Type II DNA Topoisomerase from *Saccharomyces cerevisiae* Is a Stable Dimer†

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ABSTRACT: Type II DNA topoisomerases function as homodimeric enzymes in transiently cleaving double-stranded DNA to catalyze unlinking and unknotting reactions. The dimeric enzyme creates a DNA double-strand break by forming a covalent attachment between an active site tyrosine from each monomer and a 5′-phosphate from each strand of DNA. The dimer must be very stable to dissociation or subunit exchange when covalently attached to DNA to prevent directly or indirectly catalyzed rearrangements of the genome. Past studies have indicated conflicting results for the monomer−dimer stability of topoisomerase II in solution. Here, we report results from sedimentation equilibrium studies and two different subunit exchange assays indicating that purified *Saccharomyces cerevisiae* DNA topoisomerase II exists as a stable dimer in solution, with a K_d estimated to be $\leq 10^{-11}$ M. This high dimer stability is not detectably altered by a change of ionic strength or by the presence of ATP, ADP, or DNA.

Type II DNA topoisomerases are highly conserved, essential enzymes found in both prokaryotes and eukaryotes (for a recent review, see ref 1). Eukaryotic topoisomerase II relaxes DNA supercoils, decatenates/catenates, and knots/unknots DNA. These enzymes are involved in many aspects of DNA metabolism: replication, transcription, recombination, sister chromatid segregation, chromosome structure, and condensation (*1*−*7*). Numerous antibiotics and anticancer drugs currently in use target topoisomerase II (*8*, *9*).

Eukaryotic topoisomerase II functions as a homodimer. The dimeric enzyme alters the DNA linking number in steps of two by a double-stranded DNA passage mechanism (*10*−*12*). It catalyzes these reactions in an ATP-dependent manner by transporting one DNA duplex through a transient double-strand break in a second DNA duplex. DNA cleavage occurs via a transesterification reaction between the hydroxyl group of the active site tyrosine and a 5′-phosphate of the DNA backbone, resulting in a covalent link between enzyme and DNA. Cleavage by both monomers of the dimeric enzyme results in a DNA double-strand break. A second DNA duplex is transported through the transient break in the cleaved duplex, and the cleaved DNA is religated (reviewed in ref 13). The enzyme must bind and hydrolyze ATP for DNA transport and enzyme turnover (*14*, *15*).

Topoisomerase II-cleaved DNA complexes, in which one strand of DNA is covalently attached to each monomer of the enzyme dimer, are transient intermediates in all topoisomerase II-catalyzed reactions. Dissociation of the dimeric enzyme into monomers covalently attached to cleaved DNA could result in DNA damage and rearrangements by a number of different mechanisms. Additionally, subunit exchange between dimers attached to different regions of the genome has been proposed to explain rare illegitimate recombination events involving type II DNA topoisomerases from a variety of organisms (*16*). Clearly, given the high concentration of topoisomerase II in the nucleus and the normally high genomic integrity, the topoisomerase II dimer must be very stable when covalently attached to DNA.

Despite the implications of topoisomerase II dimer stability, a consensus on this point is lacking; biochemical, structural, and sedimentation equilibrium studies from several laboratories present conflicting results. Hydrodynamic studies using glycerol gradients, sucrose gradients, and gel filtration indicate that eukaryotic topoisomerase II is a dimer in solution (*17*−*19*). No lag time in topoisomerase II relaxation or ATPase activity is seen in assays using nanomolar amounts of enzyme (T. T. Harkins, R. B. Tennyson, and J. E. Lindsley, unpublished results); a measurable lag would be expected if dimerization induced by DNA binding was the first step of the reaction pathway. Circular DNA trapping and heterodimer studies done in the presence of AMPPNP[1] show a stable dimeric topoisomerase II even at very high salt concentrations (*20*−*22*). While heterodimers of differentially tagged yeast topoisomerase II, as well as α and β isoforms of the human enzyme, can be formed *in vivo*, heterodimers have not been detected when two separate populations are mixed *in vitro* (*22*, *23*), indicating that the dimer dissociation rate is very slow. The crystal structure of a 92 kDa yeast topoisomerase II subfragment with no cofactors present reveals a dimeric protein (*24*). This structure shows two dimer interfaces, one of which buries a large hydrophobic surface area, and is therefore predicted to be the primary stable dimer interface. Recently, however, results of a sedimentation equilibrium study of yeast topoisomerase II indicated that the protein has a dimer dissociation constant of 2×10^{-6} M (*25*). This result implies that topoisomerase II has only weak dimer interactions and that it should exist primarily as a monomer in solution under most assay conditions. As sedimentation

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[1] Abbreviations: AMPPNP, adenosine 5′-(β,γ-imido)triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Ha-topo II, topoisomerase II with an amino terminal Ha tag; topo II-His, topoisomerase II in which the last 95 amino acids were replaced by a six-histidine tag; wt, wild type topoisomerase II; topo II(C→A), topoisomerase II in which all of the cysteines were changed to alanines; Y783F, topoisomerase II in which the active site tyrosine has been changed to phenylalanine; BSA, bovine serum albumin.

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equilibrium is considered one of the most thermodynamically rigorous ways of measuring the stoichiometry and association constants of self-associating protein systems (*26*), the results of this study present a serious conflict to those of previous studies.

In order to clarify what the stability of the *Saccharomyces cerevisiae* topoisomerase II dimer is in solution, we have used three independent approaches: sedimentation equilibrium and two subunit exchange assays. The only detectable species in our sedimentation equilibrium experiments is a dimer. The exchange assays were designed to determine the rate of dimer rearrangement by measuring the appearance of heterodimers after mixing two homodimeric populations. If the dimer is of only moderate stability, rearrangement should be rapid. On the basis of exchange assays, no rearrangement could be detected for up to 2 weeks. All three methods indicated that topoisomerase II exists as a stable dimer. Neither the presence of cofactors nor varying reaction conditions altered these results.

