DNA topoisomerase II catalyzes the transport of one DNA duplex through a transient break in a second duplex using a complex ATP hydrolysis mechanism. Two key rates in the ATPase mechanism, ATP resynthesis and phosphate release, were investigated using $^{18}$O exchange and stopped-flow phosphate release experiments, respectively. The $^{18}$O exchange results showed that the rate of ATP resynthesis on the topoisomerase II active site was slow compared with the rate of phosphate release. When topoisomerase II was bound to DNA, phosphate was released slowly, with a lag. Since each of the preceding steps is known to occur rapidly, phosphate release is apparently a rate-determining step. The length of the lag phase was unaffected by etoposide, indicating that inhibiting DNA religation inhibits the ATPase reaction cycle at some step following phosphate release. By combining the $^{18}$O exchange and phosphate release results, the rate constant for ATP resynthesis can be calculated as $-0.5 \text{ s}^{-1}$. These data support the mechanism of sequential hydrolysis of two ATP by DNA topoisomerase II.

DNA topoisomerase II is a ubiquitous enzyme essential for unlinking intertwined chromosomes and altering DNA topology (for reviews, see Refs. 1 and 2). It catalyzes the ATP-dependent transport of one DNA segment through a transient break in a second DNA segment (3, 4). The eukaryotic topoisomerase II enzymes are homodimers and are therefore relatively simple macromolecular machines for studying the coupling of ATP hydrolysis to protein and DNA movements. In addition to being mechanistically interesting enzymes, prokaryotic and eukaryotic type II topoisomerases are the targets of several antibiotic and anticancer drugs (5, 6).

Rapid-quench kinetic studies of topoisomerase II from the yeast Saccharomyces cerevisiae have revealed that this enzyme utilizes an unusual sequential ATP hydrolysis mechanism (7, 8). While the topoisomerase, pre-bound to DNA, is rapidly mixed with labeled ATP, followed by a chase with a large excess of unlabeled ATP, a burst of labeled ATP hydrolysis is seen within 50 ms (8). The burst amplitude equals the enzyme active site concentration. These pulse-chase results show that the dimeric topoisomerase II rapidly binds two ATPs and hydrolyzes them both before either can dissociate. By contrast, when the reactions are chemically quenched, as opposed to being chased with unlabeled ATP, the burst amplitude equals half of the enzyme active site concentration (8). This was the first evidence that topoisomerase II binds two ATPs, but only hydrolyzes one rapidly, before a rate-determining step in the mechanism. The results of studies using inhibitors and heterodimeric mutants confirmed this sequential mechanism and suggested that release of the first Pi or ADP was the rate-limiting step in the mechanism (7, 9). The rate of DNA transport was determined from rapid quench decationation experiments and is consistent with transport occurring after hydrolysis of one ATP and before hydrolysis of the second (9). Mutants that can bind two ATP but only hydrolyze one catalyze DNA transport at approximately the same rate as the wild type enzyme. Mutants that bind two ATP, but hydrolyze neither, transport DNA at a much slower rate. Taken together, these results indicate that yeast topoisomerase II binds two ATPs, hydrolyzes one, transports DNA, and releases the products of the first hydrolysis, and, finally, hydrolyzes the remaining ATP.

Two aspects of this complex ATPase mechanism that remain unknown are the rates of ATP resynthesis and Pi release. The rate of ATP resynthesis at the ATPase active site of topoisomerase II has been assumed to slow in comparison to the rate of ATP hydrolysis (7). In fact, a rapid resynthesis rate would provide an alternative explanation for the chemical quench burst amplitude equal to half of the ATP active site concentration (8). ATP resynthesis was analyzed using the classical method of $^{18}$O exchange (10). In order to interpret these results in terms of a resynthesis rate constant, the rate constant of the following step, Pi release, must be known. The rate of Pi release was measured by stopped-flow using a fluorescent Pi sensor (11). Together, these results help to complete our understanding of how DNA topoisomerase II utilizes ATP to transport DNA segments.

**EXPERIMENTAL PROCEDURES**

Materials—Bacterial purine nucleoside phosphorylase (PNPase), $^{1}$ 7-methylguanosine (MEG), myosin subfragment 1 (S1-myosin), phosphate standard, Trizma (ultrapure), and potassium acetate (ultrapure).

