On the Coupling between ATP Usage and DNA Transport by Yeast DNA Topoisomerase II*

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The initial rates of ATP hydrolysis and relaxation of negatively supercoiled DNA by highly purified wild-type and mutant yeast DNA topoisomerase II were measured under identical conditions to study the coupling between the ATPase activity of a type II DNA topoisomerase and its catalysis of the transport of one DNA segment through another. The results indicate that the binding of the enzyme to DNA stimulates its intrinsic ATPase activity by about 20-fold, and ATP binding to the pair of ATPase sites in a DNA-bound dimeric enzyme appears to be cooperative. The cooperativity in ATP binding may be significant in the coordination of the two halves of a DNA-bound enzyme dimer. At low ATP concentrations, the rate-limiting step in ATP usage appears to be slower than that in DNA transport, and DNA transport is relatively efficient in terms of ATP consumption: 1.9 ± 0.5 ATP molecules are hydrolyzed/DNA transport event. At a saturating ATP concentration, however, there appears to be a reversal of these rate-limiting steps, and DNA transport is less efficient: 7.4 ± 1.0 ATP molecules are hydrolyzed/DNA transport event. These data are interpreted in terms of a model in which a DNA-bound enzyme acts as an ATP-operated clamp for the capture and transport of a second DNA segment.

Type II DNA topoisomerases (EC 5.99.1.3) are ubiquitous enzymes that catalyze the ATP-dependent transport of one double-stranded DNA segment through an enzyme-mediated transient break in another (reviewed in Maxwell and Gellert, 1986; Hsieh, 1990; Reece and Maxwell, 1991; see also Wang, 1985; Caron and Wang, 1993). In such a reaction, the enzyme-operated gate in the double-stranded DNA is opened through the attack of a pair of active site tyrosyl hydroxy groups, one in each half of the dyadic enzyme, on a staggered pair of DNA phosphodiester bonds. Breakage of the pair of DNA backbone bonds creates a transient break or gate in the double-stranded DNA segment, and at the same time a pair of covalent links is formed between the active site tyrosines and the 5' phosphoryl ends of the severed DNA strands. Closing of the DNA gate is achieved through nucleophilic attack of the 3' hydroxyls of the severed DNA ends on the enzyme-DNA phosphotyrosine linkages. The same enzyme molecule that operates the DNA gate can transport a second double-stranded DNA segment through the DNA gate. All known type II DNA topoisomerases are structurally and evolutionarily related (Lynn et al., 1986; Uemura et al., 1986; Wyckoff et al., 1989; Huang, 1990; Caron and Wang, 1993). How a type II DNA topoisomerase couples ATP binding and hydrolysis to the transport of DNA segments through each other is a key question in mechanistic studies of this class of enzymes. We have recently used highly purified type II DNA topoisomerase from the budding yeast *Saccharomyces cerevisiae* in our mechanistic studies of this class of enzymes. *S. cerevisiae* DNA topoisomerase II is a typical eukaryotic type II DNA topoisomerase, and each polypeptide of the homodimeric enzyme (Goto et al., 1984) is 1,429 amino acids long (Giaever et al., 1986). From a comparison of the amino acid sequences of the yeast enzyme and *Escherichia coli* DNA gyrase (DNA topoisomerase II), it can be inferred that the ATPase domain of the yeast enzyme is contained within the amino-terminal 400 amino acids. This inference is based mainly on the sequence homology (Lynn et al., 1986) and the known crystal structure of a 43-kDa amino-terminal fragment of *E. coli* gyrase B-subunit complexed with a nonhydrolyzable ATP analogue AMPPNP† (Wigley et al., 1991). The active site tyrosine involved in DNA brekage and rejoicing is Tyr783 of the yeast enzyme (Worland and Wang, 1989), which corresponds to Tyr-122 of *E. coli* gyrase A-subunit (Horowitz and Wang, 1987). Recently, proteolysis studies of yeast DNA topoisomerase II by SV8 endoprotease in the presence and absence of AMPPNP or ATPγS, another nonhydrolyzable ATP analogue, have provided evidence that there are allosteric interdomainal movements in the enzyme following the binding of ATP (Lindsley and Wang, 1991). Furthermore, results on the binding of different forms of DNA to the yeast enzyme and its AMPPNP complex have led to a model in which a type II DNA topoisomerase, either free or bound to a DNA segment, acts as an ATP-dependent protein clamp: in the absence of ATP, the jaws of the clamp are open and a second DNA segment can enter the channel between them; the closure of the jaws upon ATP binding traps the second DNA segment and transports it through the first DNA segment (Roca and Wang, 1992).

To obtain a clearer picture of the roles of ATP in type II DNA topoisomerase-catalyzed reactions we have measured the number (n) of ATP molecules hydrolyzed/DNA transport event catalyzed by purified yeast DNA topoisomerase II. There have already been several measurements of n in the literature. *A priori,* it is reasonable to assume that n is greater than 1 and can easily be 2, as there are two ATPase sites/enzyme molecule. Liu et al. (1979) estimated that n is 1–2 for phage T4 DNA topoisomerase, a type II enzyme mechanistically very similar to eukaryotic DNA topoisomerase II. A

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†The abbreviations used are: AMPPNP, adenosine 5'-O-(thio)triphosphate; ATPγS, adenosine 5'-3-O-(thio)triphosphate; kb, kilobase(s).
value of $n$ around 0.8 was arrived at from a more extensive set of measurements with E. coli DNA gyrase (Sugino and Cozzarelli, 1980). Because bacterial gyrase, unlike eukaryotic DNA topoisomerase II or phage T4 DNA topoisomerase, catalyzes the endergonic negative supercoiling of DNA, the level of ATP usage can also be estimated from thermodynamic considerations of the endergonic DNA supercoiling reaction and the exergonic ATP hydrolysis reaction; at the maximal attainable level of DNA negative supercoiling by E. coli gyrase, $n$ was estimated to be about 2 (Sugino and Cozzarelli, 1980; Tamura et al., 1992). For Drosophila DNA topoisomerase II, which, like all other eukaryotic type II and T-even phage DNA topoisomerases, catalyzes the ATP-dependent relaxation of supercoiled DNA, about eight ATP molecules were found to be hydrolyzed/DNA transport event (Osheroff et al., 1983).