MATERIALS AND METHODS

Materials. Standard reagents were purchased from the following commercial sources: ATP, Pharmacia; pBluescript KS^+, Stratagene; AMPPNP and ADP, Boehringer Mannheim; leupeptin, pepstatin A, benzamidine, NADH, *p*-nitrophenyl phosphate, pyruvate kinase, lactate dehydrogenase, EDTA, EGTA, BSA, and alkaline phosphatase−agarose beads, Sigma. Anti-Ha mouse monoclonal antibodies (12CA5) were from the Harvard Monoclonal Antibody Facility. Alkaline phosphatase-conjugated antibodies were made using the ImmunoPure Maleimide Alkaline Phosphatase Conjugation Kit (Pierce). Oligonucleotides used for construction of topoisomerase II expression vectors, site-directed mutagenesis, and sequencing were made at the DNA Peptide Synthesis Facility, Huntsman Cancer Institute, University of Utah (National Institutes of Health Grant CA 42014). The same reaction buffer [50 mM HEPES-KOH (pH 7.5), 170 mM KOAc, and 10 mM $Mg(OAc)_2$] was used for all experiments, except where specifically indicated.

Construction of Expression Vectors. All vectors used for the expression of modified *S. cerevisiae* DNA topoisomerase II are derived from the plasmid YEpTOP2-PGAL1 (*27*). Derivatives of YEpTOP2-PGAL1 expressing the enzyme tagged at its amino terminus (Ha-topo II, pDAT1) or at its carboxyl terminus (topo II-His, pTCH1) were constructed by standard recombinant DNA methods. In the pDAT1 construct that expresses Ha-topo II, the first 12 codons encode Met-Ser-**Thr-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala** and are followed by the remaining 1426 codons of wild type yeast topoisomerase II. The boldface peptide is the influenza hemaglutinin (Ha) epitope (*28*) specifically recognized by the monoclonal antibody 12CA5. The plasmid pJEL163 is similar to pDAT1 except that it contains the LEU2 selectable marker instead of URA3. In the pTCH1 construct that expresses topo II-His, the last 95 amino acids of the wild type enzyme are replaced by Ala-Arg-Ala-$(His)_6$. Yeast topoisomerase II truncated by 95 amino acids at the carboxyl terminus has previously been shown to be fully active (*29*). A plasmid encoding an inactive mutant form of the yeast enzyme (Y783F) in which the active site tyrosine has been mutated to phenylalanine was obtained from Brian Davis (USDA). The single mutation at codon 783 (TAT to TTT) was reconfirmed by sequencing and subcloned back into the expression vector, to generate pJEL170. The plasmid pDAT10, encoding an active mutant form of the yeast enzyme [topo II(C→A)] in which all nine of the naturally occurring cysteines were mutated to alanines, has been previously described (*30*).

Enzyme Expression and Purification. The wild type (wt), mutant, and immunotagged forms of topoisomerase II used in these experiments were overexpressed in the protease deficient *S. cerevisiae* strain BCY123 (*31*) as described (*32*). Plasmids YEpTOP2-PGAL1, pDAT1, pTCH1, pDAT10, and pJEL170 were transformed into BCY123 for the expression of wt, Ha-topo II, topo II-His, topo II(C→A), and Y783F, respectively. Ha-topo II/topo II-His heterodimers were coexpressed in the yeast strain JEL1, doubly transformed with plasmids pJEL163 and pTCH1, in media lacking both uracil and leucine as previously described (*22*).

The wt, Ha-topo II, topo II(C→A), and Y783F proteins were purified by two different variants of the previously described method (*32*) which are described in detail elsewhere (*33*). One variant used phosphocellulose (Whatman), ammonium sulfate precipitation, and Q-Sepharose fast flow (Sigma). The second variant used phosphocellulose, POROS QE, and POROS Heparin (Perseptive Biosystems). The topo II-His and Ha-topo II/topo II-His heterodimer proteins were purified by cell disruption, ammonium sulfate precipitation, and nickel-chelating Sepharose chromatography (Pharmacia). Purified proteins were dialyzed into buffer containing 50 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 1 mM EGTA, 150 mM KCl, and either 10 or 50% glycerol. Proteins in 10 and 50% glycerol were stored at −70 or −20 °C, respectively. Protein concentrations were determined using the Coomassie Plus protein assay reagent (BioRad) or absorbance at 280 nm. An extinction coefficient (ϵ) of 0.9857 mL mg^{-1} cm^{-1} was obtained for topoisomerase II using a published procedure (*34*). All topoisomerase II concentrations are reported as the dimer concentrations.

Sedimentation Equilibrium. Sedimentation equilibrium experiments were performed in a Beckman Model XL-A analytical ultracentrifuge at 20 °C in an An-60 Ti analytical rotor. Topoisomerase II was dialyzed into two changes of 1 L of reaction buffer for 8 h at 4 °C in 200 μL microdialyzer chambers with 50 kDa molecular mass cutoff membranes (Sialomed). After dialysis, the protein concentration was determined by absorbance at 280 nm. The protein was diluted with dialysis buffer to 0.4, 0.2, and 0.1 mg/mL and loaded into charcoal-filled Epon six-sectored cells. Dialysis buffer was used as the reference solution. Initial experiments indicated that the concentration gradient did not change after 24 h. To ensure that equilibrium was reached in all cases, all spins were run for 36 h. For each run, equilibrium was verified by overlaying scans taken at 34 and 36 h. Scans were taken at 280 nm with a radial step size of 0.003 mm. Fifty scans were averaged to obtain a data set. Experiments were run at three speeds, 5500, 6000, and 7000 rpm, for all three protein concentrations. Protein samples were dialyzed and diluted for separate runs at each of the three speeds; results were found to be less reproducible if a single protein sample was run continuously for all three speeds. Experiments were done using wild type topoisomerase II purified independently using the two separate methods described above and using the topoisomerase in which all cysteines have been changed to alanines [topo II(C→A)].