$^{1}$ The abbreviations used are: PNPase, purine nucleoside phosphorylase; MEG, 7-methylguanosine; S1-myosin, myosin subfragment 1; MDCC, 7-diaminomaleimidomaleimido-3-aminocarbonyl-coumarin; PBP, phosphate-binding protein; PBP-MDCC, phosphate-binding protein with a 7-diaminomaleimidomaleimido-aminocarbonyl-coumarin attached to the single cysteine engineered at residue 197; HPLC, high performance liquid chromatography; TMS, trimethylsilyl; GC-MS, gas chromatography-mass spectroscopy.
were obtained from Sigma. HPLC-grade water and chloroform, as well as N-(trimethylsilyl)diethylamine, were from Aldrich. 7-Diethylamino-3-(2-maleimidyl) ethyl)amino-1-carboxyloumarin (MDCD) was purchased from Molecular Probes (Eugene, OR). [γ-32P]ATP was synthesized as described previously (12); the overall 32P enrichment was >95% and purity was >99%, as determined by HPLC analysis. S. cerevisiae topoisomerase II was prepared as described (9); all topoisomerase II concentrations given are for the dimer. Phosphate release reactions were performed in HEPES reaction buffer (20 mM HEPES-KOH (pH 7.0), 175 mM KOAc, and 10 mM Mg(OAc)2), except where indicated. Oxygen exchange reactions were performed in Tris reaction buffer (20 mM Tris-HCl (pH 7.0), 150 mM KOAc, and 10 mM Mg(OAc)2).

Intermediate 18O Exchange—Exchange reactions were performed with 10 μM topoisomerase, 10 μM sheared salmon sperm DNA (base pairs), and 500 μM [γ-18O]ATP at room temperature in 1 ml of Tris reaction buffer. This was used instead of HEPES, as it did not significantly add to the ionic strength of the solution, which would interfere with subsequent Pi isolation. The reactions were allowed to proceed until 90% of the ATP had been hydrolyzed, as measured by the malachite green assay (13). Similar final results were obtained when the reactions were stopped after 40% hydrolysis (data not shown). The control reactions contained 50 μM S1-myosin, 500 μM [γ-18O]ATP, 20 mM Tris-HCl (pH 8.0), 20 mM KCl, and 5 mM CaCl2. The reactions were stopped by vortexing with chloroform. Following extraction of the aqueous layer, Pi was isolated from each reaction using ion exchange chromatography (14). The reactions were diluted 10-fold (ionic strength ≤ 0.01) with HPLC-grade H2O, made basic by the addition of 1 ml of 1 M Tris-Cl (pH 8.5) and loaded onto a 0.5 × 3.0 cm AG1-X4 column (100–200-mesh, Cl- form, Bio-Rad). After the column was washed with 5 ml of H2O and 4 ml of 10 mM HCl, Pi was eluted with 10 ml of 50 mM HCl. The purified Pi samples were dried by lyophilization.

PBP was converted to the TMS derivative as described (10) and analyzed for 18O content by GC-MS. Briefly, the lyophilized samples were resuspended in 50 μl of glass-distilled CH2Cl2 and 5 μl of N-(trimethylsilyl)diethylamine. The solution was shaken occasionally for at least 1 h at room temperature. The TMS-PBP samples were analyzed by gas chromatography-mass spectrometry using an Agilent (Wilmington, DE) model 5973 GC-MS. Chromatographic separation was achieved using a 15 m × 0.32 μm (inner diameter) DB-5 column (J & W Scientific, Folsom, CA). The column temperature was programmed from 70 °C to 275 °C at 15 °C/min. The carrier gas was helium at a flow rate of 3.0 ml/min. The injection port temperature was 200 °C, and the transfer line temperature was 280 °C. For the mass spectrometry analysis, positive chemical ionization was employed. The ion source temperature was maintained at 230 °C. The argon gas was employed as the reagent gas. Selected ion monitoring for the isolation of the labeled Pi was performed at m/z 305, 317, 319, and 321 and was used to calculate the isotopic distribution of TMS-PBP. Corrections were made for the isotopic spillover due to the silicon in the TMS derivative of PBP. (10). The isotopic distribution in the original [γ-18O]ATP was determined by examining the 18O distribution in the Pi produced by the Ca2+-ATPase activity of S1-myosin. S1-myosin in the presence of Ca2+ has been shown to hydrolyze ATP to ADP and Pi, with the incorporation of only one water oxygen in the product Pi (15, 16).

