Biochemistry **2007,** *46,* 7929−7941

Current Topics

Energy Coupling in Type II Topoisomerases: Why Do They Hydrolyze ATP?[†]

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Received April 26, 2007; Revised Manuscript Received May 23, 2007

ABSTRACT: Type II topoisomerases are essential enzymes in all cells. They help to solve the topological problems of DNA by passing one double helix through a transient break in another, in a reaction coupled to the hydrolysis of ATP. Members of one class of the enzymes, DNA gyrases, are configured to carry out an intramolecular reaction, removing positive supercoiling and introducing negative supercoiling into circular DNA using free energy derived from ATP hydrolysis. The nonsupercoiling class, including bacterial topoisomerase IV and eukaryotic topoisomerase II enzymes, can carry out both intra- and intermolecular reactions, and their primary role is the unlinking (decatenation) of daughter replicons before partition. In these enzymes, ATP hydrolysis is coupled to a reduction in DNA complexity (catenation, supercoiling, and knotting) below the level expected at equilibrium. This review discusses our current understanding of the mechanisms behind the coupling of the energy of ATP hydrolysis to topological changes catalyzed by both of these classes of enzyme.

The double-helical structure of DNA is a 20th Century icon, with its capacity for encoding information in the base sequence, and with the complementary nature of the two strands providing the mechanism for its replication and hence the efficient transfer of hereditary information (*1*). However, the fact that double-stranded DNA is helical is probably an accident of the formation of a polymer of asymmetric monomers (nucleotides), rather than an evolved feature of the molecule; helices are very common in macromolecules for this reason. The double-helical configuration of the molecule means that the two strands are interlinked, a fact that underlies the "topological" properties of DNA: supercoiling, knotting, and catenation (interlinking of two separate double-stranded DNA molecules). In a closed-circular molecule, the two single strands are linked together, and this linking is quantified by the linking number (Lk),[1] an integer that counts, in simple terms, the number of double-helical turns in the molecule (*2*). A change in the linking number corresponds to a change in the level of supercoiling of the molecule. These topological properties are problematic for the cell, and a group of enzymes, the topoisomerases (topos), has evolved to deal with their consequences (*3*).

Perhaps the easiest way to visualize the basic problem is to think about what happens as the DNA strands are separated by a helicase at a replication fork. Since the whole replication apparatus has a limited capacity to rotate as it tries to follow the parental helix, the DNA must instead rotate ahead of the fork. Friction and tethering to other structures in the cell prevent the whole DNA from rotating, and the result is the compression of the helical turns or overtwisting of the DNA ahead of the fork (Figure 1). This is analogous to an attempt to pull apart the strands of a long twisted rope, which will result in overtwisting and ultimately tangling (supercoiling) of the strands (*2*). Overtwisting of the DNA helix corresponds to positive supercoiling, which must be removed for replication elongation to continue. The only effective way to resolve this problem is to break and rejoin one or both of the DNA strands, allowing the supercoiling to relax; this process is mediated by the topoisomerase enzymes. In an analogous manner, the spooling of DNA through an effectively stationary transcription complex, where the strands are transiently separated, causes the formation of positive supercoils ahead of the complex and an untwisting of the helix (negative supercoils) behind, with a similar requirement for topoisomerases to moderate this effect (*4*).

In replication, any rotation of the replication fork that does occur leads to an intertwining of the daughter molecules (to form what are known as precatenanes), which results in catenanes (links between daughter molecules, like links of a chain) once replication is complete (*5*). These catenanes are parental double-helical turns that have not been removed by relaxation of supercoils ahead of the fork and which persist after replication is complete. Catenanes must be removed before daughter replicons can be segregated.

[†] We thank BBSRC (U.K.) for financial support (Grant BB/C517376/1).

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[1] Abbreviations: topo, topoisomerase; ADPNP, 5′-adenylyl β,γ-imidodiphosphate; GyrA, gyrase A protein; GyrB, gyrase B protein; NTD, N-terminal domain; CTD, C-terminal domain; Lk, linking number.

10.1021/bi700789g CCC: $37.00

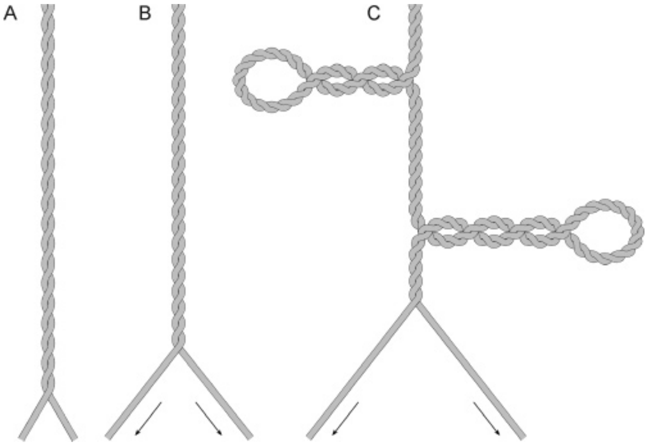

FIGURE 1: Positive supercoiling at the replication fork. As the parental strands are pulled apart, with limited rotation of the fork (A), the double-helical turns are compressed ahead of the fork (B) and would ultimately form interwound ("plectonemic") positively supercoiled regions (C), preventing replication elongation. This positive supercoiling is relieved by the action of topoisomerases. Reprinted with permission from ref *2*. Copyright 2005 Oxford University Press.