Data analysis was performed using the Optima XL-A data analysis software version 2.0 supplied by Beckman. Triple data sets, obtained by using the six-sectored cells, were edited to separate the three channels of data without cropping any of the sets. The resulting data sets were analyzed both individually and globally. Individual data sets were fit to eq 1 using the Marquardt−Levenburg (*35*) algorithm

$$A_r = A_{0,1} \exp(M\delta) + E \quad (1)$$

where $\delta = (1 - \nu\rho)\omega^2(r^2 - r_0^2)/2RT$, A_r is the absorbance at radius r, $A_{0,1}$ is the absorbance of the smallest species present at the reference radius r_0, M is the molecular mass of the smallest species present, ν is the partial specific volume of the protein, ρ is the solvent density, ω is the angular velocity, r is the radial distance from the center of rotation, r_0 is the distance from the center of rotation to the meniscus, R is the gas constant, T is the temperature in kelvin, and E is the baseline offset. A value of 1.01 g/mL was used for the solvent density. This value was determined from the mass of 1 mL of the reaction buffer and was verified by calculating the value for similar buffers as previously described (*35*). A partial specific volume of 0.7316 mL/g was calculated for yeast topoisomerase II by the method of Cohn and Edsall using partial specific volumes of the amino acids as presented (*36*). For all data presented, E was allowed to vary and the calculated value was comparable to that measured by meniscus depletion as described (*35*). Data sets were pooled by speed or protein concentration for global analysis using the "self" model to fit the data to monomer, monomer−dimer, dimer, or dimer−tetramer models. The fitting equation used by the self model is the following:

$$A_r = A_{0,1} \exp(M\delta) + (A_{0,1})^{n_2}K_{a2} \exp(n_2M\delta) + (A_{0,1})^{n_3}K_{a3} \exp(n_3M\delta) + (A_{0,1})^{n_4}K_{a4} \exp(n_4M\delta) + E \quad (2)$$

where n_2, n_3, and n_4 are the stoichiometries of species 2, 3, and 4, respectively, and K_{a2}, K_{a3} and K_{a4} are the association constants for the equilibria of species 2, 3, and 4 with the smallest species present, respectively. All other variables are as described for eq 1. Association constants calculated by this program are in absorbance units. They were converted to molar units using the equation K_a (M^{-1}) = $K_a(\epsilon l)^{n-1}/n$, where ϵ is the extinction coefficient for the smallest species present (162 150 M^{-1} cm^{-1} for the monomer, 324 300 M^{-1} cm^{-1} for the dimer), l is the path length (1.2 cm), and n is the stoichiometry for the association (e.g. $n = 2$ for monomer−dimer). The dissociation constants reported (K_d) are equal to $1/K_a$. The goodness of fit values (GOF) were calculated using eq 3:

$$\text{GOF} = (1/\text{DOF})[\Sigma(\text{residuals/standard error})^2] \quad (3)$$

where DOF is the degrees of freedom or the number of observations minus the number of fitting parameters and the residuals are the observed values minus the calculated values for all data points.

Inactivation Assay. This assay is based on the fact that topoisomerase II needs two active site tyrosines, one from each monomer, to function. To assay subunit exchange, wt and Y783F proteins were mixed in reaction buffer at a 1:5 (60 nM:300 nM) ratio of wt/Y783F. This mixture and controls containing either 60 nM wt or 300 nM Y783F alone were preincubated at room temperature. DNA relaxation assays were used to measure any changes in enzyme activity over time. Assays were performed either immediately after mixing the proteins for preincubation ("0" time) or after 24 or 48 h. These preincubation mixes were diluted to either (a) 3.2 nM wt, (b) 20 nM wt, (c) 20 nM wt/100 nM Y783F, or (d) 100 nM Y783F, in reaction buffer plus 200 μg/mL BSA immediately prior to the reaction. Equal volumes of 20 nM supercoiled plasmid (pBluescript KS^+) and diluted protein solutions were mixed and held at 30 °C. After a 5 min incubation, an aliquot was placed in another tube to serve as a −ATP control (0 time). ATP (1 mM final) was mixed with the remaining reaction mixture. The final reaction mixture contained 10 nM plasmid, 50 mM HEPES-KOH (pH 7.5), 10 mM $Mg(OAc)_2$, 170 mM KOAc, 200 μg/mL bovine serum albumin, 1 mM ATP, and either (a) 1.6 nM wt, (b) 10 nM wt, (c) 10 nM wt/50 nM Y783F, or (d) 50 nM Y783F. Aliquots were removed at a series of time points and quenched with an equal volume of 10% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol, and 0.2% SDS. Samples were electrophoresed in a 0.8% agarose gel at 10 V/cm for 6 h in TBE (89 mM Tris-borate and 2 mM EDTA). Gels were stained in ethidium bromide, destained in water, and photographed over a UV transilluminator.

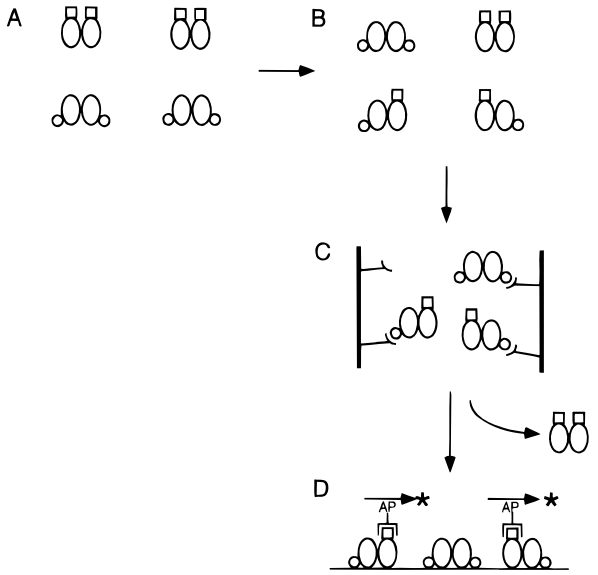

FIGURE 1: Strategy for detecting subunit exchange *in vitro* using immunotagged enzymes. (A) Two populations of immunotagged (□ = Ha tag, ○ = His tag) yeast DNA topoisomerase II homodimers (ovals) are mixed and allowed to preincubate under various conditions for various amounts of time. (B) Subunit exchange results in the formation of heterodimers containing both immunotags. (C) Ni^{2+} chromatography captures dimers containing one or two His tags. Dimers without His tags are washed through the column. (D) The eluted protein is bound to an ELISA plate and probed for the presence of the Ha tag using alkaline phosphatase (AP)-conjugated anti-Ha antibodies. The product of the AP reaction (*) absorbs at 405 nm.

