Pre-Steady-State Analysis of ATP Hydrolysis by *Saccharomyces cerevisiae* DNA Topoisomerase II. 1. A DNA-Dependent Burst in ATP Hydrolysis†

Timothy T. Harkins‡ and Janet E. Lindsley*

Department of Biochemistry, University of Utah School of Medicine, 50 North Medical Drive, Salt Lake City, Utah 84132

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ABSTRACT: When bound to DNA, topoisomerase II from *Saccharomyces cerevisiae* exhibits burst kinetics with respect to ATP hydrolysis. Pre-steady-state analysis shows that the enzyme binds and hydrolyzes two ATP per reaction cycle. Our data indicate that at least one of the two ATP is rapidly hydrolyzed prior to the rate-determining step in the reaction mechanism. When DNA is not bound to topoisomerase II, the rate-determining step shifts to become either ATP binding or hydrolysis. Two possible mechanisms are proposed that agree with our observations.

Type II DNA topoisomerases are ubiquitous and essential enzymes that catalyze the ATP-dependent transport of one segment of duplex DNA through an enzyme-mediated transient break in another DNA duplex [for a recent review, see (*1*)]. Due to their ability to change the topology of DNA, these enzymes influence many aspects of DNA metabolism, including their essential function of separating intertwined daughter chromosomes (*2*). Additionally, type II topoisomerases are the targets of several antibiotic (*3*) and anticancer agents (*4*, *5*).

All type II topoisomerases are structurally and mechanistically related (*6*). They are dyadic, where the subunit arrangement can be A_2 homodimer, A_2B_2 tetramer, or $A_2B_2C_2$ hexamer. Each half of the enzyme possesses two distinct catalytic domains: one for transient DNA cleavage and the other for ATP hydrolysis. The two segments of DNA involved in topoisomerase II catalyzed reactions have been termed the "G" segment, for gate, and the "T" segment, for transport (*7*). Topoisomerase II makes a double-stranded break in the G segment by attack of a pair of active site tyrosines on a staggered pair of phosphodiester bonds. This results in a pair of transient, 5′-phosphotyrosyl covalent attachments between dimeric enzyme and cleaved DNA. At this point in the reaction cycle if the enzyme is also bound to ATP, it can transport a T segment of DNA through the break in the G segment. After the ATP-dependent transport of the T segment, topoisomerase II reseals the break in the G segment by an essentially isoenergetic back-attack of the 3′-hydroxyl groups on the phosphotyrosyl linkages. The ATPase reaction is not required for G segment cleavage or religation, but is coupled in an unknown fashion to the transport of the T segment.

While all type II topoisomerases have the key mechanistic features described above, they can be divided into two subfamilies based upon their biological functions and differences in DNA interaction. Enzymes in the first subfamily are primarily required to decatenate interlinked DNA and include all eukaryotic type II topoisomerases as well as bacterial topoisomerase IV and phage T4 topoisomerase II. Enzymes in this subfamily can also relax positively and negatively supercoiled DNA (*8*−*10*) and require ATP for all of these reactions. The second subfamily is comprised of bacterial gyrases that negatively supercoil DNA in the presence of ATP (*11*), and can slowly relax supercoils in its absence (*12*, *13*). While gyrases clearly utilize the energy of ATP to increase the free energy of DNA, the role of ATP for the decatenating enzymes has been less clear. It had been previously assumed that the decatenating enzymes simply passed one duplex of DNA through another DNA duplex with no preference for directionality, resulting in DNA topological equilibrium. By this scenario, the enzymes perform no work. It has recently been demonstrated, however, that these enzymes simplify the DNA topology beyond equilibrium, showing that they use ATP to do work (*14*). Consequently, based on these studies, all type II topoisomerases can be classified as motor proteins that utilize the energy stored in ATP to alter the topological state of DNA away from that at equilibrium.

Our knowledge of how type II topoisomerases use ATP in their reaction pathways is incomplete. Previous studies to address the mechanism of ATP hydrolysis have been limited to steady-state kinetics and the use of nonhydrolyzable ATP analogues. The ATPase activity of the enzyme isolated from several different organisms is stimulated by binding to DNA (*8*, *15*); yeast topoisomerase II is stimulated about 20-fold by DNA, regardless of the DNA's topological state (*16*). When bound to DNA, the yeast enzyme binds ATP with positive cooperativity, and hydrolyzes ATP with an approximate k_{cat} of 5−10 s^{-1} per enzyme dimer. When the concentration of ATP is well below $S_{0.5}$ ($S_{0.5}$ is similar to K_m for cooperative enzymes), making the binding of ATP rate-determining, two ATP are hydrolyzed for every measur-

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* To whom correspondence should be addressed. E-mail: jlindsley@msscc.med.utah.edu. FAX: (801) 581-7959.

