20 μl of buffer (50 mM Tris-HCl, pH 7.9, 2 mM MgCl2, 2.5 mM CaCl2, 20 mM NaCl, 0.1 mM EDTA) at 37 °C. After incubation for 60 min, the cleavage reactions were stopped by the addition of NaCl to 0.4 M, thereby preventing further cleavage during the ligation reaction. Ligation was initiated by the addition of 4 pmol of a 45-mer ligation substrate. After further incubation (with incubation times as indicated in the figure legends), the reactions were stopped by the addition of SDS to 1% and subjected subsequently to SDS–polyacrylamide gel electrophoresis. Covalent cleavage complexes were revealed by the transfer of the radiolabeled oligonucleotide to the topoisomerase polypeptide, and ligation was measured as the reduction in the amount of cleavage complexes with time. Reaction products were visualized and quantified using a Molecular Imager (Bio-Rad).

Clamp Closing Analyzed by Analytical CsCl Density Gradient Ultra-centrifugation—The technique used is essentially that described previously by Morris et al. (20). Reaction mixtures of 30 μl containing 150 nm topoisomerase II, 250 nm relaxed pBR322 (topoGEN), or linearized pUC19 plasmid DNA in sample buffer (50 mM Tris-HCl, pH 8, 60 mM NaCl, 1 mM EDTA, 8 mM MgCl2) were incubated for 5 min at 30 °C. AMP-PNP was added to a final concentration of 1 mM, and incubation was continued for 10 min. For each sample, 130 μl of 100 mM MgCl2 and 334 μl of saturated CsCl were added. The samples were run at 20 °C and at 40,000 rpm in a Beckman XL-A analytical ultracentrifuge (Beckman Instruments). Scans were taken at 260 and 280 nm until equilibrium was reached (~40 h).

Topoisomerase II-mediated Hydrolysis of ATP—The ATPase assays were performed using an ADP-kinase-linked enzyme assay (14). Reactions contained 30–200 nm topoisomerase II and, when indicated, 20 nm of negatively supercoiled pUC19 plasmid DNA. Reactions were carried out in volumes of 60 μl containing 1.25 mM ATP, 50 mM Tris-HCl, pH 8, 140 mM KCl, 1 mM EDTA, 8 mM MgCl2, and 0.4 mM phosphoenolpyruvate, and 0.2 mM NADH, 1.5 μM of pyruvate kinase/facade dehydrogenase (Sigma). Reactions were initiated by the addition of enzyme and stopped by the addition of 100 mM ethidium bromide and visualized by UV light.

Topoisomerase II-mediated Cleavage of Circular DNA—Topoisomerase II-mediated DNA cleavage was performed by incubating 100 nm topoisomerase II and 6.5 nm of negatively supercoiled pUC19 DNA in a total volume of 20 μl of buffer (50 mM Tris-HCl, pH 8, 140 mM KCl, 1 mM EDTA, 8 mM MgCl2). Samples were incubated at 37 °C for 7 min, and cleavage products were trapped by the addition of SDS to 1%. After protease K digestion (0.5 mg/ml), samples were subjected to electrophoresis in 1% agarose gels in TAE buffer (40 mM Tris acetate, pH 8.3, 2 mM EDTA). DNA was visualized by ethidium bromide staining (1 μg/ml). When topoisomerase II-mediated cleavage was carried out in the presence of ATP or AMP-PNP4 (Roche Applied Science), the concentration of the nucleotide was 1 mM.