Immunotagged Subunit Exchange Assay. Ha- and His-tagged proteins were mixed in reaction buffer plus 10% glycerol at a 1:2 ratio of topo II-His/Ha-topo II and a total protein concentration of 0.1 mg/mL. Two separate aliquots of the preincubation mixes containing 5 μg of Ha-topo II and 2.5 μg of topo II-His were taken at each time up to 2 weeks and assayed separately for subunit exchange. Subunit exchange was measured in a two-step process depicted in Figure 1. First, any His tag-containing protein was allowed

to bind to 100 μL of Ni^{2+}-chelating sepharose in a 1 mL spin column by incubation for 30 min at 4 °C in binding buffer [5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9), plus 0.5 mg/mL BSA]. Unbound protein was removed by spinning the column at 700*g* for 1 min. The column was then washed with 2 mL of binding buffer and 15 mL of wash buffer [60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9), plus 0.5 mg/mL BSA] to remove any non-His-tagged protein. Bound protein was eluted with 500 μL of strip buffer [100 mM EDTA, 500 mM NaCl, and 20 mM Tris-HCl (pH 7.9)]. A 20 μL aliquot of protein was diluted 10-fold in coating buffer (50 mM sodium bicarbonate at pH 9.6) and incubated overnight in a 96-well ELISA plate (Corning) at 4 °C. Wells were washed four times with 200 μL of PBST [10 mM potassium phosphate (pH 7.4), 150 mM NaCl, 0.1% Tween 20, and 0.02% sodium azide] and blocked with 200 μL of gelatin wash buffer (0.5% gelatin in PBST) for 1 h at room temperature. Ha-tagged protein was detected using the alkaline phosphatase-conjugated monoclonal antibody (12CA5-AP). Antibody was diluted 500-fold in gelatin wash buffer, and 200 μL was added to each well and incubated for 1 h at room temperature. Wells were washed four times with 200 μL of PBST. The detection signal was produced by adding 200 μL of substrate buffer [1 μg/mL *p*-nitrophenyl phosphate in 1 M diethanolamine (pH 7.4), 0.5 mM $MgCl_2$, and 0.02% sodium azide] to each well. The plates were incubated for 30 min at 37 °C, followed by addition of 50 μL of 3 M NaOH to each well to stop color development. Absorbance of each sample well was read at 405 nm using an SLT Rainbow microtiter plate reader (Phoenix Research Products).

Both negative and positive controls were performed for this assay. The negative control involved preincubating 0.1 mg/mL Ha-topo II alone. Aliquots of the preincubation mix containing 5 μg of protein were processed as described above. The positive control utilized Ha-topo II/topo II-His heterodimeric protein produced *in vivo*. Aliquots containing 2.5 μg of heterodimer were processed as described above. The results for the positive control were similar regardless of whether the aliquots were processed immediately after thawing or after a 2 week incubation at room temperature.

The degree of subunit exchange under a variety of reaction conditions was probed using this assay. For the results of the assay shown in Figure 5, the preincubation buffer is reaction buffer plus 10% glycerol and the preincubation temperature was room temperature. For the results of the assays shown in Figure 6, the preincubation buffers were altered to contain either (a) a total of 500 mM KOAc, (b) a total of 50 mM KOAc, (c) 0.2 mM AMPPNP, (d) 2 mM ADP, (e) 2 mM ATP, (f) 60 nM plasmid DNA, or (g) 2 mM ATP plus 60 nM plasmid DNA, and the preincubation temperature was 30 °C. For assays that contained ATP, 5 mM phosphoenolpyruvate, 6.4 units of pyruvate kinase, 5 mM NADH, and 8.8 units of lactate dehydrogenase were added. These amounts of pyruvate kinase and phosphoenolpyruvate will regenerate ATP for up to 1 h in the presence of DNA and for the entire length of the reaction in the absence of DNA [topoisomerase II is a more rapid ATPase when bound to DNA (*37*)]. NADH and lactate dehydrogenase were added to convert pyruvate, a strong product inhibitor of pyruvate kinase, to lactate, a poor inhibitor of both enzymes.

Table 1: Calculated Molecular Mass Based on the Sedimentation Equilibrium

speed (rpm)	[protein][a] (mg/mL)	calcd molecular mass[b] (kDa)	GOF[c]	*n*-mer[d]
7000	all[e]	333 ± 8	0.374	2.02 ± 0.05
6000	all	351 ± 10	0.236	2.13 ± 0.06
5500	all	346 ± 11	0.117	2.10 ± 0.07
all[f]	0.4	338 ± 9	0.425	2.05 ± 0.05
all	0.2	340 ± 8	0.231	2.07 ± 0.05
all	0.1	337 ± 11	0.100	2.05 ± 0.07

[a] Concentration of protein loaded into the centrifuge cells. [b] The molecular mass calculated for topoisomerase II, as described in Materials and Methods. Values are rounded to the nearest kilodalton. The error estimate represents the 95% confidence interval. [c] Goodness of fit (GOF) is calculated using eq 3 (see Materials and Methods). [d] Stoichiometry was calculated using a value of 164.5 kDa for the topoisomerase II monomer. [e] Data from six samples (two each at 0.4, 0.2, and 0.1 mg/mL) at the indicated rotor speed were fit globally. [f] Data from six samples (two each at 5500, 6000, and 7000 rpm) at the indicated concentration were fit globally.