A variety of strategies are used by topoisomerases to resolve these topological problems. Type I topos break and rejoin one of the two DNA strands, forming a transient phosphodiester intermediate between an active site tyrosine and the 5′ (type IA) or 3′ (type IB) side of the break (*6*). Type IB topoisomerases (for example, eukaryotic topo I and vaccinia virus topo I) allow the free 5′ end at the break point to rotate around the intact strand, dissipating either positive or negative supercoiling in a processive, energetically favorable reaction. Type IA enzymes (prokaryotic topo I/topo III and eukaryotic topo III) use a different mechanism. Binding only to negatively supercoiled DNA, they use the energy of supercoiling to facilitate the unwinding of a segment of DNA. They break one strand and pass the intact strand through the break into a cavity in the enzyme's interior; resealing the break results in a change in linking number of ±1. The enzymes are distributive and relatively inefficient and can incompletely remove only negative supercoils. However, type IA enzymes can also manipulate catenanes, providing one of the partner DNAs has a preexisting single-strand break, or nick. Breaking the second strand at the same point allows passage of another double-stranded molecule through the break, thereby removing a catenane. Prokaryotic topo III is an effective decatenase of linked nicked-circular DNAs (*7*).

On the other hand, the type II topoisomerases, the subject of this review, are more sophisticated molecular machines that break both strands of the DNA helix and pass a second double helix through the break (gate) in a reaction that is dependent on the binding and hydrolysis of ATP (*8*). If the strand-passage reaction on a circular DNA is intramolecular, the result is a change in the linking number of ±2 (or, potentially, the introduction or removal of a knot), but if intermolecular, the result is the formation or resolution of catenanes. Type II topoisomerases can be divided into two subtypes, IIA and IIB, on the basis of structural and evolutionary considerations (*9*); this review relates to type IIA enzymes, although we expect the IIB group to exhibit similar properties. The best-known type IIA enzyme, bacterial DNA gyrase [now also identified in plants (*10*, *11*)], is actually atypical in its ability to introduce negative supercoiling, an endergonic reaction introducing torsional and bending energy into the DNA (*12*). DNA gyrase thus clearly couples the free energy of ATP hydrolysis to the introduction of elastic strain into the DNA molecule. The other homologous type IIA enzymes, bacterial topo IV and eukaryotic topo II (the nonsupercoiling type II enzymes), have basically the same strand-passage mechanism but can only relax supercoiling and carry out the apparently exergonic decatenation reaction, so their requirement for ATP was for a long time mysterious. The type IIB topos are a distantly related group identified in Archaea, and more recently in plants, and have only recently been characterized structurally and biochemically (*13*).

In vivo, the type IIA topoisomerases are believed to have distinct but overlapping roles. In bacteria, gyrase is responsible for removing positive supercoils ahead of replication and transcription complexes (*14*) and maintaining a steady state of negative supercoiling, whereas topo IV is a specialized decatenase responsible for the resolution of catenated daughter replicons (*15*), although it may also contribute to the relaxation of positive supercoils (*16*). In eukaryotes, the enzymes may be more promiscuous, although decatenation of un-nicked double-stranded daughter replicons is the only role that cannot also be carried out by type I enzymes (*14*).

A Generic Mechanism for Type II Topoisomerases

The menu of reactions catalyzed by type II DNA topoisomerases includes supercoiling and relaxation of DNA, knotting and unknotting, and catenation and decatenation, although not all enzymes can carry out all these reactions (*2*). It is assumed, and there has been no evidence to the contrary, that all these processes are carried out by the same core mechanism. Consideration of the catenation and decatenation reactions suggests that this must involve passage of one double-stranded segment of DNA through another, and this mechanism, termed strand passage, was proposed to account for all the reactions of type II enzymes (*17*). Following this, a combination of structural and biochemical studies, particularly using *Escherichia coli* DNA gyrase and *Saccharomyces cerevisiae* topoisomerase II, has been used to derive a common general catalytic mechanism for the type IIA topos; this has been reviewed in detail elsewhere (*3*, *6*, *8*). The enzymes are homodimeric, although in the bacterial enzymes two distinct subunits correspond to the single eukaryotic protein, and they are $(AB)_2$ dimers (Figure 2). They operate on two distinct segments of DNA (Figure 3). The enzymes bind the G (gate) segment close to the active site tyrosines responsible for breaking and rejoining the DNA backbones, and then the N-terminal domains act like a clamp, or bear trap, closed by the binding of ATP, to capture the T (transported) segment. The G segment is transiently broken in both strands by attack of a tyrosine residue on a backbone phosphate in each strand, with phosphodiester exchange forming a 5′-phosphotyrosine intermediate and a free 3′-OH, which is tightly bound to the enzyme. The T segment is then passed through the break in the G segment, and hence between the subunits of the enzyme, to leave by a gate at the bottom of the complex, as drawn (Figure 3). The G segment is resealed by the reverse phosphodiester exchange reaction. As part of this cycle, the bound ATPs are

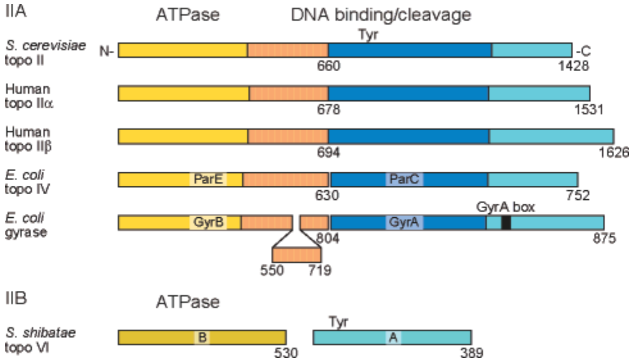

FIGURE 2: Domain structures and alignment of type II topoisomerases. The “GyrB-like” part of the proteins is colored yellow (ATPase domain) and orange, and the “GyrA-like” part is colored dark blue (DNA cleavage domain) and light blue. The approximate positions of the active site tyrosine (Tyr) and the GyrA box are indicated.