4 The abbreviation used are: AMP-PNP, adenosine 5′-(β,γ-imino)-triphosphate; k-DNA, kinetoplast DNA.
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RESULTS

Insertion of Two Amino Acids at Position 351 in Human Topoisomerase IIα Abolishes the Strand Passage Activity of the Enzyme—In a previous study performed by Jensen et al. (8), it was observed that insertion of two amino acids at position 351 in human topoisomerase IIα disrupted the ability of the enzyme to complement growth of a yeast strain in which the endogenous topoisomerase II gene was deleted. Fig. 1A verifies the complementation inability of the mutant enzyme (351i). The alignment with the crystallized S. cerevisiae topoisomerase II fragment suggests that the insertion in 351i is situated at the inner wall of the transducer domain in the DNA transport path of the enzyme as shown in Fig. 1B. To determine the underlying causes of the complementation inability, 351i was purified to near homogeneity as seen from the Coomassie Blue-stained gel in Fig. 1C and subjected to biochemical analysis. To investigate the DNA strand passage activity of the mutant enzyme, a decatenation assay was performed first (Fig. 1D, upper panel). Whereas wild-type topoisomerase IIα under the given reaction conditions decatenated k-DNA, no release of mini-circles occurred with 351i even at high enzyme concentrations. A similar lack of DNA strand passage activity was observed in a DNA relaxation assay (Fig. 1D, lower panel) and in a DNA cleavage assay performed in the presence of ATP (Fig. 2A, lane 6). In the latter experiment, the enzyme was present in an ~15-fold excess, which excludes that 351i can perform even a single round of strand passage. Thus, consistent with the lack of in vivo complementation, 351i has lost its overall catalytic activity.

351i Has Retained DNA Cleavage Activity—Topoisomerase II is a multidomain enzyme, which is capable of both ATP hydrolysis and DNA cleavage/ligation. The two activities reside in different regions of the enzyme and can work independent of each other; however, a strict coordination is required to obtain topoisomerase activity. Coordination of the two catalytic activities of topoisomerase II is obtained through domain communication. When investigating topoisomerase II-
To test whether the lack of topoisomerase II activity seen with 351i is caused by an inability of the enzyme to perform DNA cleavage, 351i and the wild-type enzyme were incubated first with supercoiled plasmid DNA in the absence of nucleotide. The reactions were stopped by SDS, which immediately denatures the enzyme. After proteinase K treatment, cleavage products were separated from uncleaved plasmid DNA by agarose gel electrophoresis (Fig. 2A, upper panel, compare lanes 2 and 5). In the absence of the nucleotide, 351i actually displays a DNA cleavage level higher than that of the wild-type enzyme as also shown in the histogram in Fig. 2A, lower panel. The result shows that the mutant enzyme indeed has retained its cleavage activity.

The addition of the ATP analog, AMP-PNP, to the cleavage reaction increases the DNA cleavage level obtained by 351i (Fig. 2A). However, the stimulatory effect of the nucleotide is low compared with the effect obtained with the wild-type enzyme. In the presence of ATP, the wild-type enzyme relaxes the DNA. Even with these high enzyme concentrations, ATP only allows 351i to cleave DNA in support of its lack of overall catalytic activity.

In the assay described above, the DNA cleavage level reflects an equilibrium between topoisomerase II-mediated DNA cleavage and ligation. To investigate the DNA ligation ability of 351i, an advantage was taken of the topoisomerase II suicide system, which allows a separation of the DNA cleavage and ligation half-reactions (Fig. 2B, upper panel) (22). Since the topoisomerase II cleavage complex generated upon cleavage of a suicide substrate is kinetically competent (22), it is able to perform ligation if a suitable ligation substrate is added to the cleavage mixture.

Topoisomerase II-DNA cleavage complexes were prepared for the ligation assay as described under “Experimental Procedures.” After an increase in the salt concentration to inhibit further cleavage, ligation was initiated by the addition of a 45-mer DNA oligonucleotide able to hybridize to the bottom strand of the cleaved substrate. Ligation reactions were stopped with SDS at different time points and subjected to electrophoresis in an 8% SDS gel. The cleavage complexes were identified because of the covalent linkage of labeled DNA, whereas ligation was revealed as a disappearance of cleavage products upon the addition of ligation substrate (Fig. 2B, middle panel).