RESULTS

Sedimentation Equilibrium. Topoisomerase II was analyzed by sedimentation equilibrium because it is a thermodynamically rigorous method for determining the native molecular mass and interactions of biological molecules (*26*). Purified topoisomerase II was sedimented to equilibrium at 0.4, 0.2, and 0.1 mg/mL at 5500, 6000, and 7000 rpm to measure the degree of self-association. These conditions were used to sample a larger range of concentrations and to verify that neither nonideality nor association was being masked in the data (*35*). The experimental results showed equilibrium distributions over a range of absorbance from 0.02 to 0.95 at 280 nm, corresponding to 62 nM to 3 μM topoisomerase II. Additionally, each experiment was done in duplicate using protein prepared by two separate methods. Individual data sets were initially fit to eq 1 (see Materials and Methods) using the Marquardt−Levenburg (*35*) algorithm. The individual data sets fit well to this single-species model in which the calculated molecular mass approximates that of the topoisomerase II dimer (not shown). For a more rigorous analysis, six data sets obtained at a given concentration and three different speeds or at a given speed and three different concentrations were pooled. These pooled data were globally fit to eq 2 (see Materials and Methods) using the algorithm of Johnson et al. (*35*). Global fitting to multiple data sets improves one's ability to statistically differentiate between possible models. In all cases, the best global fit was to a single-species model. The molecular mass was allowed to float in the calculations. The calculated molecular masses for topoisomerase II are reported in Table 1. Under all conditions tested, the calculated molecular mass is very close to that of a topoisomerase II dimer (329 kDa).

Examples of the globally fit data at different speeds or different concentrations are shown in Figures 2A and 3A, respectively. In all cases, at the lowest protein concentration used, the residuals are small and randomly distributed. At the highest protein concentration used (0.4 mg/mL) and the fastest rotor speed (7000 rpm), the residuals are somewhat larger and less random (Figure 3A). However, no other model tried (monomer, monomer−dimer, or dimer−tetramer) improved the fit (not shown). The difficulty in fitting this data may result from 7000 rpm being at the upper limit of usable speeds for a 300 kDa species (*26*).

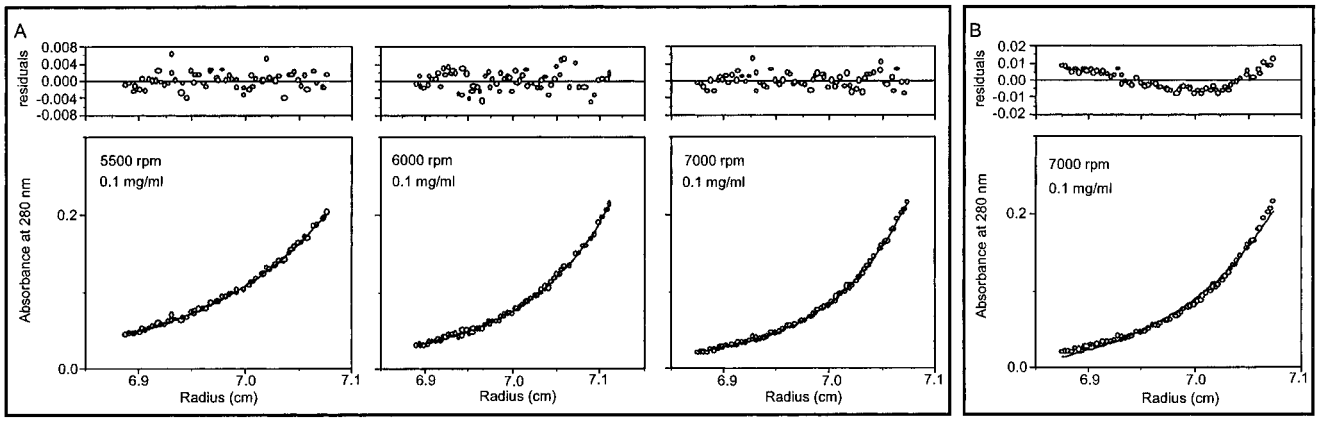

FIGURE 2: Sedimentation equilibrium data globally fit by concentration. Concentration distribution and residual plots of data for topoisomerase II fit to either (A) a single-species dimer or (B) a monomer−dimer model are shown. Data were analyzed using the Optima XL-A data analysis software version 2.0 supplied by Beckman. (A) Six data sets from two preparations of enzyme at 0.1 mg/mL and spun at 5500, 6000, and 7000 rpm were pooled and globally fit to a single-species dimer model. Three data sets (one at each speed) are shown. Calculated molecular masses are presented in Table 1. (B) Six data sets were pooled as in panel A, but were fit to a monomer−dimer model in which the monomer molecular mass was set to 164.5 kDa and K_d was set to the previously reported value of 2×10^{-6} M (*25*). Note that the scale of the residual plot is larger than that shown in panel A.