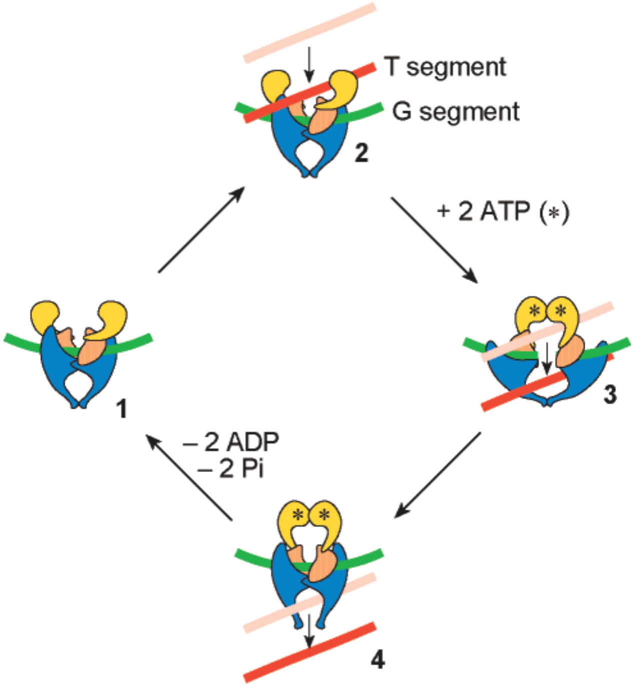

FIGURE 3: Generic core mechanism for type II topoisomerases. The enzyme domains are color-coded as they are in Figure 1; the CTD is not shown. The enzyme binds a DNA G segment (green, **1**), and then a T segment (red) binds between the N-terminal domains (yellow, **2**). ATP binding triggers the dimerization of the NTDs, trapping the T segment, which is passed through a transient break in the G segment (**3**). The T segment exits through the bottom of the protein dimer (**4**). ATP hydrolysis and product release reset the enzyme for a further reaction (**1**).

hydrolyzed to ADP and P_i, and the products are released, allowing the N-terminal clamp to open and the enzyme to be reset for further catalytic cycles.

Energy Coupling in DNA Gyrase

Mechanism of Supercoiling by Gyrase. E. coli DNA gyrase, the first type II topoisomerase to be identified, was characterized in 1976 as a host factor that introduces negative supercoils into circularized phage λ DNA before integration (*12*). It was immediately apparent that the enzyme transduced the free energy of ATP hydrolysis into the endergonic introduction of negative supercoils. The basic outline of the mechanism was fairly soon discovered. The enzyme was shown to make double-stranded breaks in DNA and to operate by the passage of one double strand through another (*18*). Liu and Wang (*19*) showed that the enzyme wraps DNA with a positive handedness, suggesting a mechanism whereby the enzyme converts a positive crossover, or node (*2*), to a negative one by strand passage, resulting in a linking number change of −2 (+1 → −1). More recently, this process has been described in further detail. The key difference between the mechanism of gyrase and that of other type II topoisomerases (described in outline above) is the ability of gyrase to wrap a segment of DNA around itself with a prescribed handedness. This is a property of the C-terminal domain of GyrA (GyrA-CTD), which is the region that shares the least sequence similarity with other topo II enzymes (*20*). Indeed, deletion of this domain results in an enzyme with topo II-like properties (*21*); i.e., it cannot supercoil DNA but can carry out ATP-dependent relaxation. Moreover, whereas gyrase is rather inefficient at decatenation (*22*), the truncated enzyme can decatenate well enough to complement a topo IV temperature-sensitive mutant in vivo (*21*). Recent structures of GyrA-CTD (*23*, *24*) have shown that this domain, which has a novel β-propeller structure, is indeed able to wrap DNA around itself in a manner appropriate for initiating the supercoiling reaction.

Biophysical studies of DNA wrapping by gyrase using footprinting and microscopy techniques have established that ∼130 bp of DNA is wrapped around the enzyme with a positive handedness that presents a T segment over a G segment (*25*, *26*). Capture of the T segment was proposed to lead to the establishment of an “on-enzyme” equilibrium across the open DNA gate (Figure 4, **3**−**4**), where the T segment is trapped within the enzyme following nucleotide binding (*27*). This capture event occurs irrespective of the superhelical state of the DNA [as evidenced by the observation that DNA-dependent ATP hydrolysis is independent of the level of supercoiling (*28*); see below], but the superhelical state was proposed to determine the position of the on-enzyme equilibrium and hence the probability of strand passage (*27*). Work using atomic force microscopy and reanalysis of earlier hydroxyl radical footprinting data strongly supports modifications of this model in which an asymmetric wrap positions the T segment above the G segment, and nucleotide binding establishes the on-enzyme equilibrium of the T segment, ultimately leading to the release of the T segment from the complex and loss of the DNA wrap (*26*). Recent determination of the low-resolution structures of full-length GyrA and GyrB by small-angle X-ray scattering (*29*, *30*) has enabled incorporation of these data into a coherent mechanism for ATP-driven strand passage by gyrase (Figure 4). Recent single-molecule experiments have confirmed the basic features of this mechanism (*31*−*33*) and shown that there is a competition between DNA wrapping and DNA dissociation that determines the processivity of the enzyme, at least when the DNA is under tension. Additionally, it has been shown that gyrase can relax positive supercoils under conditions where the DNA is not wrapped (*33*).