The results obtained in the ligation assay shows that 351i and the wild-type enzyme have very similar rates of ligation (Fig. 2B, lower panel). Thus, the slightly increased DNA cleavage level obtained by 351i is probably not the result of a lower ligation rate of this enzyme.

The results obtained in the DNA cleavage and ligation assays revealed that the insertion of two amino acids at position 351, although detrimental to overall enzyme catalysis, does not stop the addition of NaCl to 0.4 M, and ligation was initiated by the addition of a 45-mer ligation substrate. Samples were withdrawn at the indicated time points and subjected subsequently to electrophoresis in an 8% SDS gel and visualized on a Molecular Imager (Bio-Rad). DNA ligation is revealed as a disappearance of cleavage complexes due to release of topoisomerase II from the labeled oligonucleotide upon ligation to the 45-mer ligation substrate. Cl, cleavage complexes; S, substrate; C, DNA control. Lower panel, graphic illustration of the topoisomerase II-mediated DNA ligation experiment presented in the upper panel. The level of DNA cleavage at time point zero for each enzyme is set to one. Results are means ± S.D. of three independent experiments.

Fig. 2. 351i retains DNA cleavage/ligation activity. A, upper panel, 351i or wild-type human topoisomerase IIα was incubated with supercoiled plasmid DNA at 37 °C in the absence or presence of ATP or the ATP analog, AMP-PNP. The reactions were stopped after 7 min by the addition of SDS to 1%. After proteinase K digestion, the samples were subjected to electrophoresis in a 1% agarose gel. The enzyme and nucleotide used in the reactions are indicated above the lanes. C, DNA control. SC, supercoiled plasmid DNA; L, linear DNA; R/N, relaxed or nicked plasmid DNA; Cat, catenated plasmid DNA. Lower panel, the histogram of the cleavage reactions performed with the wild-type enzyme and 351i in the absence or presence of AMP-PNP. The results are the means ± S.D. of three independent cleavage experiments. wt, wild-type. B, upper panel, schematic illustration of the topoisomerase II suicide system. Middle panel, topoisomerase II-mediated DNA ligation was tested using the topoisomerase II suicide system. In this system, kinetically competent topoisomerase II-DNA cleavage complexes were formed by incubating topoisomerase II and a substrate designed to prevent ligation due to the release of a trinucleotide 5′-deligation position on the top strand upon DNA cleavage. Cleavage reactions were mediated DNA cleavage, domain communication can be revealed by the addition of a nucleotide that binds to the N terminus of the enzyme and thereby increases the DNA cleavage activity of the cleavage/ligation domain (19, 25).

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Fig. 3. 351i retains a functional N-terminal clamp. A, 351i or wild-type human topoisomerase IIα was preincubated for 5 min with plasmid DNA before the addition of AMP-PNP. After 5 min of further incubation, the reactions were stopped by the addition of either NaCl or SDS to 800 mM and 1%, respectively. The samples then were phenol-extracted. The waterphase was removed and ethanol-precipitated, proteinase K-digested and subjected to electrophoresis in a 1% agarose gel containing ethidium bromide next to the waterphase samples. Enzyme, nucleotide, and stop solutions used in the samples are indicated above the gel. i and w denote interphase and waterphase, respectively. SC and Cat indicate the position of supercoiled and catenated plasmid DNA, respectively. Catenated DNA also becomes topologically trapped by topoisomerase II because of its circular form. B, for the analysis of clamp closure by analytical ultracentrifugation, enzyme, DNA, and AMP-PNP were incubated as described under “Experimental Procedures.” The samples were loaded on a CsCl gradient and spun for 40 h. Absorbance traces at 260 nm of the CsCl density gradients are shown for each reaction. The absorbance trace of the linear DNA preparation. wt, wild-type.

disrupt the DNA cleavage/ligation domain of topoisomerase IIα and does not abolish interdomain communication as measured by nucleotide-stimulated DNA cleavage. Thus, the lack of strand transfer in 351i is not because of an inability of the enzyme to mediate DNA cleavage/ligation.