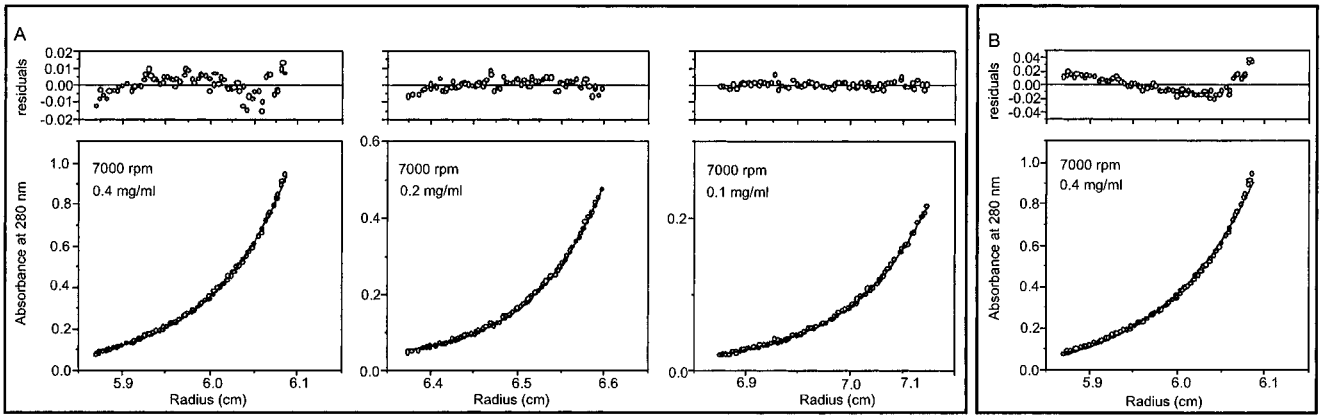

FIGURE 3: Sedimentation equilibrium data globally fit by speed. Concentration distribution and residual plots of data for topoisomerase II fit to either (A) a single-species dimer or (B) a monomer−dimer model are shown. (A) Six data sets from two preparations of enzyme at 0.4, 0.2, and 0.1 mg/mL and run at 7000 rpm were pooled and fit to a single-species dimer model. Three data sets (one at each speed) are shown. Calculated molecular masses are presented in Table 1. (B) Six data sets were pooled as in panel A but were fit to a monomer−dimer model in which the monomer molecular mass was set to 164.5 kDa and K_d was set to 2×10^{-6} M. Note that the scale of the residual plot is larger than that shown in panel A.

The present pooled data were also globally fit to a monomer−dimer model using eq 2, where the monomer molecular mass was set at 164.5 kDa. The calculated K_d values ranged from 10^{-33} to 10^{-60} M. These values are clearly unrealistic for this system and the protein concentrations used and indicate that virtually all of the protein present is dimeric. Previously published sedimentation equilibrium data for yeast topoisomerase II were fit to a monomer−dimer model with a K_d of 2×10^{-6} M (*25*). Setting the K_d at 2×10^{-6} M and the monomer molecular mass at 164.5 kDa, the global fits to our data were in all cases considerably worse than those obtained from the single-species dimer model. Representative examples are shown in Figures 2B and 3B. Note that the scale on the residual plots is larger than that shown for the single-species dimer models in Figures 2A and 3A. Therefore, for all of the models tested, the single-species model, in which the calculated molecular mass approximates that of the topoisomerase II dimer, produced the best fit to our data.

The presence of cysteine and the phosphorylation state of topoisomerase II on the dimer stability were also tested by sedimentation. Topoisomerase which lacked cysteines [topo II(C→A)] gave results similar to those of the wild type protein, indicating that intradimeric disulfide bonds are not responsible for the dimer stability (data not shown). Additionally, topoisomerase II which was dephosphorylated by incubation with alkaline phosphatase−agarose beads also sedimented as a dimer (data not shown). Topoisomerase II with an increased level of phosphorylation precipitated upon dialysis and was not analyzed further. Aggregation of highly phosphorylated topoisomerase II is consistent with previously published results (*38*).

Inactivation Assay. On the basis of the well-studied mechanism of topoisomerase II, this enzyme must be able to cleave both strands of DNA to show supercoil relaxation activity. Since two active site tyrosines, one from each subunit, are required for double-strand cleavage, subunit exchange occurring between wild type and Y783F mutant topoisomerase dimers will produce inactive heterodimers. Because this experiment was performed with a 5-fold excess of Y783F to wild type protein, most subunit exchange that occurs will result in heterodimer formation and subsequent loss of enzyme activity as determined by DNA relaxation or decatenation.

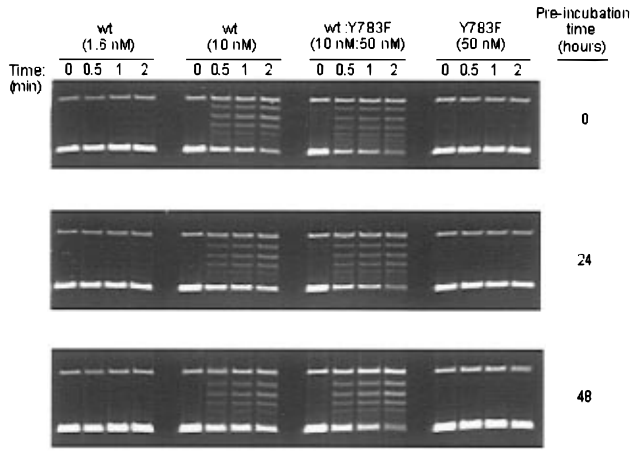

FIGURE 4: Relaxation of negatively supercoiled DNA by wild type (wt), active site mutant (Y783F), or a 1:5 mixture of wt/Y783F topoisomerase II was analyzed to detect subunit exchange. Preincubation mixes containing 60 nM wt, 300 nM Y783F, or 60 nM wt/300 nM Y783F in reaction buffer plus 200 μg/mL bovine serum albumin were placed at room temperature for the indicated preincubation time. After the preincubation, the protein was diluted and mixed with DNA substrate to give the final concentrations of protein indicated in the figure and 10 nM plasmid. ATP was added to 1 mM to start the reaction (0 time points contained no ATP and were incubated for 10 min). Aliquots were taken at the times indicated and quenched. Supercoiled substrate and relaxed product DNA were separated in a 0.8% agarose gel.

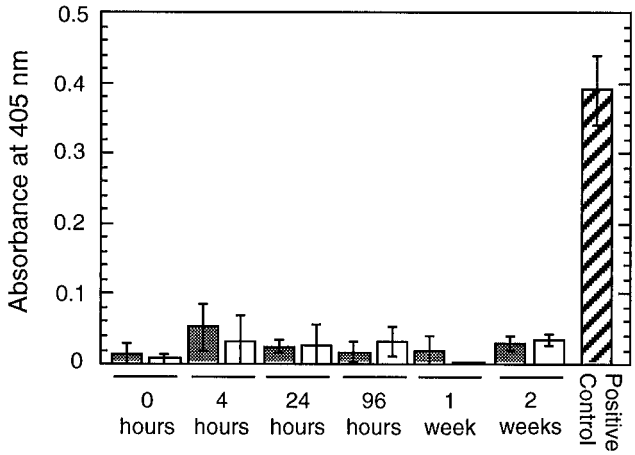

FIGURE 5: Absorbance values for immunotagged subunit exchange assays performed on samples containing a 1:2 ratio of His- to Ha-tagged topoisomerase II (open bars) or Ha-tagged protein alone (shaded bars). Preincubation mixes containing 0.1 mg/mL protein in reaction buffer plus 10% glycerol were placed at room temperature. Two-step assays were performed as illustrated in Figure 1. Ha-tagged protein, and therefore absorbance, should be present only if subunit exchange has occurred among topoisomerase II dimers. The open bars indicate absorbance values obtained over a 2 week period for samples taken from preincubation mixes containing both Ha- and His-tagged proteins. The shaded bars indicate absorbance values obtained over two weeks for samples taken from preincubation mixes containing only Ha-tagged protein. These results represent a negative control for the assay. The striped bar marked positive control shows the absorbance value expected if complete subunit exchange had occurred and was obtained using a sample of heterodimeric protein produced *in vivo* by coexpressing the two proteins (*22*) and assayed as described above.