‡ Present address: Molecular Informatics, Incorporated, 1800 Old Pecos Trail, Suite M, Santa Fe, NM 87505.

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able DNA transport event (*16*). When the concentration of ATP is saturating, many more ATP are hydrolyzed per transport event. These observations suggest that ATP hydrolysis and DNA transport are coupled, but the degree to which they are linked can vary based upon the reaction conditions.

Although steady-state data have provided much useful information about the ATPase activity of topoisomerase II, they cannot provide direct mechanistic information on how ATP is used by the enzyme. The steady-state rate of a reaction reflects only the slowest steps in the reaction cycle. Indeed, for a reaction such as the hydrolysis of ATP, the rates of all potentially interesting steps that occur between substrate binding and product release are often not reflected by the k_{cat} and K_m values (*17*).

To identify individual steps in the topoisomerase II mechanism, a single enzyme turnover must be studied. Previously this has been attempted by using nonhydrolyzable ATP analogues, particularly AMPPNP[1] (*7, 18*−*21*). AMPPNP stabilizes a major conformational change in the enzyme that most likely involves the dimerization of the ATPase domains (*22*−*24*). It has been proposed that free enzyme resembles an "open-clamp" to which DNA can bind (*7*). Upon binding of ATP or AMPPNP, the enzyme closes down around the DNA, forming a new dimer interface near its amino terminus (*23*). This process has been called "closing of the clamp" (*7*). Binding of AMPPNP to only half of the enzyme appears sufficient to stabilize the "closed clamp" conformation (*25*). This suggests that the positive cooperativity observed in steady-state ATPase kinetics can be explained by an ATP-induced shift in equilibrium between different conformational states of topoisomerase II. Most importantly, in the presence of AMPPNP, type II topoisomerases allow at most one DNA transport event per enzyme dimer (*18, 19, 26*). After the AMPPNP-induced reaction, the enzyme remains in the "closed clamp" conformation, surrounding one segment of DNA, presumably the G segment (*20*). Based on these studies, it has been assumed that topoisomerase II undergoes all of the conformational changes required for DNA transport upon the binding of two ATP. The hydrolysis of both ATP and release of the four products of hydrolysis (two ADP and two inorganic phosphates) were thought to be coupled to opening of the enzyme clamp as the last step of the reaction cycle. Recently it has been shown that nonhydrolyzable NTP analogues can allow reaction events to occur that normally are observed only after hydrolysis (*27*). If this is shown to be a universal phenomenon, then reliance on nonhydrolyzable analogues may lead to overly simplified reaction mechanisms.

To avoid the interpretive problems inherent with steady-state assays and nonhydrolyzable ATP analogues, we have begun a pre-steady-state analysis of ATP hydrolysis by yeast topoisomerase II. Observing a reaction before the steady state has been reached allows the researcher to follow events as they occur on the enzyme active site in a single turnover (*17*). We describe the use of rapid chemical quench techniques to follow the production of ADP by yeast topoisomerase II from 15 ms to 3 s after addition of ATP.

[1] Abbreviations: AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEP, phospho(enol)pyruvate; P_i, inorganic phosphate.

MATERIALS AND METHODS

Materials. Standard reagents were purchased from the following commercial resources: ATP, Pharmacia; [α-^{32}P]-ATP (3000 Ci/mmol), New England Nuclear; AMPPNP and ultrapure HEPES, Boehringer Mannheim; [2,8-^{3}H]AMPPNP (21 Ci/mmol), ICN; NADH, phospho(enol)pyruvate (PEP, trisodium salt hydrate), and pyruvate kinase (700 units/mL)/lactate dehydrogenase (100 units/mL) mixture from rabbit muscle, Sigma. All buffers were filtered (0.45 μm).