The observed 18O distributions in the product Pi were compared with those of the Ca-ATPase activity of S1-myosin, and theoretical distributions were calculated based on transition probability functions with a computer program kindly provided by David Hacksney (Carnegie Mellon University).

Phosphate Release Assay—PBP release from topoisomerase II was measured using an assay developed by Webb and co-worked by us (11, 17). The Escherichia coli strain ANCC25 transformed with a plasmid containing the A197 mutant phoS gene (pSN51827) was a kind gift of M. Webb (Medical Research Council, London, United Kingdom). The single cysteine mutant phosphate-binding protein (PBP) was expressed and purified as described (11, 18). Two liters of cells yielded 150 mg of PBP at a concentration of 170 μM as determined by absorbance at 280 nm (εmax, 60,880 M-1 cm-1). Protein was frozen in liquid nitrogen in 1-ml aliquots. Labeling of the single-cysteine containing PBP with MDCC and purification of the labeled protein were performed as described except that the "phosphate mop" included only PNase and MEG. The 280/430 nm absorbance ratio of the final protein was 1.6, indicating that the majority of the PBP was labeled (11). When PBP was added to this preparation of PBP-MDCD, the emission maximum shifted from 475 nm to 467 nm and the fluorescence increased 6-fold. When PBP was titrated into 5 μM PBP-MDCD, the fluorescence responded linearly to 3.5–4.0 μM PBP, suggesting that 70–80% of the protein was active, labeled PBP. These measurements are in agreement with those reported previously (11).

Phosphate Release Kinetics of Topoisomerase II—Phosphate release kinetics were measured using a KinTek model SF-2001 stopped-flow apparatus with excitation at 425 nm through 4-nm slits. Emission was measured after a 450-nm cutoff long-wave pass filter (Corion, LL-450-F). Since PBP-MDCC is sensitive to nanomolar quantities of Pi, great care was taken to remove contaminating Pi from all solutions and glassware. In addition to using ultrapure chemicals and plastic vessels whenever possible, a coupled enzymatic phosphate mop was added to all solutions to remove contaminating Pi. This phosphate mop uses purified nucleoside phosphorylase (PNPase) with 7-methylguanosine (MEG) to sequester Pi, as ribose 1-phosphate. Assay conditions were adjusted to ensure the phosphate mop did not compete with PBP-MDCC for phosphate (kcat/KM for the PNPase reaction with Pi is 3.2 × 108 M-1 s-1); all reaction solutions contained 0.1 units/ml PNPase and 0.15 mM MEG. Prior to the experiment, the stopped flow was preincubated with a concentrated phosphate mop (0.5 units/ml PNPase, 0.5 mM MEG) in HEPES reaction buffer. Topoisomerase II (0.5 or 2 μM, as indicated) and PBP-MDCC (10 μM) in HEPES reaction buffer were loaded in one syringe. ATP (100–2000 μM) in the same buffer was placed in a second syringe. Where indicated, sheared salmon sperm DNA (400 μg) was added to the topoisomerase II/PBP-MDCD solution. The relative change in fluorescence of PBP-MDCD as it bound Pi, released from the hydrolysis of ATP by topoisomerase II was measured. The traces shown were averaged from three time courses. To relate the relative change in fluorescence to concentration of Pi released, the topoisomerase II/PBP-MDCD enzyme solution was mixed with excess KH2PO4 (200 μM). The fluorescence emission in volts at saturation was equated to the active concentration of PBP-MDCD.

Pre-steady-state data were fit to Equation 1 describing a lag phase preceding a linear steady-state phase (19).

\[
y = \frac{a}{\alpha} (t) + \frac{b}{\alpha} (e^{-\alpha t} - 1)
\]

From the solutions of a and b in this equation, the duration of the lag phase (1/α) and the steady-state rate (β/α) were calculated. The data were also fit by global analysis to the mechanism shown in Scheme 2 using the computer program DynaFit version 3.22.01 (BioKin Ltd, Madison, WI) (20).