Gyrase ATPase Reaction. The ATP-binding site of gyrase is situated within the N-terminal domain of GyrB [GyrB-NTD, a 43 kDa fragment (Figure 2) (*34*)]. The ATPase

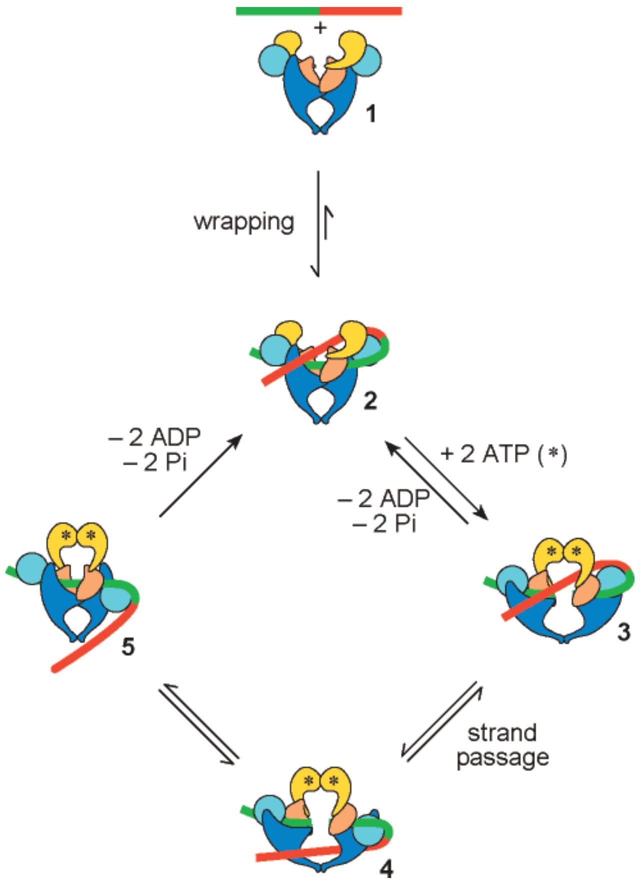

FIGURE 4: Structure and mechanism of DNA supercoiling by DNA gyrase. Enzyme domains are color-coded as they are in Figure 1. Wrapping of DNA around gyrase (**1**→**2**) presents the T segment over the G segment with a positive crossover. For clarity, only half of the wrapped region and one potential T segment are shown. Upon ATP binding (**2**→**3**) GyrB dimerizes, captures the T segment, and the G segment is transiently cleaved (at this stage ATP can be hydrolyzed with release of the T segment, i.e., an abortive cycle). The T segment is then transported through the cleaved G segment and into the GyrA cavity (**3**→**4**). The T segment is then released (**4**→**5**). Hydrolysis of ATP allows reopening of the GyrB clamp (**5**→**2**) and resetting of the enzyme. It is likely that the two ATP molecules are hydrolyzed sequentially at different points in the cycle. Based on Costenaro et al. (*30*) and Bates (*32*).

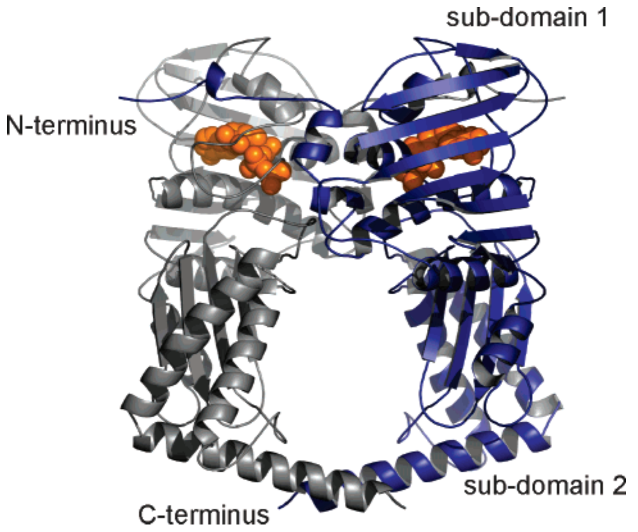

FIGURE 5: Structure of the N-terminal ATPase domain of DNA gyrase (GyrB43) complexed with ADPNP (*38*). Ribbon representation of the structure of the GyrB43−ADPNP complex. ADPNP is colored orange, and the two subdomains are indicated. The image was generated using PyMOL (*94*).