Whereas these earlier measurements and estimates were in general agreement within a factor of 10, there was a fundamental problem in the calculation of $n$ from these measurements. Because of the relatively high turnover number for type II DNA topoisomerase-catalyzed DNA transport, of the order of $1 \times 10^3$ (Higgins et al., 1978; Sugino and Cozzarelli, 1980; Osheroff et al., 1983; Maxwell and Gellert, 1984; Baker et al., 1987), all experiments cited above were carried out with a low molar ratio of enzyme to DNA molecules so that the net reaction would continue for at least several minutes to permit manual measurements. Under these conditions, however, the processive nature of the DNA-bound enzyme predicts that the linking number of a DNA with a bound enzyme would reach that of the final product in seconds; thus the measured total linking number change for the entire population of DNA molecules may depend strongly on how fast a DNA-bound enzyme can dissociate and reassociate with a different DNA molecule. The rate of linking number change measured under these conditions thus may have little to do with the enzyme-catalyzed rate of DNA transport. Theoretical estimates based on free energy considerations are not subject to this complication; these estimates can only set a minimal value for $n$ for the bacterial gyrase-catalyzed DNA supercoiling reaction, however, and even this minimal estimate is valid only at the maximal level of DNA negative supercoiling attainable.

In the measurements reported here, we have employed conditions that avoid the complication described above. Our results indicate that when the ATP concentration is in the $\mu M$ range, the slow step in the yeast DNA topoisomerase II-catalyzed reaction pathway is probably ATP binding or hydrolysis, and $n$ is $1.9 \pm 0.5$; when ATP is in excess, the rate of DNA transport is considerably slower than the rate of ATP hydrolysis, and $n$ is $7.4 \pm 1.0$. Through the use of ATPase mutants of yeast DNA topoisomerase II, in which Gly-144 of the wild-type enzyme is replaced by an isoleucine, valine, or proline, we show also that mutational inactivation of the ATPase, similar to the omission of ATP in the reaction with the wild-type enzyme, completely blocks the DNA transport activity. In terms of the ATP-modulated protein clamp model (Roca and Wang, 1992), these results suggest that conformational changes in the protein, which are associated with the closure of the clamp triggered by ATP binding, are responsible for the capture and transport of a DNA segment through the DNA gate operated by the same enzyme in another DNA segment; the rates of opening and closing of the protein clamp are likely to increase with increasing ATP concentration, but at a high ATP concentration a protein clamp may often close without trapping a DNA segment for transport.

### Experimental Procedures

**Materials**—DNA oligonucleotides used for site-directed mutagenesis were purchased from Amber. The oligonucleotide sequences and the mutations they were designed to introduce are: 5'-AGAAA(TAT)TTTATGTTG-3', Gly144Ile or G144I; 5'-ACTGTTGTAGAAC(CCA)TATGGTCAATGCTT-3', Gly144Pro or G144P; 5'-TAGAAC(TG)TTATGGTG-3', Gly144Val or G144V; 5'-GATGATGATGAGAAGGC(TCT)TGATGGTGATGAGA-3', Gly137Ala or K137A; 5'-GTCATCCTATTATCG(T)CTGCCTAT-3', Gly144Ile or G144I; 5'-GCAAAGGAGACGAGCCTGACCTATTTATGAGTGGTCTGC-3' and 5'-ACGCTTCTCTATGAGTGCATCTTATTATGAGTGGTCTGC-3', were purchased from Operon Technologies. These two oligonucleotides were designed to form a 40-base pair duplex with short hairpin loops at the ends. Other reagents were purchased from commercial sources as described below: ATP, ADP, AMPPNP, and E. coli DNA ligase, Boehringer Mannheim; E. coli DNA polymerase I, GIBCO-BRL; pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, NADH, and NAD, Sigma; pBlueScript DNA, Stratagene.

**Expression of Wild-type and Mutant S. cerevisiae DNA Topoisomerase II**—Wild-type yeast DNA topoisomerase II was overexpressed in yeast from the inducible promoter PGAL1 in a monocopy expression plasmid YEpTOP2-PGAL1, as described (Worland and Wang, 1989). The BamHI to KpnI fragment from YEpTOP2-PGAL1, encoding approximately the amino-terminal quarter of the enzyme, was first subcloned into pBluescript KS+; site-directed mutagenesis was carried out with this subclone using an oligonucleotide-directed in vitro mutagenesis kit (Amersham version 2). Each of the mutant BamHI to KpnI regions was sequenced to confirm the intended nucleotide changes and cloned back into YEpTOP2-PGAL1 for overexpression. The ability of mutant top2 to complement a conditional lethal top2 allele (Holm et al., 1985) was tested by transforming a top2-4 strain CH1106 cells (Gartenberg and Wang, 1992) with the mutant versions of YEpTOP2-PGAL1. Colonies of transformants were tested for growth at 35 °C, a nonpermissive temperature for untransformed CH1106 cells, on minimal agar plates with 2% glucose and no uracil. Even in the presence of 2% glucose, there is sufficient expression of the PGAL1-linked wild-type TOP2 gene to complement the thermal sensitive product of the top2-4 mutant allele.