Given starting concentrations of 10 nM wt and 50 nM Y783F topoisomerase II, one would expect to see the activity of 10 nM wt if no subunit exchange occurred or of 1.6 nM wt if complete subunit exchange occurred (i.e. all dimers dissociated and randomly reassociated). The results of this assay for preincubations at room temperature up to 48 h are shown in Figure 4. Under the conditions used to run these agarose gels, supercoiled substrate DNA migrates most rapidly, nicked DNA migrates most slowly, and relaxed product DNA runs as a ladder of intermediate mobility. As expected, Y783F shows no activity and wt at 1.6 nM shows barely detectable levels of activity. By contrast, 10 nM wt and 10 nM wt/50 nM Y783F each show the same high levels of activity after up to 48 h of preincubation. These results indicate that no appreciable subunit exchange occurs within 48 h. The same results were found using a kinetoplast DNA decatenation assay to measure topoisomerase II activity (data not shown).

Immunotagged Subunit Exchange Assay. In this assay, subunit exchange that produces differentially tagged heterodimers will result in an increase of absorbance, as illustrated by the scheme in Figure 1. Verification that the assay will detect heterodimers was demonstrated using purified heterodimeric protein produced *in vivo* (see Materials and Methods) (Figure 5, positive control). For the assays containing a 1:2 ratio of His-tagged to Ha-tagged protein, if no subunit exchange occurs, a background level of absorbance is expected. If complete subunit exchange occurs, absorbance approximating that of the heterodimer positive control is expected. This assay was repeated three separate times; the results shown in Figure 5 represent the mean and standard deviation from the mean at each time point of the three assays. To determine the background absorbance expected for this assay, Ha-topo II alone was taken through the entire assay procedure as for the mixture of tagged proteins. With a comparison of the results from incubations containing both tagged proteins with the control incubations containing only the Ha-topo II, it is clear that no subunit exchange between topoisomerase II dimers could be detected above background within the 2 week assay period.

To determine the effects of ionic strength and cofactors on the stability of the topoisomerase II dimer, this assay was repeated under a variety of preincubation conditions as indicated in Figure 6. The effect of ionic strength on heterodimer formation was tested by altering the reaction buffer so it contains a total of either 500 or 50 mM KOAc. The cofactors tested were 0.2 mM AMPPNP (a nonhydrolyzable analog of ATP), 2 mM ADP, 2 mM ATP, 60 nM plasmid DNA, or both 2 mM ATP and 60 nM plasmid DNA. Preincubation mixtures that contained ATP also contained the ATP-regenerating system described in Materials and Methods. This system is capable of converting ADP back to ATP for approximately 1 h in the presence of DNA, which stimulates the rate of ATP hydrolysis by topoisomerase II, or for approximately 20 h in the absence of DNA. For the results shown in Figure 6, the preincubation time was 24 h except when ATP was present, in which case the time was shortened to 5 h. These assays were each repeated twice. Under no condition was the absorbance found to be above background levels, indicating that no detectable subunit exchange occurred.

DISCUSSION

Results from sedimentation equilibrium and subunit exchange experiments all show that DNA topoisomerase II from *S. cerevisiae* is a stable dimer in solution. Sedimentation equilibrium data were fit to models of topoisomerase II as a single species and as an associating species. Regardless

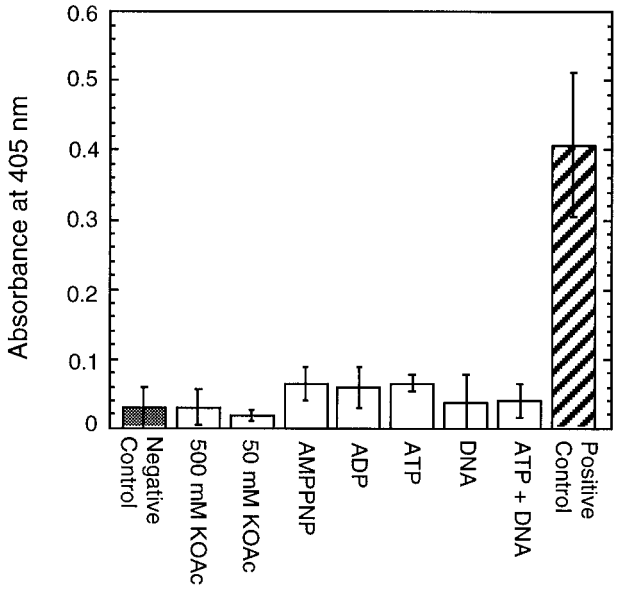

FIGURE 6: Absorbance values for immunotagged subunit exchange assays performed on samples containing a 1:2 ratio of His- and Ha-tagged proteins under various conditions or in the presence of cofactors (open bars). Preincubation mixes containing 0.1 mg/mL protein in reaction buffer containing 500 mM KOAc, 50 mM KOAc, 0.2 mM AMPPNP, 2 mM ADP, 2 mM ATP, 60 nM plasmid DNA, or 2 mM ATP plus 60 nM plasmid DNA plus 10% glycerol were placed at 30 °C. Preincubations were for 24 h, except when ATP was present in which case the time was reduced to 5 h. Two-step assays were performed as described in Figure 1. Ha-tagged protein, and therefore absorbance, should be present only if subunit exchange has occurred among topoisomerase II dimers. The shaded bar indicates the mean absorbance value obtained for samples taken from preincubation mixes containing only Ha-tagged protein and therefore represents a negative control. The bar marked positive control shows the absorbance value expected if complete subunit exchange had occurred and was obtained using a sample of heterodimeric protein produced *in vivo* by coexpressing the two proteins (*22*).

of whether data sets were analyzed individually or globally, the best fit was obtained for a single-species dimer. When a monomer−dimer model was used for the fitting, the K_d values calculated were much lower than the lowest concentration of protein in the cell, again indicating that virtually all of the protein present exists as a dimer. The same result was found for a mutant topoisomerase in which all of the cysteines were replaced by alanines, confirming that intradimeric disulfide bonds are not involved in dimer stability.