Preparation of the DNA. Because of the very large amounts of DNA required in the pre-steady-state reactions, purified and sheared salmon sperm DNA was used. After sonication, the salmon sperm DNA was sequentially extracted with buffered phenol, chloroform/isoamyl alcohol (24:1v/v), and finally ethanol-precipitated. The DNA was resuspended and dialyzed against reaction buffer [50 mM HEPES−KOH (pH 7.5), 150 mM KOAc, and 10 mM Mg(OAc)$_2$] for 48 h. The final concentration was 9.5 mM (base pair).

Expression and Purification of Saccharomyces cerevisiae Topoisomerase II. The enzyme was expressed to very high levels from the plasmid YEpTOP2-PGAL1 in the yeast strain BCY123 as previously described (*28*). The buffer used throughout purification, buffer I, was 50 mM HEPES−KOH, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol. Different concentrations of KCl were added for different steps; buffer I:100 is used to designate buffer I with 100 mM KCl. The cells were cracked as previously described (*29*). Following centrifugation of the lysate (30 min at 45000*g*), phosphocellulose (P11, Whatman; equilibrated with buffer I:100) was added at 1 mL (settled) per 10 mg of protein and stirred gently for 1 h on ice. The slurry was poured into a 5 cm diameter column and washed with 5 column volumes of buffer I:150 and 10 column volumes of buffer I:300 and eluted with buffer I:1000. Peak fractions were combined and diluted with buffer I to an ionic strength equal to buffer I:100. This was loaded onto a POROS QE column (equilibrated with buffer I:100, 1.6 mL, Perseptive Biosystems) at a flow rate of 1.6 mL/min. The column was washed with a linear gradient from 100 mM to 1 M KCl, and the topoisomerase eluted at approximately 700 mM KCl. The most concentrated fractions were pooled, diluted to an ionic strength equaling that of buffer I:100, and loaded on a POROS heparin column (equilibrated with buffer I:100, 1.6 mL, Perseptive Biosystems) at 5 mL/min. The column was washed with a linear gradient from 100 mM to 1 M KCl, and the topoisomerase eluted at approximately 700 mM KCl. The protein was stored in buffer I:700 at −70 °C. As estimated by Coomassie-stained SDS−PAGE analysis, the topoisomerase II purified by this method is greater than 95% pure. Typically, 7−8 mg of topoisomerase II could be purified from 1 L of cell growth. Each pre-steady-state time course required approximately 20 mg of pure enzyme.

Steady-State ATPase Assays. Steady-state ATPase assays were performed to determine the optimal reaction conditions for enzyme turnover. A coupled assay using pyruvate kinase, PEP, lactate dehydrogenase, and NADH was used as previously described (*16*). Reactions performed with 10 different ATP concentrations (25 μM−2 mM) were used to determine k_{cat} values. Based on studies involving varying KOAc and Mg(OAc)$_2$ concentrations, a reaction buffer was

chosen that reproducibly allows maximum enzyme turnover. This reaction buffer was used in all subsequent experiments and included 50 mM HEPES−KOH (pH 7.5), 150 mM KOAc, and 10 mM $Mg(OAc)_2$. To compare DNA stimulation of ATP hydrolysis rates by DNA from different sources, rates of hydrolysis at 1 mM ATP were observed for topoisomerase II in the presence of either plasmid or salmon sperm DNA. Assays were performed at 25 °C.

Reaction Conditions for Pre-Steady-State Assays. To achieve the high protein concentration needed to produce a large signal in the first turnover, purified topoisomerase II was precipitated in 60% ammonium sulfate and resuspended in a minimal volume of reaction buffer. DNA was added to a ratio of ∼200:1 base pairs:enzyme dimer, and the solution was dialyzed against reaction buffer for 4 h (50 000 MWCO dialysis membrane). It was important to remove all glycerol associated with the enzyme so that maximal mixing speeds could be achieved. Following dialysis, three small aliquots of the topoisomerase II/DNA mixture were removed to determine protein concentration and steady-state ATPase activity. The first aliquot was used to determine total protein concentration (Bio-Rad Protein Assay), where 1 M KCl final concentration was added to help dissociate the DNA from topoisomerase II. The second aliquot was used to determine the concentration of ATP active sites. The topoisomerase II/DNA solution was mixed with (^{3}H)-AMPPNP (1000 dpm/pmol), a control aliquot was removed, and the remaining solution was loaded into a spin filter (MWCO 10 000, ultra-free-MC, Millipore) to separate free (^{3}H)AMPPNP from topoisomerase II-bound (^{3}H)AMPPNP. The radioactivities of equal volumes containing the control, bound, and free AMPPNP were measured by liquid scintillation counting (Bio-Safe II counting cocktail, Beckman LS 5000 TD). The concentration of ATP active sites was determined using the equation:

$$[\text{active site}] = [\text{AMPPNP}_{\text{initial}}]\left(\frac{\text{cpm}_{\text{retained}} - \text{cpm}_{\text{flow-thru}}}{\text{cpm}_{\text{control aliquot}}}\right)$$

where cpm represents the counts per minute of the (^{3}H)-AMPPNP from various aliquots. This method provided estimates of the total ATP active site concentration to within ±1 μM. The third aliquot was used for steady-state ATPase assays to estimate the k_{cat} of the particular topoisomerase II/DNA solution to be used for pre-steady-state experiments.

Pre-Steady-State Assays. The KinTek Model RQF-3 rapid quench apparatus (*30*) was used to assay the ATPase activity of topoisomerase II in the time range of 15 ms to 3 s. One of the two 40 μL sample loops was filled with the topoisomerase II/DNA solution; the other was filled with [α-^{32}P]ATP (0.01 μCi/μL) in reaction buffer. The enzyme and substrates were rapidly mixed to initiate the reactions, halving their initial concentrations. In the various time courses performed, the final topoisomerase II dimer concentration ranged from 5 to 16 μM and the final ATP concentration ranged from 25 to 1200 μM, as indicated in the figure legends. For the chemical quench experiments, at the indicated time points the reactions were quenched by the rapid addition of 250 mM EDTA in 100 mM Tris base (pH 10) through the quench line of the instrument. SDS (1% final) was present in the sample collection tubes to ensure that no residual enzyme activity persisted. The substrate and product were stable in this quench solution. The efficiency of the quench solution was determined by loading the topoisomerase II/DNA solution into one sample loop, the quench solution into the other sample loop, and the [α-^{32}P]ATP solution into the quench line (*31*). In this configuration, the enzyme solution was then rapidly mixed with the quench solution for 3 ms prior to addition of the ATP solution. No ADP product formation was ever observed in these controls, indicating that the quench completely inactivates the enzyme within 3 ms. Pulse−chase experiments were performed just as the chemical quench experiments except that at the indicated time points 10.6 mM unlabeled ATP in reaction buffer was mixed into the reactions from the quench line. The final unlabeled ATP concentration always exceeded the labeled ATP concentration by at least 10-fold. The reactions were allowed to proceed for an additional 1.5 s (∼10 turnovers) before being expelled into sample collection tubes containing the chemical quench solution of EDTA/Tris/SDS. For both the chemical quench and the pulse−chase experiments, all time points were randomly ordered to remove any potential errors due to enzyme decay. All reactions were performed at 25 °C.

Product Analysis. The amount of ATP hydrolyzed to ADP at each reaction time point was determined by separating the substrate and product using thin-layer chromatography (*16*). PEI cellulose F sheets (EM Separations Technology) were cut into 5 × 20 cm strips, and 1 × 5 cm lanes were etched into them. Aliquots (0.8 μL) from each quenched time point were spotted 1 cm from the bottom of the cellulose sheet, one per lane, and allowed to dry. Separation was performed in 1 M formic acid and 0.5 M $LiCl_2$. [α-^{32}P]-ATP and [α-^{32}P]ADP spots were quantified by phosphorimage analysis [Molecular Dynamics Phosphor Imager TM Model 400 with ImageQuant (version 3.3) software]. Product formation was equal to the radioactivity corresponding to ADP divided by the total radioactivity (from both the ADP and ATP spots). All reactions were spotted 5 times, and the average of the calculated concentrations of ADP is presented. ADP concentrations for all reported reactions have a standard deviation ≤5%.

Data Analysis. Steady-state ATPase data were fit to the Michaelis−Menten equation using GraFit version 3.0 (Erithacus software). Pre-steady-state data were all fit to single-exponential equations with a linear term, $A(1 - e^{-Bt}) + Ct$, using SigmaPlot version 3.0 (Jandel software).