activity of gyrase is stimulated by DNA and is coupled to the introduction of negative supercoils. Substitution with a nonhydrolyzable analogue, ADPNP (5′-adenylyl β,γ-imidodiphosphate), leads to substoichiometric introduction of supercoils into relaxed DNA; it was hypothesized that nucleotide binding can lead to one round of strand passage but that ATP hydrolysis is required to complete the reaction cycle (*35*). With relaxed DNA, only 30% of the enzymes carry out a supercoiling reaction in the presence of ADPNP, suggesting that the coupling between nucleotide binding and strand passage is not perfect (*36*). The coupling is dependent on the level of supercoiling of the substrate, being high with positive supercoils but too low to measure at moderate levels of negative supercoiling (*36*). Consistent with this, it was shown that as relaxed DNA was supercoiled by the enzyme in the presence of ATP, the reaction became slower and reached a limit of negative supercoiling, while ATP hydrolysis continued at a rate independent of the level of supercoiling of the DNA substrate (*28*, *36*). This can be interpreted in terms of the on-enzyme equilibrium model (*27*) in which the T segment is always trapped, thus stimulating ATP hydrolysis, but the enzyme's ability to carry out strand passage is determined by the level of supercoiling of the substrate DNA.

Investigations of the DNA length dependence of the stimulation of ATPase activity demonstrated that full stimulation requires >100 bp of DNA, although shorter lengths could stimulate at high concentrations (*37*). This corresponds approximately to the length of DNA that has been shown to be wrapped around the gyrase complex [∼130 bp (*25*)]. These data suggest, in terms of the generic mechanism, that a length of DNA corresponding to a G and a T segment is required for stimulation of the ATPase activity (*26*, *37*).

In 1991, the high-resolution structure of the ATPase domain of gyrase (GyrB-NTD), complexed with ADPNP, was determined by X-ray crystallography (*38*). This clearly showed how ATP was likely to be bound by the enzyme (Figure 5). The salient features of the structure are that it is a dimer, with a central hole ∼20 Å across between the two monomers, and with an N-terminal arm from each monomer wrapped around the other. Each monomer can be subdivided into two distinct subdomains. One molecule of ADPNP is bound per monomer with additional contacts with the other monomer; i.e., the ATP-binding site is composed of residues from both monomers. Most of the contacts with the nucleotide are from the N-terminal part of the fragment (subdomain 1), although two side chains from subdomain 2 (Gln335 and Lys337) also make contacts; subdomain 2 is also termed the "transducer" domain and is proposed to communicate ATP hydrolysis to the rest of the molecule (*38*, *39*). ATPase kinetics and nucleotide binding studies confirmed that GyrB-NTD is a monomer in the absence of nucleoside triphosphate and a dimer in its presence (*34*, *40*). Subsequent site-directed mutagenesis experiments identified Glu42 from subdomain 1 as the catalytic base, which abstracts a proton from water, which in turn nucleophilically attacks the γ-phosphate of ATP (*41*). An adjacent residue (His38) was proposed to orient and polarize Glu42. Further work suggested that Lys337 from subdomain 2 has a critical role in transition-state stabilization, i.e., stabilizing the pentacoordinate phosphate intermediate (*42*).

From this work, a clear picture of how the enzyme binds and hydrolyzes ATP and how nucleoside triphosphate binding promotes dimerization of GyrB43 has emerged. This supported the concept of the "ATP-operated clamp" proposed for the nonsupercoiling yeast topo II (*43*), which traps the T segment prior to passage through the G segment. A direct test for such a function was performed by mutating an arginine residue (Arg286), which protrudes into the central hole (*38*), and showing that ATP-driven strand passage and DNA-dependent ATPase were virtually abolished (*44*). However, whether the T segment is stably bound within the cavity of the GyrB43 dimer is not known.

Energy Transduction in Gyrase. A key mechanistic question is how gyrase transduces the free energy of ATP hydrolysis into torsional stress in DNA (negative supercoiling). Early work had suggested that there is an equivalence between the limit of supercoiling that can be achieved by gyrase [a superhelix density, or specific linking difference (σ), of -0.11] and the free energy available from the hydrolysis of ATP (*45*−*47*). The free energy required for the introduction of the final two supercoils in plasmid pBR322 can be calculated as $\sim$114 kJ/mol (*48*) and compared to the free energy liberated from the hydrolysis of two ATP molecules, approximately -120 kJ/mol (*49*). Bates and Maxwell (*45*) investigated the supercoiling of small DNA circles in the range 116−427 bp to determine whether supercoiling by gyrase is limited by steric or thermodynamic constraints. They found that the constraint was thermodynamic, supporting the idea that the limit of supercoiling is determined by the free energy available from ATP hydrolysis. Further, using ATPαS as a substrate, Cullis et al. (*50*) showed that gyrase could introduce an extra supercoil into plasmid pBR322 compared with ATP; the difference in the free energy of hydrolysis of these two nucleoside triphosphates is approximately equivalent to the additional free energy required to introduce the additional supercoil. Using a mutant gyrase in which one GyrB subunit was unable to hydrolyze ATP, Kampranis and Maxwell (*51*) showed that the enzyme could still supercoil DNA but that the limit of supercoiling was reduced. Taken together, these results strongly support a direct link between the free energy of ATP hydrolysis and the free energy of supercoiling DNA.