Expression vectors were transformed into the yeast strain JEL1 (a leu2 trpl ura3-52 pdr1-1122 pep4 Δhis3:PGAL1-GAL4). In this strain, the expression of a mutant protein is also induced by galactose; the higher level of the GAL4 protein upon induction with galactose in turn improves the expression of genes linked to GAL4-activated promoters such as PGAL1 (Schultz et al., 1987). Strain JEL1 was derived from the protease deficient strain NKY879 (a leu2 trpl ura3-52 pdr1-1122 pep4) by the use of an integrating plasmid pHINT-C, encoding approximately the amino-terminal quarter of the enzyme, into the HIS3 sequences (a kind gift of Dr. James E. Hopper, Hershey Medical School). Integration of the plasmid into the chromosomal HIS3 locus inactivates the HIS3 gene and introduces a URA3 marker, which is present on the integration plasmid; the URA3 marker was subsequently disrupted by targeted gene replacement (Rothstein, 1983), using a ΔURA3 gene missing 250 base pairs in the middle section of the marker gene, to restore JEL1 to ura- for transformation with the URA3-marked overexpression plasmids. The levels of expression of DNA topoisomerase II from YEpTOP2-PGAL1 are typically 5–10 times higher in the JEL1 strain than that in the NKY879 parent strain (data not shown). Cell growth and induction with galactose were done as described previously (Worland and Wang, 1989).

Protein purification was done essentially as described (Worland and Wang, 1989), except that two additional protease inhibitors, leupeptin (0.5 μg/ml) and pepstatin (0.7 μg/ml), were included in all buffers and dialysis buffers. In addition, the crude extract was dialyzed against a high concentration of ATP molecules hydrolyzed/DNA transport event, the protein was further purified on an high pressure liquid chromatography MATQ anion exchange column (Bio-Rad). Yeast DNA topoisomerase II came off at 0.36–0.4 M KCl upon eluting the column with a 0.15–0.5 M KCl linear gradient. All proteins were stored at −70 °C and protein concentrations of 1 mg/ml buffer, in 20% glycerol for 1 month, 0.5% HCl (pH 7.5), 150 mM KCl, and 5 mM 2-mercaptoethanol. All experiments were done with preparations stored for no more than 3 months.
Protein concentrations were determined with a Coomassie Plus protein assay reagent (Pierce), using bovine serum albumin as a standard.

**Enzyme Assays**—The standard reaction buffer consisted of 50 mM Tris acetate (pH 7.8), 150 mM potassium acetate, 6 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 250 μg/ml bovine serum albumin (BSA). In the determination of the number of ATP hydrolyzed/DNA translocated, reactions were carried out in 750 μl at 37 °C. The reaction was stopped by the addition of EDTA to 50 mM, and then 50 μl of samples were loaded on a 15% nondenaturing polyacrylamide gel. Electrophoresis was performed in TBE buffer at 250 V for 3 h. The protein/DNA molecule concentration was found to be about one-third to one-half of the total protein concentration. In most of the experiments, the active enzyme concentration was found to be about one-third to one-half of the total protein concentration. In most of the experiments, the active enzyme concentration was found to be about one-third to one-half of the total protein concentration.

**ATPase Assays**—The ATPase activity of yeast DNA topoisomerase II was measured by two methods. In the spectrophotometric assay, rapid conversion of ADP back to ATP by pyruvate kinase and phosphoroclypuryvate, a reaction coupled to NADH oxidation, was used to measure the rate of ATP hydrolysis; the procedure described in Morricci et al. (1986) was followed (see also Tamura and Gelbart, 1990). In this assay, the concentration of ATP remains at its initial concentration. Each reaction mixture (1 ml) contained 2 mM phosphorylpyruvate, 5 units of pyruvate kinase, 8 units of lactate dehydrogenase, and 0.16 mM NADH in addition to yeast DNA topoisomerase II, DNA, and ATP, as indicated in the appropriate figure legends, and the reaction temperature was 30 °C unless otherwise indicated.

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**Determination of the Number of ATP Molecules Hydrolyzed/DNA Transport Event**—Purified, supercoiled pBluescript KS+ DNA (50 μg) was nicked by pancreatic DNase I in the presence of excess endonuclease HindIII (Promega, Madison, WI) and 10 μg/ml bovine serum albumin (BSA) for 2 h at 37 °C. The reaction was stopped by the addition of EDTA to 50 mM, and the reaction was purified by two phenol-chloroform extractions followed by ethanol precipitation. A coupled nick translation/ligation reaction of phosphoethidium bromide density gradient centrifugation (Radloff et al., 1987). Less than 10% of the total DNA used in these reactions was nicked, and therefore no correction was made for this contaminant in the quantitation of the rate of relaxation of the DNA.