RESULTS

Optimizing Reaction Conditions. Prior to examining the pre-steady-state ATPase activity of yeast topoisomerase II, the optimal reaction conditions were established using steady-state analysis. The optimal concentration of KOAc was determined as shown in Figure 1A. The ATPase activity measured in the presence of DNA is very sensitive to KOAc concentration, peaking at approximately 200 mM. The ATPase rate in the absence of DNA is effectively insensitive to KOAc concentration. All subsequent experiments were performed at 150 mM KOAc. At 150 mM KOAc, optimal ATPase activity was observed at $Mg(OAc)_2$ concentrations ranging from 5 to 12 mM (data not shown); 10 mM $Mg(OAc)_2$ was used in all subsequent experiments. The pre-steady-state experiments require a very large amount of

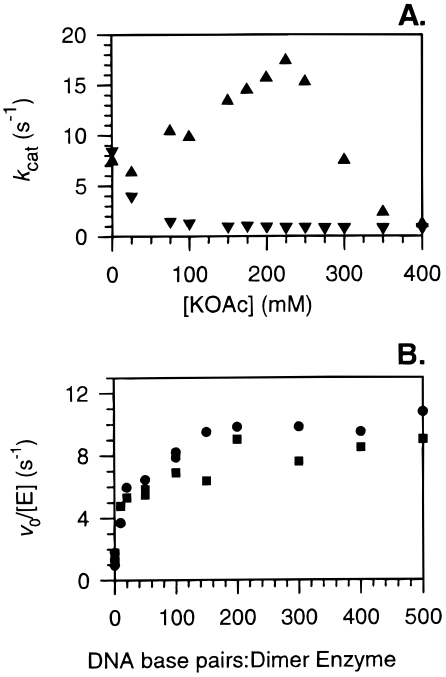


FIGURE 1: Defining optimal reaction conditions for ATP hydrolysis by yeast topoisomerase II. (A) The ATPase activity as a function of KOAc concentration is shown for topoisomerase II in both the presence (▲) and absence (▼) of DNA. The values of k_{cat} were determined from initial velocities at 10 ATP concentrations. (B) The stimulation of ATPase activity by two different DNA substrates, sheared salmon sperm DNA (■) and supercoiled plasmid DNA [pHC624, (*29*)] (●), was examined at various ratios of DNA base pairs to dimeric topoisomerase II concentration.

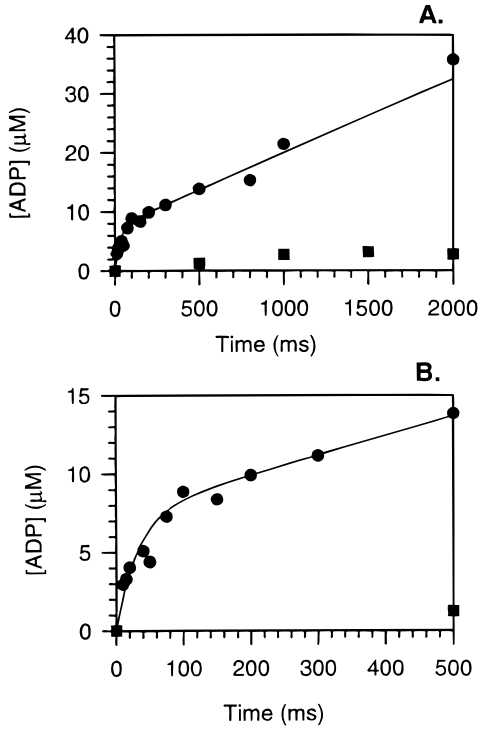


FIGURE 2: Pre-steady-state ATP hydrolysis by topoisomerase II in the presence (●) and absence (■) of DNA. The full time course is shown in (A), while an expanded time scale is shown in (B). The reaction time course performed in the presence of DNA (1.7 mM base pairs, final) contained final ATP and topoisomerase II concentrations of 300 and 8.7 μM, respectively. These data were fit as described under Materials and Methods with $A = 8.0 \pm 0.6$ μM, $B = 31 \pm 4$ s^{-1}, and $C = 13 \pm 1$ μM s^{-1}. In the absence of DNA, the reaction contained final ATP and topoisomerase II concentrations of 1200 and 6.1 μM, respectively. In the absence of DNA, the full time course included data up to 8 s; for clarity, only the first 2 s is shown. At the later time points, the production of ADP continued in a linear fashion.

DNA, making plasmid DNA an impractical source. To ensure that sheared salmon sperm DNA would stimulate the ATPase activity of topoisomerase II as well as plasmid DNA, these two DNA cosubstrates were compared, as shown in Figure 1B. Both forms of DNA stimulate the ATPase activity of topoisomerase II equally well. A hyperbolic trend in the initial ATPase rate was observed as either DNA cosubstrate concentration was increased. Once the DNA base pairs:enzyme dimer ratio reached ~200:1, the ATPase rate no longer increased with increasing DNA. This is the ratio used in the pre-steady-state experiments.