However, a more challenging issue is how the free energy is actually transduced; i.e., what is the mechanism of coupling? Clearly, this will involve conformational changes in the gyrase proteins, and there is already ample evidence that these occur (*52*). One clue has come from studies with ATPβS, which is a good substrate for the gyrase ATPase reaction but drives supercoiling very inefficiently (*53*); i.e., the supercoiling and ATPase reactions are somewhat uncoupled with this analogue. One possible explanation for these data is that the T segment is captured by the ATP-operated clamp, thus stimulating the ATPase reaction, but is released prematurely prior to strand passage. This could be ascribed to rapid release of product (ADPβS) compared to the normally slow release of ADP. However, the ordered series of conformational changes in gyrase driven by ATP binding and/or hydrolysis that must occur during supercoiling are not known, although there is good evidence for their existence. Type II topoisomerases are described as being constructed as a series of gates that control the strand-passage process (*54*). The G segment sits across the "DNA gate", which in gyrase is formed, at least in part, by the N-terminal domain of GyrA; the structure of the 59 kDa N-terminal fragment (GyrA-NTD) reveals a positively charged "saddle" region, which is likely to be the G segment binding site (*55*). The GyrB-NTDs form the ATP-operated clamp, which controls the delivery of the T segment to the G segment (*38*). GyrA-NTD also contains the "exit gate", which is responsible for the release of the T segment at the end of the strand-passage process (*55*). Cross-linking experiments have been used to probe the function of each of these gates and have shown that they fulfill their predicted roles (*56*−*58*). This parallels work carried out with yeast topo II (*59*, *60*), which has provided strong evidence for the role of protein gates in that enzyme.

Work with yeast topo II has provided some insight into the finer details of ATP hydrolysis and energy coupling issues (*61*−*64*). These studies suggest that the two ATPs are hydrolyzed sequentially and that the hydrolysis steps occur at different stages of the topoisomerase reaction cycle and could be used to drive different processes. Specifically, it has been shown that one ATP is rapidly hydrolyzed and its products are released before the second ATP is hydrolyzed (*61*, *63*, *64*). It is proposed that hydrolysis of the first ATP precedes and accelerates DNA strand passage (*62*). It may be that such sequential hydrolysis events and differential utilization of the free energy of the two ATPs also occur with gyrase; this idea could be incorporated into the model in Figure 4.

In experiments with gyrase in which one GyrB subunit could bind but not hydrolyze ATP (*51*), the topoisomerase reaction could still occur, as could that of DNA-dependent ATPase. In similar experiments with a heterodimer of human topo IIα in which only one subunit could bind ATP, this enzyme was also shown to retain topoisomerase activity, albeit at a reduced rate (*65*); however, in this case, the ATPase activity of the enzyme could not be stimulated by DNA. The authors speculate that one ATP is sufficient for catalyzing strand passage and the other is required for communication between the ATPase domain and the cleavage/religation domain to promote efficient catalysis. Interestingly, a mutation in the transducer domain of human topo IIα results in an enzyme incapable of strand passage, but able to carry out DNA-dependent ATP hydrolysis, albeit at a reduced rate (*66*). This implies a defect subsequent to T segment capture but prior to strand passage, suggesting that these are separable events.

Recent work using topo VI from the archeon *Sulfolobus shibatae* has revealed the range of conformational changes that can occur in the ATPase domain of type II topos (*67*, *68*). Topo VI, from Archaea, is a member of the type IIB topoisomerases, which exhibit only weak sequence similarity to type IIA enzymes like gyrase and yeast topo II (*9*). However, its ATPase domain is very similar to that of GyrB-NTD, and both are members of the GHKL family of ATPases (*69*). Structures available for this domain include the apoprotein and complexes with ADPNP (substrate analogue), ADP•AlF_4^- (transition-state analogue), and ADP and ADP•P_i (product complexes) (*67*, *68*). These data have revealed a detailed outline of the nucleotide hydrolysis cycle and the associated protein conformational changes. It appears that

ATP hydrolysis itself does not directly drive structural changes within the enzyme and that it may be the trapped T segment that plays a direct role in powering the conformational changes that result in strand passage (*68*). It is likely that a similar series of events will occur with type IIA topos, including DNA gyrase, and other GHKL proteins.

Nonsupercoiling Type II Topoisomerases

Substrate Specificity. Although much was known about the structure and basic mechanism of type II topoisomerases by the mid-1990s, relatively little was known about the specificity (or selectivity) of their reactions. While gyrase has a very clear specificity for intramolecular strand-passage reactions, for reasons elaborated above, at first it appeared that the nonsupercoiling type II topos might operate as rather indiscriminate strand-passage enzymes, binding and transporting whichever T segment first comes to hand, i.e., converting DNA molecules into “phantom chains”, with the apparent ability to pass freely through themselves (*70*). However, a number of aspects of the specificity of type II topoisomerases have been identified and are being investigated. In general, type II topoisomerases are highly specific for decatenation and unknotting reactions, relative to the relaxation of negative supercoils. With a negatively supercoiled catenated substrate, topo IV is 30-fold more efficient at decatenation than relaxation (*71*), despite the presence of an apparently high density of potentially competing “supercoiled” T segments (that is, T segments that if transported would lead to relaxation). Indeed, it appears that decatenation is actually stimulated ∼4-fold by negative supercoiling (*71*). The same is true of *S. cerevisiae* topoisomerase II (*72*, *73*), and it has additionally been shown that, relative to relaxed DNA, decatenation is inhibited, and catenation stimulated, by positive supercoiling (*72*). A possibly related finding is that some of the enzymes have a preference for the relaxation of positive over negative supercoils. In both ensemble and single-molecule experiments, topo IV is approximately 20-fold more efficient at the relaxation of positive supercoils than negative, with a much more processive reaction (*74*). However, the same specificity is not exhibited by yeast topo II (*73*) and *Drosophila* topo II (*75*), which relax positive and negative supercoils equally well. It has recently been shown that the two human type II enzymes differ in this aspect of specificity, topo IIα exhibiting a >10-fold specificity for relaxation of positive supercoils, while topo II*β* shows little or no preference (*76*).