The N-terminal Clamp of 351i Is Functional—One of the essential steps in the catalytic cycle of topoisomerase II involves the closure of the ATP-operated N-terminal clamp, whereby a T-segment may be trapped for subsequent transport through the G-segment. To test whether 351i has retained this essential function, a clamp-closing assay was performed (modified from Bjergbaek et al. (4)). In the assay, topoisomerase II was preincubated with supercoiled plasmid DNA followed by the addition of the non-hydrolyzable ATP analog, AMP-PNP. When the wild-type enzyme was used in the assay, this led to the formation of a salt-stable enzyme-DNA-interlinked complex, which was collected readily from a phenol-water interphase (Fig. 3A). A similar result was obtained with 351i, showing that it is able to keep circular DNA trapped in high concentrations of salt. Thus, the insertion of two amino acids at position 351 does not disturb the ability of human topoisomerase IIα to undergo stable clamp closure. None of the enzymes allowed the trapping of DNA upon the addition of SDS, which disrupts the interlink between topoisomerase II and DNA, or in the absence of AMP-PNP where stable clamp closure does not occur.

To confirm that 351i retains a normal clamp function, clamp closure was analyzed using analytical CsCl density gradient centrifugation as described under “Experimental Procedures.” The assay takes advantage of the sedimentation differences of clamp complexes and free DNA in CsCl because of the different densities of protein and DNA. Free DNA will migrate to the bottom of a CsCl gradient, whereas the lighter species of DNA complexed with protein will sediment in the upper part of the gradient. In the presence of AMP-PNP, a peak was revealed in the upper part of the CsCl gradient both with 351i and the wild-type enzyme, demonstrating that both enzymes under these conditions form a stable complex with circular DNA (Fig. 3B, upper panel). Two control reactions also were performed. In one reaction, the circular DNA was substituted with linear DNA, which cannot be linked topologically to topoisomerase II (Fig. 3B, middle panel) and in another, AMP-PNP was excluded, preventing stable closure of the topoisomerase II clamp (Fig. 3B, lower panel). The assay revealed that the wild-type enzyme and 351i are able to generate similar amounts of the salt-stable closed clamp complex in the presence of the non-hydrolyzable ATP analog, AMP-PNP, and therefore confirmed that 351i retains a functional N-terminal clamp. Thus, the lack of strand transfer observed in 351i is not the result of a malfunction of the N-terminal clamp.

351i Has Retained Intrinsic as Well as DNA-stimulated ATPase Activity—The catalytic activity of topoisomerase II is dependent on ATP hydrolysis. Thus, even though a non-hydrolyzable ATP analog can lead to clamp closure, hydrolysis is required for consecutive strand passage events. To test whether the strand passage defect of 351i is attributed to an inability of the enzyme to hydrolyze ATP, 351i and wild-type
human topoisomerase IIα were subjected to an NADH-coupled ATPase assay (Fig. 4A, upper panel) (23, 24). The intrinsic ATPase activities of the enzymes were measured first in the absence of DNA. The results, which are shown in Fig. 4A, lower panel (columns 1 and 3), demonstrate that this activity is not influenced by the two-amino acid insertion at position 351.

Optimal coordination of the catalytic steps in DNA topoisomerase II requires a two-way communication between the N-terminal and core domains. Besides the ATP-stimulated DNA cleavage, normal topoisomerase II operation also requires a DNA-stimulated ATPase activity. To test whether DNA still stimulates the ATPase activity of 351i, the ATPase activities of 351i and the wild-type enzyme were also probed in the presence of supercoiled plasmid DNA. Both enzymes displayed a DNA-mediated stimulation of the ATPase activity. In the present study, the addition of DNA stimulated the ATPase activity of the wild-type topoisomerase IIα ~14-fold, whereas 351i displayed a ~4-fold stimulation (Fig. 4A).