Chemical Quench Analysis. The observed signal during the first turnover of an enzyme-catalyzed reaction cannot exceed the number of enzyme active sites present. The maximum signal will also be generated when the enzyme is saturated with substrate. Topoisomerase II is thought to bind ATP with positive cooperativity, such that the enzyme is predicted to have at least a 10-fold greater affinity for the second ATP in comparison to the first ATP bound (*16*). The $S_{0.5}$ for ATP, the ATP concentration at half-maximal velocity, in the presence of DNA is ~130 μM. Therefore, at concentrations of only a few fold greater than $S_{0.5}$, topoisomerase II should be nearly saturated with ATP. Because it is difficult to accurately measure <0.5% conversion of ATP to ADP, it was essential to perform these experiments at the highest possible topoisomerase II concentration (5−16 μM dimer) and the lowest possible ATP concentration where the enzyme is still nearly saturated with ATP (generally 300−400 μM).

Chemical quench ATPase time courses for topoisomerase II prebound to DNA (●) and in the absence of DNA (■) are shown in Figure 2. A rapid burst in ADP production is seen in the first 100 ms of the reaction containing DNA. This burst indicates that the rate-determining step for ATP turnover is after ATP hydrolysis. By fitting the data from the reaction containing DNA to a simplified, two-step mechanism, the initial burst phase is calculated to be ~18 times faster than the slower second phase. The rate of the slow phase is approximately equal to the steady-state rate of the reaction at 300 μM ATP. No detectable burst is seen in the absence of DNA. The $S_{0.5}$ for ATP in the absence of DNA is approximately 5-fold higher than in the presence of DNA. Consequently, higher concentrations of ATP were used in attempts to saturate the enzyme. The highest ATP concentration at which a signal could be detected above the noise is 1200 μM; this is the ATP concentration used in the minus DNA reaction time course shown in Figure 2. It is unclear if the lack of a burst is the result of topoisomerase II remaining unsaturated at the highest experimental ATP concentration or if it is due to a reduced rate of ATP hydrolysis in the absence of DNA. However, it is clear that in the absence of DNA either ATP binding or hydrolysis is rate-determining. DNA binding, therefore, stimulates the ATPase activity of topoisomerase II primarily by increasing the affinity for ATP and/or the rate constant for ATP hydrolysis.

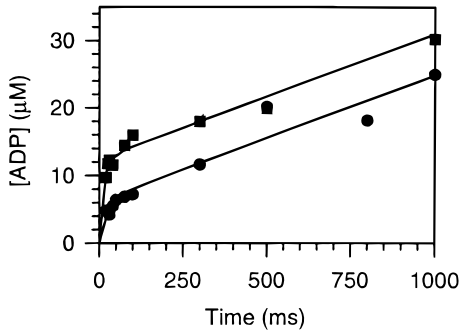


FIGURE 3: Pulse−chase versus chemical quench time courses for ATP hydrolysis for DNA-bound topoisomerase II. In both reaction time courses, 6.75 μM topoisomerase II dimer prebound to 1.35 mM DNA (base pairs) was reacted with 300 μM radiolabeled ATP. For the chemical quench reaction (●), the data points indicate the times at which the reaction was quenched. For the pulse−chase reaction (■), the time points indicate when 10.6 mM unlabeled ATP was added; these reactions were chemically quenched 1.5 s later. The data were fit as described under Materials and Methods; for the chemical quench time course, $A = 6.3 \pm 0.7$ μM, $B = 37 \pm 4$ s^{-1}, and $C = 18 \pm 1$ μM s^{-1}, while for the pulse−chase time course $A = 12.4 \pm 0.6$ μM, $B = 80 \pm 10$ s^{-1}, and $C = 18 \pm 1$ μM s^{-1}.