Two series of reactions were carried out. When the rate of ATP hydrolysis was followed spectrophotometrically, ATP hydrolysis and DNA linking number measurements were performed separately but always on the same day. The DNA used in the ATPase assays contained no radiolabeled tracer, and the DNA relaxation experiment was carried out in a smaller volume; otherwise the two sets of measurements were carried out exactly as described above. When the ATPase rate was followed by TLC, the same reaction mixture was used in both the ATPase assays and the DNA relaxation measurements; reactions were performed in the standard buffer except that the potassium acetate concentration was 120 mM and that magnesium acetate was added (to 8 mM final concentration) with ATP to initiate the reactions. Topoisomerase and DNA concentrations used are indicated in the table and figure legends. Prior to these experiments, the fraction of active topoisomerase in each protein preparation was determined by titrating 20 nM supercoiled DNA with topoisomerase under highly processive reaction conditions (50 instead of 120 mM potassium acetate). Assuming a Poisson distribution of protein dimers/plasmid, the concentration of protein that relaxes 64% of the plasmids was taken to correspond to one active protein/DNA molecule. In most of the enzyme preparations used, the active enzyme concentration was found to be about one-third to one-half of the total protein concentration. In most of the enzyme preparations used, the active enzyme concentration was found to be about one-third to one-half of the total protein concentration. In most of the enzyme preparations used, the active enzyme concentration was found to be about one-third to one-half of the total protein concentration.
RESULTS

DNA-independent ATPase Activity of Yeast DNA Topoisomerase II—Purified yeast DNA topoisomerase II exhibits a weak but readily detectable ATPase activity in the absence of DNA. Hydrolysis of ATP by this activity is adequately represented by Michaelis-Menten kinetics, as shown by the linear $v/[\text{ATP}]$ versus $v$ plot depicted in the inset of Fig. 1 ($v$ being the rate of ATP hydrolysis). From the data shown, which were obtained from four independent sets of measurements using the same enzyme preparation, values for the maximal velocity $V_{\text{max}}$ and the Michaelis constant $K_m$ are calculated to be 1.1 $\mu$M/min and 0.3 mM, respectively. From the total amount of yeast enzyme in the reaction mixture, the apparent turnover number $k_{\text{cat}}$ can be estimated to be 0.4/s/dimeric enzyme at 30 °C (in a medium containing 50 mM Tris acetate (pH 7.8), 150 mM potassium acetate, 6 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 250 $\mu$g/ml bovine serum albumin). Comparable $V_{\text{max}}$ and $K_m$ values were obtained with a different preparation of the same enzyme. The absolute $k_{\text{cat}}$ value is likely to be two to three times higher or around 1/s/dimeric enzyme, as estimated from the fraction of active enzyme in a typical preparation (see "Experimental Procedures"). As will be discussed in a later section, this DNA-independent ATPase activity is not caused by a contaminating ATPase in the enzyme preparations but is largely intrinsic to the topoisomerase.

Strong Dependence of the ATPase Activity of Yeast DNA Topoisomerase II on DNA and Cooperativity between the two ATPase Sites in a DNA-bound Enzyme—The addition of DNA to yeast DNA topoisomerase II greatly stimulates the ATPase activity, as expected from previous studies with other type II DNA topoisomerases (see for example Mizuuchi et al., 1978; Liu et al., 1979; Osheroff et al., 1983). At a total enzyme concentration of 50 nM, half-maximal stimulation of ATPase by DNA was reached at 5 $\mu$M base pairs or an enzyme dimer to DNA base pair ratio of 1:100 (data not shown). No difference in the rate of ATP hydrolysis was observed in the presence of 30 $\mu$M (in base pairs) of negatively supercoiled, linear, or relaxed covalently closed DNA; this lack of dependence on the topological form of the input DNA effector is expected, as a supercoiled DNA would be completely relaxed in the first few seconds under these conditions (see below).

In reactions used to determine the steady-state kinetic constants of DNA-dependent hydrolysis of ATP by yeast DNA topoisomerase II, the dimeric enzyme to DNA base pair ratio was set at 1:600 to ensure DNA excess and a maximal level of ATPase stimulation. In Fig. 1, the rate $v$ of ATP hydrolysis is plotted as a function of [ATP] for four sets of experiments carried out at 30 °C and in the 150 mM potassium acetate medium described above. The maximal velocity, $V_{\text{max}}$, at high ATP concentrations is approximately 21 $\mu$M min$^{-1}$, which is 20 times higher than the value 1.1 $\mu$M min$^{-1}$ in the absence of DNA. Significantly, the $v$ versus [ATP] plot is sigmoidal in shape in the presence of DNA, suggesting cooperativity in the ATPase kinetics (Fig. 1). Deviation from Michaelis-Menten kinetics is also evident when $v/[\text{ATP}]$ is plotted versus [ATP], which shows distinct curvature at ATP concentrations below 125 $\mu$M (plot not shown). Digestion of the DNA with AluI and HhaI restriction endonucleases, so as to generate DNA fragments shorter than 400 base pairs, had no effect on the sigmoidal shape of the curve shown in Fig. 1. Thus the cooperativity is probably a result of interaction between the two ATPase sites in each DNA-bound dimeric enzyme rather than interaction between two dimeric enzyme molecules bound to the same DNA fragment.

The experimental ATPase data in the presence of excess DNA can be fitted by a simple model in which the rate of hydrolysis of ATP occupying an ATPase catalytic pocket is the same whether one or both pockets of the enzyme dimer are occupied, but the dissociation constant is $K_1$ for E·ATP and $K_2$ for E·(ATP)$_2$ (see Segel, 1975). The curve drawn in Fig. 1 is the one calculated for this model with a value of $r = 0.01$. However, because $v$ is sensitive to the value of $r$ only at low ATP concentrations at which a significant fraction of the enzyme is in the E·ATP form, the relatively large experimental error in $v$ when [ATP] is low makes it difficult to evaluate the cooperativity parameter accurately; the value $r = 0.01$ should therefore be viewed as an order of magnitude estimate. It is straightforward to show that $K_1 = [\text{ATP}]_{0.5}/r^{1/2}$, where [ATP]$_{0.5}$ is the ATP concentration at half-maximal velocity; from the data shown in Fig. 1, $K_1$ is estimated to be about 1.2 mM.