RESULTS AND DISCUSSION

Analysis of ATP Synthesis by DNA Topoisomerase II Using 18O Exchange—Direct measurement of ATP synthesis by DNA topoisomerase II has not been successful, probably because of its complex reaction mechanism and high Kd for Pi (7). Therefore, the less direct method of 18O exchange was used. This technique has been used to probe the mechanism of many ATPases and was instrumental for Boyer and colleagues (21) to determine the binding-change mechanism of the F1F0 ATPase. ATP labeled with 18O at the γ-phosphate ([γ-18O]ATP) is incubated with the ATPase in buffer made from [18O]H2O. The initial hydrolysis of the [γ-18O]ATP results in the incorporation of one unlabeled, water-derived oxygen into the resulting Pi (see Scheme 1 for a simplified mechanism). If ATP is resynthesized on the enzyme before Pi is released, then the subsequent hydrolysis event will introduce another unlabeled oxygen into Pi, with a constant probability.

In Scheme 1, E, S, and D represent topoisomerase II, ATP, and ADP, respectively. Although this is clearly a simplified scheme, showing binding and hydrolysis of only one ATP, it is adequate to describe the topoisomerase II reaction from the perspective of 18O exchange. The average number of unlabeled oxygens that appear in each Pi formed (18O/Pi ratio) is a function of the partitioning coefficient (Pp) (22). The partitioning
ATP Resynthesis and $P_i$ Release by Topoisomerase II

The topoisomerase ATP active site. Essentially the same results were found when the ATPase reaction was performed in the absence of DNA (data not shown). The rate constant for ATP resynthesis could be estimated from the $P_i$ value if the rate constant of $P_i$ release were known. Therefore, the rates of $P_i$ release were next measured.

Kinetics of Phosphate Release—A sensitive fluorescent sensor for inorganic phosphate has been developed by Webb and colleagues (11, 18). The sensor is a labeled derivative of the E. coli phosphate-binding protein (PBP-MDCC). $P_i$ binding to PBP-MDCC is rapid (1.36 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$) and tight ($K_p$ = 0.1 $\mu$M) and increases the fluorescence emission 5-fold at 470 nm (11). Because of this tight and rapid binding, the kinetics and magnitude of observed fluorescence changes can be directly related to the rate and stoichiometry of $P_i$ release from the examined ATPase.

Fig. 2 shows the changes in fluorescence with time as PBP-MDCC bound the $P_i$ released from topoisomerase II. In these experiments the topoisomerase II was pre-bound to DNA. An initial lag in $P_i$ release is observed under all ATP concentrations tested (50, 350, 500, and 1000 $\mu$M are shown). All four data sets are shown fit to Equation 1 (see “Experimental Procedures”), an equation that describes a lag phase followed by a linear, steady-state phase. The initial lag in $P_i$ release lasts 48–125 ms, depending on the ATP concentration and directly precedes a linear phase. The same reaction mixes were also analyzed for steady-state ATPase activity by a coupled spectrophotometric assay (23). The rates estimated from the linear phase of $P_i$ release approximate those calculated for the steady-state ATP hydrolysis.

The $K_p$ for ATP under these reaction conditions is $-130$ $\mu$M, and a clear burst in ADP production is seen in rapid chemical quench studies when the initial ATP concentration exceeds 100 $\mu$M (8). This burst in ADP production was shown previously to be caused by the rapid hydrolysis of one of the two bound ATP (7). Results of experiments with the inhibitor vanadate and of experiments measuring the concentration of ADP bound to the enzyme during the first few turnovers suggest that the products of the first ATP hydrolysis dissociate prior to the second ATP hydrolysis event (7). Release of either the first $P_i$ or ADP formed was predicted to be the rate-determining step. The lag in $P_i$ release presently seen indicates that the rate-determining step is indeed associated with $P_i$ release. The rate-determining step could be a conformational change that occurs between ATP hydrolysis and $P_i$ release. However, since there is no evidence for such a step, and inclusion of a conformational step does not change the analysis of the present data, the simplest mechanism of rate-determining $P_i$ release is utilized. Therefore, the rate constant for $P_i$ release can be estimated from the steady-state rate of ATP hydrolysis under conditions of saturating ATP.

In the HEPES reaction buffer used for these studies, and all of the previous pre-steady state topoisomerase II experiments, the rate constant for $P_i$ release is $-4.6$ s$^{-1}$. Under the buffer conditions required for the $^{18}$O exchange experiments, the rate constant for $P_i$ release is slightly lower (3 s$^{-1}$).