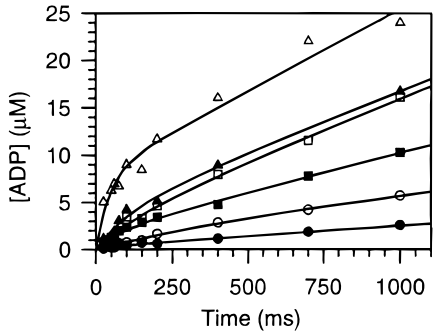


FIGURE 4: Chemical quench reaction time courses of ATP hydrolysis by DNA-bound topoisomerase II at various ATP concentrations. The final ATP concentrations were 25 μM (●), 50 μM (○), 100 μM (■), 150 μM (□), 200 μM (▲), and 400 μM (△). The final topoisomerase II dimer and DNA concentrations were 8.2 μM and 2 mM, respectively. All data were fit as described under Materials and Methods.

Topoisomerase II is a homodimer, such that the number of ATP active sites is twice the active enzyme concentration. It is striking that the amplitude of the burst phase (8 μM) for the reaction containing DNA is approximately equal to half of the ATP active sites (17.2 μM) as measured by (^{3}H)-AMPPNP titration (see Materials and Methods). This experiment has been repeated a minimum of 12 times, and the burst amplitude is always within 15% of half the active site concentration. There are at least three possible explanations for these results: (1) although essentially 100% active in ATP binding, 50% of the enzyme population is inactive in ATP hydrolysis; (2) the rate of ATP hydrolysis at the enzyme active site equals the rate of ATP synthesis; and (3) the topoisomerase II dimer rapidly hydrolyzes only one of the two bound ATP. The following set of experiments limits these possibilities.

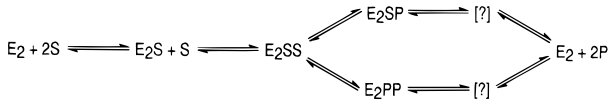
Pulse−Chase Analysis. If instead of chemically quenching the ATPase reaction a large excess of unlabeled ATP, a chase, is added at the indicated time point, the enzyme can either continue to hydrolyze any labeled ATP that it had bound prior to the chase or release the unhydrolyzed ATP. Any new ATP that the enzyme binds will be unlabeled and therefore undetectable in the assay system. The reaction is allowed to continue for greater than 10 turnovers before it is chemically quenched. Pulse−chase (■) and chemical quench (●) time courses using the same topoisomerase II/DNA preparation are shown in Figure 3. The chemical quench results are similar to those observed in Figure 2, where the burst amplitude (6.3 μM) is approximately equal to half the ATP active site concentration (13.5 μM). In the pulse−chase experiment, there is again a rapid burst in ADP production, followed by a slower, second phase of the reaction. The burst rate is at least 2-fold faster for the pulse−chase results in comparison to the chemical quench results. The burst amplitude in the pulse−chase experiment (12.4 μM) approximately equals the enzyme active site concentration, twice that observed for the chemical quench experiment. This result indicates that two ATP bind to each topoisomerase II dimer, and each is hydrolyzed within a single reaction cycle. Under these reaction conditions, close to 100% of bound ATP partitions to hydrolysis, and almost none dissociates from the enzyme. These results also indicate that the enzyme is nearly 100% active, eliminating half inactive enzyme as a possible explanation for the chemical quench burst amplitude.

Pre-Steady-State Analysis of ATP Binding. To analyze the steps of ATP binding to the topoisomerase II/DNA complex, chemical quench reactions were carried out in the presence of various ATP concentrations. Using one topoisomerase II/DNA preparation, a series of chemical quench experiments was performed with ATP concentrations varying between 25 and 400 μM as shown in Figure 4. These results show that an obvious burst in ADP production is only observed if the ATP concentration is greater than 100 μM. At ATP concentrations below 100 μM, the rate of ATP binding becomes at least partially rate-determining, similar in magnitude to the rate of the slow step after hydrolysis. It is clear that a transition between no clear burst and a burst occurs over a small range of ATP concentrations (100−300 μM), consistent with the positive cooperativity in ATP binding noted previously. This transition occurs at ATP concentrations very close to the $S_{0.5}$ (~130 μM). A simple K_d for the binding of two ATP cannot be extracted from these data because neither an accurate estimate of K_d nor a quantitative measure of the positive cooperativity of binding two ATP exists. Consequently, replots of neither the burst rates nor burst amplitudes vs ATP concentrations can be used to determine K_d and are therefore not shown (*32*). A plot of the burst amplitudes versus the ATP concentration squared is roughly hyperbolic, consistent with the binding of two ATP.