Number of ATP Hydrolyzed/DNA Transport Event Catalyzed by Yeast DNA Topoisomerase II—Because the DNA-
dependent ATPase activity of the enzyme does not require the transport of DNA through the DNA gate, the value of n, the number of ATP hydrolyzed/DNA transport event catalyzed by a type II DNA topoisomerase, is mechanistically meaningful only under conditions when DNA transport readily accompanies ATP hydrolysis; otherwise n would approach infinity. Furthermore, as described in the Introduction, n must be measured under conditions such that the rate-limiting step is not the dissociation of a DNA-bound enzyme or its reassociation with a different DNA substrate. At a turnover rate of DNA transport around 1/s/enzyme molecule, a typical 5-kb supercoiled DNA with a specific linking difference of -0.06 would be relaxed by a single bound enzyme in about 15 s. To slow down this rate without resorting to the use of a low enzyme to DNA molar ratio, which may make the dissociation of a DNA-bound enzyme or its reassociation with a different DNA the rate-limiting step, we either lowered the temperature or the ATP concentration.

There is a technical problem in measuring the DNA linking number change by a type II DNA topoisomerase under conditions such that the enzyme is processive and that the enzyme to DNA molar ratio is around 1. Because the number of enzyme molecules/DNA ring follows a Poisson distribution, under these conditions different DNA molecules are relaxed at different rates, and the linking number distribution of the DNA substrate becomes very broad shortly after its incubation with the enzyme, which makes the quantitation of the linking number changes more difficult. To overcome this problem, we used two-dimensional agarose-gel electrophoresis to resolve all 32P-labeled DNA topoisomers of different linking numbers; the overall linking number change was then calculated by quantitating the relative amounts of all DNA topoisomers using a Phospho-Imager (see “Experimental Procedures”).

Fig. 2 depicts a typical experiment on the rate of relaxation of a negatively supercoiled DNA by yeast DNA topoisomerase II at 7 °C and a saturating level of ATP (1 mM). In this experiment a negatively supercoiled DNA was incubated with the yeast enzyme, and aliquots of the reaction mixture were sampled at various times for the analysis of DNA linking number distributions by two-dimensional agarose-gel electrophoresis. The rate of ATP hydrolysis was followed spectrophotometrically through an NADH-coupled reaction (see “Experimental Procedures”) in a parallel experiment carried out under conditions identical to those of the DNA relaxation experiment. The rate of ATP hydrolysis remained constant during the entire time course, and no significant change in the rate was detectable as the DNA became progressively less negatively supercoiled. It was reported previously that ATP hydrolysis by Drosophila DNA topoisomerase II was four times faster in the presence of a negatively supercoiled rather than a linear or relaxed DNA, under otherwise identical conditions (Osheroff et al., 1983). The half-time for the removal of negative supercoils in the experiment shown in Fig. 2 can be estimated to be about 1 min (see below), and a 4-fold drop in the rate of ATP hydrolysis in the first couple of minutes would have been detectable.

From this pair of experiments the rate of ATP hydrolysis was calculated to be 43 nM s⁻¹, and the rate of DNA transport at time zero was calculated to be 5 nM s⁻¹; the ratio of the rates indicates that 8.6 ATP are hydrolyzed/DNA transport event under the experimental conditions employed. From the concentration of active enzyme and the concentration of DNA rings in the reaction mixture, the measured rates corresponds to 1.3 ATP hydrolyzed/s/dimeric enzyme and 0.15 DNA transport event/s/dimeric enzyme. These results and those from similar experiments, measured at a saturating ATP concentration of 1 mM and temperatures from 7 to 17.5 °C, are tabulated in Table I. The average number of ATP hydrolyzed/DNA transport event, n, is 7.4 ± 1, where the error indicated is for a particular measurement of n. The Arrhenius activation energy is estimated to be 20 and 24 kcal mol⁻¹, respectively, for DNA transport and ATP hydrolysis from these data; the difference between these values is insignificant.
TABLE I
Rates of yeast DNA topoisomerase II-catalyzed ATP hydrolysis and DNA transport at low temperatures

Reactions were performed at the indicated temperatures in the standard reaction buffer, as described under “Experimental Procedures.” Each reaction contained 100 nM enzyme dimers (one-third of which was active), 33 nM pBluescript monomeric DNA, and 1 nM ATP. ATP hydrolysis was monitored spectrophotometrically. The experimentally measured rates of linking number changes were divided by 2 and the active enzyme concentration to give the rates of DNA transport/enzyme; similarly, the rates of ATP hydrolysis were expressed in units of ATP hydrolyzed/s/active enzyme. The quantity n is the number of ATP hydrolyzed/DNA transport event and is obtained directly from the ratio of the rates; this quantity is independent of the fraction of active enzyme molecules in the preparation, as the same preparation was used in both rate measurements.

| Reaction | Temperature | DNA transport | ATP hydrolyzed | n |
|----------|-------------|---------------|----------------|---|
| °C       | events s⁻¹  | s⁻¹ enzyme⁻¹  |                |   |
| 1        | 7           | 0.15          | 1.30           | 8.6 |
| 2        | 9.5         | 0.30          | 2.15           | 7.1 |
| 3        | 10          | 0.53          | 2.12           | 6.4 |
| 4        | 12          | 0.45          | 3.10           | 6.8 |
| 5        | 14.5        | 0.51          | 4.54           | 7.1 |
| 6        | 17.5        | 0.73          | 6.00           | 8.3 |

**Fig. 3.** Simultaneous determination of the rates of ATP hydrolysis and DNA relaxation from a reaction at 30 °C with 10 μM ATP. A reaction containing 150 nM [³²P]-labeled pBluescript DNA, with an average of 20 negative supercoils/plasmid, and 400 nM topoisomerase II dimer in a total volume of 200 μl was started by the simultaneous addition of magnesium acetate (to 8 mM final), ATP (to 10 μM final), and [α-³²P]ATP (10 nM final). For the zero time point, an aliquot of the reaction without Mg(II) and ATP was simultaneously mixed with magnesium acetate and ATP and a stop solution (to give 0.5% sodium dodecyl sulfate and 25 mM EDTA final concentration). All other points were taken by removing aliquots from the original reaction and quenching with the stop solution at the indicated times. The concentration of ATP hydrolyzed at each time point was measured by the TLC method as described under “Experimental Procedures.” Three two-dimensional agarose gels were used to separate the topoisomers of all quenched samples. Electrophoresis and quantitation of topoisomers were performed as described under “Experimental Procedures.”

relative to the uncertainties in the experimental data.