Two different types of subunit exchange assays were used to probe the frequency at which the monomers of topoisomerase II dimers reassociate. One assay probed for the disappearance of activity over time, while the other measured development of a positive signal upon subunit exchange. Both assays gave consistent results; no subunit exchange was detected, even at the longest time point tested (2 weeks). Altering the reaction conditions by increasing or decreasing the ionic strength, including ATP, ADP, and/or DNA, had no effect on these results.

The association of two monomers to form a dimer is described by Scheme 1 below.

Scheme 1

$$M + M \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} D$$

The two monomers associate with the bimolecular rate constant k_{on} and dissociate with the unimolecular rate constant k_{off}. The dissociation constant for the dimer, K_d, equals $2k_{off}/k_{on}$; the factor of 2 comes from one dimer dissociating into two monomers. If one assumes that the rate-limiting step of subunit exchange is k_{off}, a reasonable assumption given the relatively high protein concentrations used, then an upper limit on this rate can be determined. Given that no signal for exchange is detected for up to 2 weeks, it is reasonable that the half-life ($t_{1/2}$) of the dissociation reaction is $\geq$4 weeks, which corresponds to a k_{off} of $\leq 3 \times 10^{-7}$ s^{-1} ($k_{off} = \ln 2/t_{1/2}$). The association rate for protein multimerization reactions has been estimated to be on the order of 10^5-10^6 M^{-1} s^{-1} (*39*). Using these approximations for k_{on} and k_{off}, an upper limit on K_d for topoisomerase II can be roughly estimated to be 10^{-11} M. This value for K_d is in the range of those for other tightly associating protein complexes (*40*).

Our results are in direct conflict with sedimentation equilibrium results previously published (*25*). These authors report a dissociation constant for the topoisomerase II dimer of 2×10^{-6} M that increases as the ionic strength is increased. We obtain significantly better fits to our data by using a single-species, dimer-only model than by using a monomer−dimer equilibrium. In addition, having a K_d of 2×10^{-6} M predicts that one should readily be able to detect subunit exchange between dimers. The lack of any detectable exchange of monomers between topoisomerase II dimers supports the model of a stable dimer with a low K_d as discussed above.

A tight association between topoisomerase monomers is necessary to prevent the enzyme from rearranging the genome. When topoisomerase II is covalently attached to cleaved double-stranded DNA, the dimer interface or interfaces are predicted to be very strong. It is these interfaces that hold the complex together, preventing its disintegration to broken DNA with covalently attached monomers at the 5′ ends or exchange with monomers covalently attached to other regions of the genome. *In vitro* illegitimate recombination assays have predicted that subunit exchange between topoisomerase II dimers covalently attached to DNA can occur at a very low frequency (*41*). These assays involve mixing purified topoisomerase II, ATP, and two heterologous DNA substrates; recombination between the DNA substrates is tested genetically. These genetic assays are much more sensitive than the biochemical assays described in the present report; results from both types of assays agree in showing that, if subunit exchange occurs, it occurs rarely.

Very rare dissociation events of the topoisomerase II dimer would be even less frequently detected as disruptions of the DNA because normally the protein is covalently attached to the DNA for only a small percentage of the entire reaction cycle. However, the presence of drugs that increase the lifetime of the covalent protein−DNA complex may increase the frequency of detecting separated DNA breaks or DNA recombinants caused by dimer dissociation. Several such drugs, including VP-16 and VM-26, increase the frequency of *in vitro* and *in vivo* catalyzed illegitimate recombination events (*41*−*45*). This is particularly intriguing in light of recent reports linking the use of topoisomerase II-targeting anticancer drugs to the development of leukemia associated with DNA translocations (*46*−*48*).

Type II DNA topoisomerases are predicted to function by transporting a DNA duplex completely through themselves

(see ref 24 for illustration). This means that all of the dimer interfaces must be transiently disrupted during the reaction cycle of the enzyme. Since we know that the dimer does not readily come apart during enzyme turnover, new highly stable interfaces must be formed as others are broken. The turnover cycle in which one DNA duplex is transported and the enzyme is reset for another round takes approximately 200 ms (*37*). How can the enzyme break and reform at least two highly stable interfaces so quickly? This dilemma between stability of dimer interfaces and fast turnover rate may explain why eukaryotic type II topoisomerases require ATP to function. Unlike prokaryotic DNA gyrase, eukaryotic type II topoisomerases do not increase the energetic state of the DNA even though they absolutely require ATP. The enzyme may instead use the energy of ATP binding and hydrolysis for rapid, transient disruption of dimer interfaces that naturally have very slow dissociation rates. ATP usage may be necessary to have both a fast turnover rate and a very low frequency of dimer dissociation. In other words, ATP may be used to keep the fidelity of the topoisomerase II reaction high, by preventing the dimer from dissociating during the reaction cycle, while allowing turnover to occur rapidly. This model predicts that the energy derived from ATP binding and hydrolysis will be coupled to an increased rate of protein dimer interface formation and breaking. There is ample evidence that this is true for the amino terminal domain of topoisomerase II that appears to dimerize specifically when bound to ATP (*49*). It will be interesting to learn what other intradimeric topoisomerase II contacts are affected by steps of the ATPase reaction cycle.

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