DISCUSSION

Yeast topoisomerase II bound to DNA displays burst kinetics in hydrolyzing ATP. When the ATP concentration is saturating, this enzyme binds two ATP and hydrolyzes them both before either can dissociate. These results directly show that at least one ATP is rapidly hydrolyzed before the rate-determining step in the reaction cycle is encountered. In the absence of DNA and at the highest possible experimental ATP concentrations, no burst in ADP production by topoisomerase II is detected. This indicates that when topoisomerase II is not bound to DNA, the rate-determining

Scheme 1



step in the ATPase reaction cycle is either ATP binding or hydrolysis. Therefore, DNA binding by topoisomerase II increases the rate of either ATP binding or ATP hydrolysis such that some step after these becomes rate-determining.

Our pre-steady-state chemical quench and pulse−chase results show directly that the topoisomerase II/DNA complex binds two ATP and hydrolyzes both per enzyme turnover. In the chemical quench experiments, the burst amplitude is equal to half the enzyme active site concentration, while in the pulse−chase experiments the burst amplitude is equal to the total ATP active site concentration. There are two possible mechanistic interpretations of these data, as illustrated in Scheme 1.

In this scheme, E_2 represents dimeric topoisomerase II bound to DNA, S represents ATP, and P represents ADP·P_i. One possible interpretation, as illustrated by the top pathway of Scheme 1, is that the chemical quench burst amplitude is produced by the rapid hydrolysis of one of the two bound ATP. By this pathway, the doubling of burst amplitude seen in the pulse−chase results is explained by the hydrolysis of the second ATP later in the reaction pathway. The present results do not address whether the products of the first hydrolysis would be released prior to the second being hydrolyzed, hence the question mark. By this mechanism, the rate-determining step could be associated with either release of the first ADP·P_i or hydrolysis of the second ATP. The second possible interpretation is illustrated by the bottom pathway. This simultaneous hydrolysis mechanism could only fit the chemical quench and pulse−chase data if all steps following formation of the E_2SS complex are highly reversible under our reaction conditions. This means that the rate constants for ATP hydrolysis and ATP synthesis must be equal. Additionally, if P_i is rapidly released from the E_2PP complex, it must rapidly rebind, even though the free P_i concentration is near zero in these experiments. Only if these two constraints are true could the burst amplitude double in the pulse−chase as compared to the chemical quench data. Although these constraints seem unlikely, they cannot be ruled out with the present data.

Steady-state ATPase analysis of yeast topoisomerase II (*16*) and AMPPNP binding studies with DNA gyrase (*33*, *24*) and yeast topoisomerase II (*25*) suggest that these enzymes bind two ATP with positive cooperativity. This is confirmed by the pre-steady-state chemical quench results at various ATP concentrations. By varying the ATP concentration over only a narrow range (100−300 μM), the concentration of enzyme saturated with ATP changes dramatically. ATP binding is partially rate-determining at 100 μM ATP, as indicated by the lack of a burst in ADP production. However, at 300 μM ATP, a clear burst is observed. At this higher ATP concentration, the rate of the burst phase is ∼15-fold faster than the rate of the steady-state phase, indicating that ATP binding is now much faster than the rate-determining step in the mechanism.

The pre-steady-state results described in this paper were made possible by the development of methods to purify very large amounts of yeast topoisomerase II (*29*). The starting and final enzyme concentrations in these experiments were on average 5 and 2.5 mg/mL, respectively; at lower enzyme concentrations, the burst in ADP production is obscured. We know that yeast topoisomerase II is only detectably present as a dimer up to concentrations of 0.4 mg/mL (*28*); above this concentration, it is unknown if the dimers interact to form higher order multimers. Although we cannot rule out the possibility that topoisomerase II dimers are interacting at the high required concentrations, the pre-steady-state data described in this and the following paper are together most consistent with the enzyme acting as a dimer.

The results described in this paper indicate that topoisomerase II rapidly hydrolyzes at least one ATP. These findings are not in obvious agreement with models of the enzyme mechanism predicted from results of experiments using nonhydrolyzable ATP analogues. These models were often drawn with the enzyme bound to two ATP throughout most of the reaction cycle. The present pre-steady-state results show that the enzyme in fact spends only a small fraction (<10%) of the ATPase reaction cycle bound to two ATP. It seems unlikely that all of the protein and DNA conformational changes that are required for DNA transport will all occur within the rapid phase of the reaction, prior to ATP hydrolysis.

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