In a second set of measurements, low ATP concentrations in the range of 5–25 μM were employed, and the yeast DNA topoisomerase II-catalyzed reduction in ATP concentration was measured at 30 °C either spectrophotometrically as described above or by thin layer chromatographic analysis of [α-³²P]ATP in the reaction mixture. The results of a typical experiment by TLC analysis of radiolabeled ATP are shown in Fig. 3. In such an experiment, ATP was not regenerated, and its depletion was responsible for the nonlinearity of the plot of [ATP] hydrolyzed versus time. From the ratio of the slopes of the two curves shown in Fig. 3 as time approaches zero, n is calculated to be 2.6. Data from the low [ATP] set of measurements are listed in Table II; the average value of n is 1.9 ± 0.5 from this data set.

**Mutations in the ATP Binding Domain of Yeast DNA Topoisomerase II and the Absolute Dependence of DNA Passage on ATP**—Several mutations were introduced into a putative ATP binding region of yeast DNA topoisomerase II. Table III summarizes the results obtained for five mutants with amino acid substitutions within a short stretch of yeast DNA topoisomerase II, from Asn-130 to Gly-146; the counterpart of this stretch in E. coli GyrB protein, from Lys-103 to Gly-119, appears to be involved in ATP binding (Tamura and Gellert, 1990; Wigley et al., 1991). Expression of a plasmid-borne K137A mutant top2, in which Lys-137 in the wild-type codon is replaced by alanine codon, or the D132–134A mutant top2, in which the three aspartic acid codons 132–134 are replaced by alanines, was found to complement a temperature-sensitive yeast top2 strain carrying a top2–4 mutation in the chromosomal copy of the gene. The lethal phenotype of the top2–4 strain at 35 °C was not rescued, however, by the expression of the G144I, G144V, or G144P

**TABLE II**
Rates of topoisomerase II-catalyzed ATP hydrolysis and DNA transport at low concentrations of ATP

Reactions were performed at 30 °C in the standard reaction buffer, as described under “Experimental Procedures.” The molar ratio of active enzyme dimer to pBluescript monomeric DNA was approximately 1 in all reactions except reaction 6, for which the ratio was about 0.5; the pBluescript DNA concentration was 150 nM (monomer rings) for reaction 5 and 66 nM for all others. The asterisk denotes reactions for which ATP hydrolysis was measured by TLC; otherwise the rates were followed spectrophotometrically. See the Table I legend for the conversion of the experimentally measured rates to those in units indicated in the table and the calculation of n, the number of ATP hydrolyzed/DNA transport event.

| Reaction | [ATP] | DNA transport | ATP hydrolyzed | n |
|----------|-------|---------------|----------------|---|
| μM      | events s⁻¹ enzyme⁻¹ | s⁻¹ enzyme⁻¹ |                |   |
| 1        | 5     | 0.045         | 0.061          | 1.3 |
| 2        | 10    | 0.24          | 0.29           | 1.2 |
| 3*       | 10    | 0.15          | 0.32           | 2.1 |
| 4*       | 10    | 0.12          | 0.29           | 2.4 |
| 5        | 10    | 0.11          | 0.27           | 2.6 |
| 6        | 25    | 0.58          | 0.97           | 1.7 |
| 7        | 25    | 0.61          | 1.03           | 1.7 |

**TABLE III**
Mutations in a predicted ATP binding region of yeast DNA topoisomerase II

Amino acids 130–146 of the yeast enzyme are shown. Boldface letters represent amino acids that are conserved among all or most of the type II DNA topoisomerases of known sequences; in the regions of other type II DNA topoisomerases corresponding to the DDD triplet shown in the yeast enzyme sequence, there is at least one aspartic acid. Bold underlined letters represent the amino acids that have been altered by site-directed mutagenesis. The ability of the enzyme to complement a temperature-sensitive top2–4 mutant allele at 35 °C when expressed from a multicopy plasmid is indicated. The activity of the enzyme in crude lysates of yeast cells overexpressing it was determined by the DNA unknotted assay (Liu et al., 1981).

| Enzyme | Amino acid sequence | Complementation | Activity |
|--------|---------------------|-----------------|----------|
| Wild-type | NTIDDEKERVTVGRNRYG | +               | +        |
| G114I | NTIDDEKERVTVGRNLYG | -               | -        |
| G144P | NTIDDEKERVTVGRNRFY | -               | -        |
| G144V | NTIDDEKERVTVGRNFGY | -               | -        |
| K137A | NTIDDEKERVTVGRNYG  | +               | +        |
| D132–134A3 | NTAAEKERVTVGRNYG | +               | +        |
The purified Gly-144 series of mutant enzymes showed no ATP-independent or ATP-dependent activity in the relaxation of supercoiled DNA. A comparison of the rates of relaxation of negatively supercoiled DNA by wild-type and G144I mutant enzyme is shown in Fig. 5. In the presence of ATP, the wild-type topoisomerase relaxed fully half of the supercoiled plasmids within 30 s. There was no detectable relaxation by the mutant protein, however, after 60 min of incubation. Similar results were obtained for G144V and G144P mutants (data not shown). In all cases, the relaxation activity of the wild-type topoisomerase was more than 100-fold higher than that of mutant enzymes. The same result was obtained by measuring the topoisomerase II-catalyzed unknotting of knotted phage P4 DNA rings (data not shown). Both wild-type and mutant enzyme preparations contained a low level of a contaminating endonuclease, and after prolonged incubation there was an increase of nicked DNA (Fig. 5).