The results show that 351i has retained intrinsic ATPase activity and is also able to respond to the presence of DNA with increased ATP hydrolysis. Therefore, the lack of topoisomerase II activity in 351i is not due to a defective ATPase activity. Taken together, the insertion at position 351i specifically hinders the DNA strand passage step of human topoisomerase IIα without disturbing DNA cleavage, operation of the N-terminal clamp, and ATP hydrolysis.

DNA Strand Passage and Not DNA Cleavage Is Important for the DNA-mediated Stimulation of ATP Hydrolysis in Topoisomerase II—The exact events leading to DNA-mediated stimulation of the ATPase activity of topoisomerase II are not known. However, the ATPase activity of a cleavage-deficient yeast topoisomerase II has been shown to display a reduced stimulation upon the addition of DNA compared with that of the wild-type enzyme (20). This finding suggests that either DNA cleavage or the DNA strand passage reaction is required to obtain efficient stimulation.

To test whether DNA cleavage or DNA strand passage plays a role in the stimulatory mechanism, the ATPase activity of the strand passage-deficient but cleavage-competent 351i enzyme was compared with that of a cleavage-deficient human topoisomerase IIα enzyme, Y805S. In the latter enzyme, the active site tyrosine has been substituted with a serine and the enzyme can neither perform DNA relaxation nor DNA cleavage to a detectable level (29). The extent of stimulation obtained with Y805S is very similar to that obtained with 351i, showing that the DNA cleavage reaction per se does not contribute significantly to the stimulation of topoisomerase II-mediated ATP hydrolysis (Fig. 4B). Taken together with the results obtained with the wild-type enzyme (Fig. 4A), which on top of the activities held by 351i can perform strand passage, efficient DNA-mediated stimulation of ATP hydrolysis in topoisomerase II requires the DNA strand passage reaction per se.

DISCUSSION

Topoisomerase II is a multidomain enzyme, which is capable of both ATP hydrolysis and DNA cleavage/ligation. The two activities reside in different regions of the enzyme and can work independent of each other. However, a coordination is required to obtain topoisomerase activity (4). Communication between the ATPase domain and the central domain of the enzyme responsible for DNA cleavage/ligation seems to go through the transducer domain (4, 13–15). The conformational changes caused by ATP binding to the N-terminal region thus reach all the way to the core domain and also change the catalytic features of the DNA cleavage/ligation reaction of the enzyme. Likewise, DNA interactions stimulate the ATPase activity of topoisomerase II, but in this case, the scenario seems more complex because DNA binding per se is not sufficient to efficiently increase the ATPase activity (20). The shift in ATPase turnover rather requires an enzymatic action on DNA.

In this study, we have characterized a human topoisomerase IIα enzyme bearing a two-amino acid insertion at position 351 in the transducer domain. The insertion is situated in the core proximal part of the inner wall suggested to interact with the T-segment prior to its passage through the G-segment (10, 27). The characterization has revealed that this enzyme, although capable of N-terminal clamp closure, ATP hydrolysis, and DNA cleavage/ligation, still lacks strand passage activity. 351i has retained a certain extent of interdomain communication. First, AMP-PNP stimulates 351i-mediated DNA cleavage slightly, demonstrating that the enzyme has preserved a tendency to increase the cleavage level upon nucleotide binding, either by
stabilizing the cleaved conformation or by increasing the forward cleavage rate. Second, DNA stimulates the ATPase activity of 351i.