The anticancer drug etoposide inhibits DNA religation by topoisomerase II (24). It was shown previously that etoposide also inhibits the ATPase reaction cycle sometime after hydrolysis of the first ATP, and before release of the second ADP (25). Additionally, this drug does not block the rate of single turnover decatenation, but it does inhibit multiple turnover reactions (25). In order to narrow down which step or steps in the ATPase reaction pathway are coupled to DNA religation, etoposide was included in a set of the $P_i$ release assays (Fig. 3).

The length of the lag phase was unaffected by the presence of saturating etoposide (71 $\pm$ 15 ms in the presence and 72 $\pm$ 11 ms in the absence of etoposide). The steady-state rate of $P_i$ release was decreased 2-fold, as expected from previously re-

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**Fig. 1. Comparison of $^{18}$O exchange between Ca$^{2+}$/S1-myosin and DNA topoisomerase II.** The numbers of $^{18}$O atoms per $P_i$ generated from ATP hydrolysis by Ca$^{2+}$/S1-myosin (dark bars) and topoisomerase II (white bars) are shown. The reactions were performed, and the data were analyzed as described under “Experimental Procedures.” The topoisomerase and S1 myosin data were averaged from three and two separate experiments, respectively; the errors shown are the standard deviation.

The number of $^{18}$O atoms per $P_i$ generated from ATP hydrolysis by S1-myosin in the presence of calcium was calculated from the distribution of $^{18}$O species in the product phosphate analyzed by mass spectrometry. Therefore, one can determine the $P_i$ experimentally using Equation 3, and if $k_3$ is known, the rate constant for $P_i$ resynthesis ($k_{-2}$) can be calculated using Equation 2.

$$P_i = \frac{k_{-2}}{k_{-2} + k_3} \quad \text{(Eq. 2)}$$

$P_i$ can also be described in terms of the average number of $^{16}$O in each $P_i$ ($^{16}$O/$P$) as shown in Equation 3 (21). The $^{16}$O/$P$ ratio can be calculated from the distribution of $^{18}$O species in the product phosphate analyzed by mass spectrometry. Therefore, one can determine the $P_i$ experimentally using Equation 3, and if $k_3$ is known, the rate constant for $P_i$ resynthesis ($k_{-2}$) can be calculated using Equation 2.

$$P_i = \frac{(4 \times (^{18}\text{O}/P) - 4)}{3 \times (^{18}\text{O}/P)} \quad \text{(Eq. 3)}$$

A $P_i$ approaching 1 would indicate that nearly all of the original $^{18}$O had been lost from the $P_i$ as a result of many rounds of ATP hydrolysis and resynthesis prior to $P_i$ release. This would occur if the rate of ATP resynthesis was much faster than the rate of $P_i$ release ($k_{-2} \gg k_3$). A $P_i$ of 0 indicates that all but one of the original $^{18}$O’s remain on the $P_i$ and ATP is not resynthesized on the enzyme before $P_i$ is released. Intermediate $P_i$ values give a predictable statistical distribution of $^{18}$O/$P$ species containing 0–3 $^{18}$O atoms per $P_i$ (21).

The ATP hydrolysis/synthesis reaction of myosin has been well characterized using $^{18}$O exchange methods (12, 15, 16). In the presence of magnesium, myosin has a complex mechanism for ATP hydrolysis and synthesis that consists of an extensive exchange pathway with a $P_i$ close to 1 and a non-exchange pathway with a $P_i$ near 0. On the other hand, S1-myosin in the presence of calcium hydrolyzes ATP by a single nonexchanging pathway producing a near-zero $P_i$ (15, 16). Because Ca$^{2+}$/S1-myosin is known to incorporate only a single $^{18}$O into the product $P_i$, it is commonly used as a control in $^{18}$O exchange experiments. Additionally, the exchange results determined with Ca$^{2+}$/S1-myosin are generally used to determine the starting distribution of $^{18}$O in the ATP substrate and to correct for the fact that the ATP is never completely or uniformly labeled (21).