Two distinct models have been proposed to explain the specificity effects described above. Cozzarelli and co-workers (*74*, *77*) suggested that topo IV can differentiate between positively and negatively supercoiled DNA by the angle and handedness of the crossing of the G and T segments. In plectonemically interwound DNA, the apparent crossings of the DNA strands subtend an angle of ∼60° measured across the superhelix axis. If the topo IV enzyme is configured to accept a G segment and a T segment preferentially with that crossing angle, and with the handedness appropriate for positive supercoils (Figure 6A), then binding of a T segment in positively supercoiled DNA would be facilitated, whereas in negatively supercoiled DNA, considerable rotation of the potential T segment would be required (Figure 6A). This model might also account for the stimulation of decatenation in negatively supercoiled substrates, since the relatively ineffective binding of a supercoiled T segment in a negatively supercoiled substrate might facilitate the alternative binding of a catenated (or knotted) T segment, leading to preferential decatenation (or unknotting) (Figure 6A). Subsequent investigations of the structural origins of these preferences of topo IV have suggested that the specificity is mediated through the C-terminal domains of the ParC protein (*23*). This domain, which is homologous to the “wrapping” domain of GyrA, is a DNA-binding protein, and it is proposed that the domain is responsible for the initial selective binding of potential T segments with a specific angular relationship to the previously bound G segment; deletion of these domains abolishes the selectivity for relaxation of positive supercoils (*78*). This idea has been incorporated into Figure 6A. This conclusion is supported by the fact that two viral type II topoisomerases, PBCV-1 and CVM-1, which naturally lack CTDs, show no selectivity for the relaxation of positively over negatively supercoiled substrates, although they do show a 2−4-fold preference for cleavage of negatively supercoiled DNA (*79*).

The problem with the “angular selection” model is that yeast topo II has a similar specificity for decatenation of negatively supercoiled catenane substrates but no strong selectivity for the relaxation of positive supercoils over negative (*73*). Instead, Roca (*72*) has suggested what might be a more generally applicable model. In this version, the enzyme is proposed to bind two potential, or proto-T, segments (pT), in other words, three DNA segments in all (Figure 6B). There are two alternatives for the handedness of the crossing of the two pT segments, and one of these has been chosen by Roca so that the binding of a negative interwound strand as pT_1 will facilitate the binding of a catenated strand as the second pT segment (pT_2), whereas binding of a positive supercoil will tend to exclude the binding of a catenated strand as pT_2 but instead promote the binding of an unlinked strand. If efficient strand passage can occur with either pT segment, then this can neatly explain the dependence of the decatenation reaction on negative and positive supercoiling. This model can be elaborated to explain the selectivity of some of the enzymes for relaxation of positive supercoils by assuming that in the yeast enzyme, the G and pT segments cross at or close to 90°, giving no angular preference, but that in topo IV or human topo IIα, angular selection occurs as described above. One of the attractive features of this model is its similarity to the gyrase situation. As Roca points out, it is clear that gyrase also has two pT segments, one of which ultimately undergoes strand passage as the true T segment. This idea can now be combined with our knowledge of the structure and function of the C-terminal domains. In gyrase, they mediate the wrapping of DNA contiguous with the G segment to deliver two pT domains (one wrapped around each CTD) close to the N-terminal ATP clamp, whereas in topo IV, the domains lack a region (the GyrA box) that specifies that contiguity (*23*, *80*); instead, the domains may be free to select noncontiguous pT segments on geometrical grounds that underlie the specificity of topo IV.

In the cases of the eukaryotic type II enzymes, the corresponding C-terminal domains appear not to be homologous with the gyrase/topo IV proteins and may consist of rather disordered and repetitive sequences (J. M. Berger, personal communication; A. D. Bates, unpublished observa-