We previously characterized a human topoisomerase IIa enzyme bearing an insertion at position 408 in the transducer domain (15), which based on alignment with the crystallized N-terminal fragment from yeast topoisomerase II is located close to position 351 in the tertiary structure. However, the two insertions result in enzymes with very different phenotypes where only 351i is able to perform clamp closure. This finding suggests that the defects observed in the two mutant enzymes do not result from a misfolding of the entire transducer domain. Rather, the two insertions cause a specific local disturbance resulting in distinct enzymatic defects. Whereas the insertion at position 408 interferes with the clamp function of topoisomerase II, the insertion at position 351 abolishes strand passage activity and leaves the clamp unaffected. Together with the proposed localization of 351 at the inner part of the clamp arms, our results suggest that the insertion disturbs the strand passage reaction per se. This can be attributed to a lack of T-segment interaction of the enzyme in the open clamp conformation, but for two reasons, we find it more likely that it is the result of a direct impair of the transport event. First, according to alignment with the structure of the N-terminal yeast fragment, position 351 in human topoisomerase IIα is located at the bottom of the cavity formed upon clamp closure in close proximity to the same position in the other subunit.

The T-segment normally can be transported through this narrow passage, but insertion of two amino acids is likely to cause a sterical hindrance of this event. Second, the N-terminal part of topoisomerase II only has very weak DNA interactions that are difficult to detect by biochemical analyses (23). Thus, it seems that the T-segment is presented to the clamp mainly by coincidence, and therefore, a disrupted T-segment interaction would not be expected to result in the complete inhibition of strand passage as is the case in 351i.

351i is able to cleave plasmid DNA, showing that the G-segment can easily access the DNA binding site in the core of the enzyme. Thus, the insertion apparently only introduces a hindrance to the transport of the T-segment, probably because this event occurs right upon clamp closure where the transducer domain of each subunit is oriented differently from what it is during G-segment entering. The DNA cleavage level displayed by 351i in the absence of nucleotide slightly exceeds that of the wild-type enzyme. This catalytic trait is shared with the enzyme bearing an insertion at position 408 in the transducer domain (15) and indicates that an important function of the transducer domain is to control the cleavage level of the core domain.

DNA only stimulates the ATPase activity of 351i −4-fold as compared with the −14-fold stimulation seen with wild-type topoisomerase II. However, the level of DNA-mediated stimulation is not significantly different from that obtained with a cleavage-deficient topoisomerase II. With the latter enzyme a −3-fold stimulation was obtained, which is similar to the stimulation seen with a cleavage-deficient yeast topoisomerase II as reported by Morris et al. (20). Because these cleavage-deficient enzymes can neither perform DNA cleavage nor strand passage, the remaining stimulatory effect of DNA on the ATPase activity in these enzymes probably reflects the stimulatory effect of DNA binding per se. Our observations that 351i and Y805S have similar ATPase activities lead us to conclude that the ATPase activity is not stimulated through the DNA cleavage reaction. This is in agreement with studies of a DNA gyrase enzyme in which the DNA gate was locked by cytotoxic cross-linking. The gyrase enzyme was able to cleave DNA, but still no DNA stimulation of the ATPase activity of the enzyme occurred (28). Thus, the step(s) leading to the pronounced increase in ATP hydrolysis is limited to the events that are very tightly coupled to DNA strand passage, most probably T-segment interactions that occur during the transport event. Alternatively, G-segment opening may be essential for the stimulatory effect of DNA, because we cannot completely rule out that 351i is unable to enter this conformation.

In conclusion, we have identified a human topoisomerase IIα mutant enzyme, where the DNA strand passage reaction is inhibited, neither due to a lack of clamp closure, a defect in the ATPase domain or due to an active site mutation that inhibits DNA cleavage. Rather, the strand passage reaction per se has been abolished by a two-amino acid insertion at position 351, which seems to block the normal DNA transport path in topoisomerase II by a sterical hindrance. The mutant enzyme thus may have further applications in studies where a clear distinction between DNA cleavage and strand passage is desired. Our results with 351i have suggested strongly that the strand passage reaction of topoisomerase II is required for efficient DNA-mediated stimulation of the ATPase activity of topoisomerase II, whereas this stimulation is independent of topoisomerase II-mediated DNA cleavage. Furthermore, the studies have confirmed that changes in the transducer domain are likely to influence the DNA cleavage level of the enzyme.

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