The extent of $^{18}$O exchange during the hydrolysis of $[^{18}\text{O}]$ATP by topoisomerase II was compared with that of Ca$^{2+}$/S1-myosin. Fig. 1 shows the averaged results of three separate topoisomerase II/DNA and two separate Ca$^{2+}$/S1-myosin experiments. A visual inspection of these data shows that topoisomerase II catalyzes only slightly more $^{18}$O exchange than Ca$^{2+}$/S1-myosin. The $P_i$ calculated from these data for topoisomerase II is 0.137. This result indicates that the rate of ATP resynthesis is slow relative to the rate of $P_i$ release from the topoisomerase ATP active site. Essentially the same results were found when the ATPase reaction was performed in the absence of DNA (data not shown). The rate constant for ATP resynthesis could be estimated from the $P_i$ value if the rate constant of $P_i$ release were known. Therefore, the rates of $P_i$ release were next measured.
of 18O atoms per Pi are essentially identical to the theoretical comparison. The experimentally determined values for the number 0.93); the experimental values for topoisomerase II are shown for 15 ms and 0.33

54, and 48 ms for 50, 350, 500, and 1000 Tris reaction buffer of 3 s

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one ATP, blocking hydrolysis of the second (7). We had previously interpreted these results to suggest that P$_i$ release occurs rapidly, and the first ADP release is the rate-determining step. The present data clearly show that this interpretation was wrong. A simplified mechanism for vanadate inhibition is depicted in Scheme 3, where $E$ is topoisomerase II, $S$ is ATP, $D$ is ADP, and $V$ is vanadate.

$$E + S + S \rightarrow ES \rightarrow ES^* \rightarrow ESDP \rightarrow ESD + P \rightarrow E + 2D + 2P;$$

$V$ $\parallel$ $E$ $SDV$

SCHEME 3

Using the rate constants shown in Table I, the previously determined $K_v$ for vanadate of 4 $\mu$m (7) and the assumption that vanadate binds at close to the rate of diffusion, this mechanism was simulated (data not shown). Vanadate was found to potentially inhibit the reaction, regardless of the fact that P$_i$ release is slow. Therefore, the present data are fully consistent with those published previously.

Nucleotide triphosphate binding and P$_i$ release are thought to trigger or accompany the critical structural changes in the reaction cycles of myosins, kinesins, GTPases, and other NTP-hydrolyzing proteins (26). The same may be true for DNA topoisomerase II. ATP binding is absolutely required for the dimerization of the ATPase domains and DNA transport (27–30). Although DNA transport is possible in the absence of ATP hydrolysis, it occurs much more slowly (9, 31, 32). The rate constant for P$_i$ release (4.6 s$^{-1}$; see Table I) and DNA transport when ATP hydrolysis occurs (7 $\pm$ 1 s$^{-1}$; Ref. 9) by topoisomerase II are similar, and probably within error. Although DNA transport was not originally seen to occur with a lag, this could be due to the fact that few early times points were analyzed. When the DNA transport reactions were repeated using radioactively labeled catenanes such that small quantities of product could be accurately measured at short time points, a lag in transported product was detected. Therefore, it is plausible that P$_i$ release triggers the conformational change required for DNA transport by topoisomerase II. However, the data are also consistent with DNA transport occurring just prior to P$_i$ release. Although simplicity would suggest that the two events are coupled, a more complex mechanism cannot be ruled out at this time.

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* The rate constants correspond to those in Scheme 2.

The extremely slow dissociation rate of non-hydrolyzable ATP analogs (7). The final two steps are shown as irreversible because the present reactions were analyzed under initial velocity conditions.

The P$_i$ release data from the topoisomerase II pre-bound to DNA at four different concentrations of ATP are shown globally fit to Scheme 2 in Fig. 6. The rate constants used in this fit are shown in Table I.

The combination of $^{18}$O exchange and P$_i$ release data presented following ATP hydrolysis by topoisomerase II, P$_i$ release occurs at a slow conformational change. Vanadate potently inhibits DNA topoisomerase II after hydrolysis of one ATP, blocking hydrolysis of the second (7). We had previously interpreted these results to suggest that P$_i$ release occurs rapidly, and the first ADP release is the rate-determining step. The present data clearly show that this interpretation was wrong. A simplified mechanism for vanadate inhibition is depicted in Scheme 3, where $E$ is topoisomerase II, $S$ is ATP, $D$ is ADP, and $V$ is vanadate.

Using the rate constants shown in Table I, the previously determined $K_v$ for vanadate of 4 $\mu$m (7) and the assumption that vanadate binds at close to the rate of diffusion, this mechanism was simulated (data not shown). Vanadate was found to potentially inhibit the reaction, regardless of the fact that P$_i$ release is slow. Therefore, the present data are fully consistent with those published previously.

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