In contrast to the elimination of the DNA transport activity, replacing Gly-144 by isoleucine, valine, or proline does not appear to abolish the DNA breakage and rejoining activity of the type II enzyme. Fig. 6 illustrates DNA cleavage by wild-type and mutant yeast DNA topoisomerase II in the presence of the eukaryotic type II DNA topoisomerase drug etoposide (VP-16). In the absence of the enzyme, little cleavage of the DNA was observable (lane 1). When 500 nM wild-type topoisomerase II was included in a reaction mixture containing VP-16, approximately 50% of the plasmid DNA was nicked or linearized (lane 4). The same level of DNA cleavage was found for each of the Gly-144 mutant enzymes (lanes 7, 10, and 13).

We have also examined the effects of ATP, ADP, and AMPPPNP on the cleavage of a DNA oligomer by wild-type and mutant yeast DNA topoisomerase II in media containing Ca(II). It has been reported previously that Ca(II) favors the trapping of covalent DNA-type II topoisomerase-covalent complex (Osheroff and Zechiedrich, 1987) and that ATP and AMPPPNP stimulate DNA cleavage by eukaryotic DNA topoisomerase II (Sander and Hsieh, 1983; Robinson and Osheroff, 1991). As shown in Fig. 7, for either wild-type or G144I mutant yeast DNA topoisomerase II, replacing Mg(II) by Ca(II) stimulates the cleavage of the DNA by several orders of magnitude (compare the patterns in lanes 1 and 2 for the wild-type enzyme and lanes 7 and 8 for the G144I mutant enzyme). In a medium containing a 4 mM concentration each of Ca(II) and Mg(II), cleavage of the DNA oligomer by either the wild-type or the G144I mutant enzyme was readily observable (lanes 3 and 9). As expected, however, stimulation of yeast DNA topoisomerase II-mediated cleavage of DNA by ATP, ADP, or AMPPPNP was only observed with the wild-type enzyme (lanes 4–6) and not with the G144I mutant enzyme (lanes 10–12). Measurements of protein-mediated retention of DNA to nitrocellulose membranes (Riggs et al., 1970) indicate that the presence of ATP, ADP, or AMPPPNP did not significantly alter the fraction of yeast DNA topoisomerase II bound to the DNA oligomer under the reaction conditions of the samples shown in Fig. 7 (data not shown).

**Discussion**

The experiments described above indicate that in the absence of DNA highly purified yeast DNA topoisomerase II has a weak but readily detectable ATPase activity. This activity exhibits the classical Michaelis-Menten kinetics, with $K_m$ and $v_{max}$ values around 0.3 mM and 1/s/dimeric enzyme, respectively, at 30 °C in a pH 7.5 medium containing 150 mM potassium and 6 mM magnesium acetate. Binding the enzyme to DNA greatly enhances its ATPase activity; in the presence...
FIG. 5. Relaxation of negatively supercoiled DNA by wild-type and G144I yeast DNA topoisomerase II. Supercoiled DNA substrate and relaxed DNA products were resolved by electrophoresis in a 1% agarose gel. Reactions contained 50 nM pH6824 supercoiled DNA, 80 nM topoisomerase, and 0 or 1 mM ATP indicated by the minus or plus sign above each lane, in the standard reaction buffer. The reactions were stopped at the times indicated above the lanes (in min) by the addition of sodium dodecyl sulfate to 0.5% and EDTA to 25 mM.

FIG. 6. Etoposide (VP-16) enhanced DNA cleavage by wild-type and Gly-144 mutant yeast DNA topoisomerase II. Electrophoresis in a 1% agarose gel was used to separate the supercoiled DNA substrate from the nicked and linear products. Supercoiled pH624 (100 nM plasmid), VP-16 (500 µg/ml where indicated), and the indicated concentration of topoisomerase I1 (dimer) were incubated at 30 °C for 10 min in the standard reaction buffer without bovine serum albumin. Sodium dodecyl sulfate was added to a final concentration of 0.5%, and the reaction was incubated for an additional 10 min. Proteinase K (200 µg/ml final concentration) and EDTA (10 mM final concentration) were subsequently added, and the reactions were incubated at 55 °C for 1 h; deproteination was then carried out as described under "Experimental Procedures" prior to loading of the samples for gel electrophoresis.

FIG. 7. ATP, ADP, and AMPPNP stimulation of DNA cleavage by wild-type and Gly-144 mutant yeast DNA topoisomerase II. A 15% nondenaturing polyacrylamide gel was used to separate the substrate DNA oligomer from its faster migrating cleavage products. Only double-stranded DNA cleavage events were detected. Reactions contained 2 µM DNA (oligonucleotide), 2 µM topoisomerase (dimer), and either no added nucleotide (lanes 1-3 and 7-9) or 1 mM ATP (lanes 4 and 10), ADP (lanes 5 and 11), or AMPPNP (lanes 6 and 12). The reactions were in the standard buffer with either 8 mM calcium acetate (lanes 1 and 7), 8 mM magnesium acetate (lanes 2 and 8), or 4 mM calcium acetate and 4 mM magnesium acetate (lanes 3-6 and 9-12). Reactions were incubated for 30 min at 30 °C before stopping with EDTA (25 mM final concentration) and sodium dodecyl sulfate (0.5% final concentration) and digestion with 200 µg/ml of proteinase K at 55 °C for 1 h.

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of excess DNA, $k_{cat}$ is increased 20-fold to about 20/s/dimeric enzyme. The rate of ATP hydrolysis by yeast DNA topoisomerase II in the presence of DNA is no longer represented by the Michaelis-Menten equation; instead, ATP binding to the two ATPase sites in each DNA-bound dimeric enzyme appears to be cooperative.

This homotropic cooperativity ensures the coordination between the two halves of a DNA-bound dimeric enzyme: when ATP concentration is above a couple of tenth mM, essentially all enzyme molecules have either both of the ATPase sites occupied or unoccupied. Because a single mutation changing Gly-144 of the yeast enzyme to isoleucine, valine, or proline greatly diminishes both the ATPase activity in the presence of DNA and the ATPase activity in the absence of DNA, both activities are most likely manifestations of the same ATPase catalytic pockets. The cooperativity between the pair of ATPase sites in a DNA-bound dimeric enzyme, but not in a free enzyme, is probably because of ATP-mediated conformational changes in the DNA-enzyme complex. Previously, a similar suggestion was made based on the stimulation of AMPPNP binding by ATP to DNA-bound but not to free E. coli gyrase (Tamura et al., 1992).

The above interpretation poses an apparent dilemma. Using mixed dimeric yeast DNA topoisomerase II consisting of one wild-type and one epitopically tagged G144I mutant polypeptide, we have shown that the binding of AMPPNP to the wild-type polypeptide can induce the same concerted confor-
matical change in the entire molecule, whether the enzyme is DNA-bound or not (Lindsley and Wang, 1991, 1993). This AMPPNP-induced concerted conformational change in the DNA-bound or free enzyme seems to contradict the idea of an ATP-mediated conformational change only in the DNA-bound enzyme. It is plausible, however, that this apparent dilemma reflects differences in the rates of the various ATP-dependent steps in the presence and absence of DNA: in the absence of DNA, for example, conformational change in the enzyme induced by ATP binding might be too slow relative to ATP-hydrolysis, in which case no homotropic cooperativity would be observable; on the other hand, with a nonhydrolyzable ATP analogue, even a very slow conformational change can be easily detected.

Not only is the ATPase of yeast DNA topoisomerase II inactivated by mutations changing Gly-144 to isoleucine, valine, or proline, the DNA transport activity of the enzyme is also abolished. These results demonstrate a striking dependence of DNA transport by the eukaryotic type II enzyme on ATP, in contrast to the ATPase activity of the enzyme, which, although enhanced by the binding of the enzyme to DNA, does not appear to require the transport of DNA through the enzyme-operated DNA gates. According to the protein clamp model described in the Introduction, a key step in a type II DNA topoisomerase-catalyzed DNA transport is the ATP binding-triggered closure of the DNA-bound clamp for the capture of a second DNA segment, termed the T-segment; conformational changes in the protein clamp in its closed state promote the transport of the T-segment through the DNA gate operated by the same enzyme molecule in the other DNA segment, termed the G-segment (Roca and Wang, 1992). Without the conformational changes triggered by ATP binding, enzyme-catalyzed transport of the T-segment through the G-segment is too slow to be detectable, as indicated by our data for the three Gly-144 mutants.

Further insights on the mechanism of coupling DNA transport to ATP hydrolysis by yeast DNA topoisomerase II are gained from the ratio of the rates of these processes measured under identical conditions. At 30 °C and low ATP concentrations, 1.9 ± 0.5 ATP are hydrolyzed/DNA transport event. It is likely that under these conditions, the rate-limiting step in ATP usage is slower than the rate-limiting step in the capture and transport of the T-segment through the DNA gate; thus the efficiency of coupling is relatively high in terms of ATP consumption. Based on our kinetic data for the DNA-dependent ATPase activity, at the low ATP concentrations of 5–25 μM, a significant fraction of the dimeric enzyme has only one bound ATP at any given time. The observation that there is a concerted conformational change of the entire enzyme as a result of the binding of one nonhydrolyzable ATP analogue suggests that the binding of a single ATP to a dimeric enzyme might be sufficient for DNA transport (Lindsley and Wang, 1991, 1993). Thus when ATP usage and DNA transport are very efficiently coupled, at a low ATP concentration the expected value of υ should be between 1 and 2, which is to be compared with the experimental finding of υ = 1.9 ± 0.5.

At a high ATP concentration condition, homotropic cooperativity in the dimeric enzyme assures a coordinated action of the two halves of the enzyme. Nevertheless, at a saturating ATP concentration and low temperature the measured υ is 7.4 ± 1.0. We attribute this much higher value of υ to a reversal of the relative magnitude of the two rate-limiting steps under the new set of conditions; namely, in the presence of a saturating amount of ATP the rate-limiting step in ATP usage is faster than that in the capture and transport of DNA. In terms of the ATP-modulated protein clamp model, there are two plausible scenarios for the high value of υ. In one, the clamp opens and closes rapidly but only one out of several times is a T-segment captured; once captured, the T-segment is transported efficiently through the DNA gate in the enzyme-bound G-segment. In the other scenario, the clamp is very efficient in trapping the T-segment at high as well as low ATP concentrations, but the catch does not get transported efficiently at a high ATP concentration. We favor the first scenario because of its conceptual simplicity and because it can encompass the low as well as the high ATP concentration results.

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