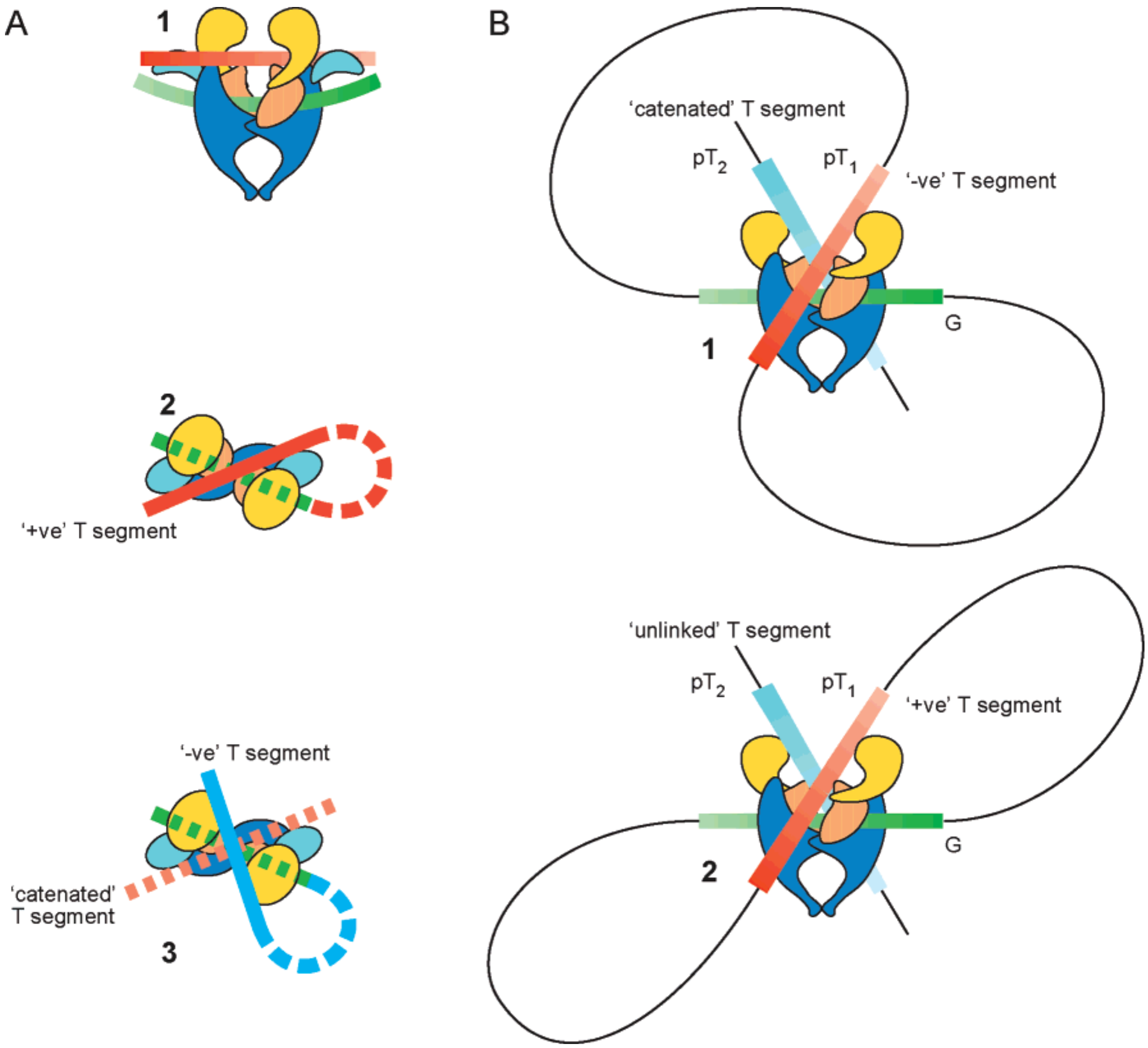

FIGURE 6: Models for selection of the T segment by type II topoisomerases. (A) Topoisomerase IV (*23*, *74*). The enzyme is optimized to select a G and T segment with a specific angular relationship (**1**). Viewed from above (**2**), a positively interwound DNA molecule presents a T segment (red) over a G segment (green) with the correct angle to be captured between the NTDs (yellow), so relaxation of positive supercoils is favored. In contrast (**3**), a negative interwound molecule presents a putative T segment (blue) at the wrong angle, and relaxation is slow. However, a "catenated" strand crossing the contour of the negatively supercoiled molecule (dashed red) could be captured with high efficiency, so negative supercoiling stimulates decatenation. (B) Yeast topo II (*72*). The enzyme binds two putative T segments (pT$_1$ and pT$_2$), before capturing and transporting one of them. The handedness of crossing of the two pT segments has been chosen such that if pT$_1$ forms a negative node with the G segment as in a negatively supercoiled substrate (**1**), pT$_2$ must cross the DNA contour; capture of pT$_2$ would favor decatenation. Conversely, if pT$_1$ forms a positive node, with positively supercoiled DNA (**2**), pT$_2$ is likely to be an unlinked strand, and capture would lead to catenation. In either case, capture of pT$_1$ will lead to relaxation.

tions). Nevertheless, the models described above would predict that these domains are likewise involved in the geometrical selection of a T segment or two pT segments.

One difficulty of a three-segment model is the lack of direct evidence for topo II and topo IV interacting with three segments of DNA. Electron microscopy studies have not apparently revealed the enzymes bound to three DNA segments. For example, Zechiedrich and Osheroff (*81*) found *Drosophila* topo II generally bound at nodes, i.e., the crossing of two segments of DNA. However, there have been observations of gyrase bound to three loops of DNA [and sometimes up to four or five (*82*)]; the significance of such observations is not clear. A recent study has investigated the catenation by gyrase and topo IV of two tethered single-stranded DNA circles incorporating short double-stranded regions designed to form G and T segments (*83*). These experiments support the idea that strand passage can occur with one T segment only. However, these catenation reactions are not highly efficient, and Roca (*72*) does not suggest that involvement of two pT segments is obligatory.

"Rybenkov" Effect. A further fundamental aspect of type II topoisomerase action remained obscure until the mid-1990s: the enzymes require the hydrolysis of ATP, although the reactions they perform (relaxation of supercoils, decatenation, and unknotting) are normally exergonic and should not require an energy input. In contrast, the requirement for energy input in gyrase was apparent from the beginning: the free energy of ATP hydrolysis is transduced into the torsional energy of negatively supercoiled DNA. In topo II enzymes, one proposal was that ATP binding and hydrolysis drove enzyme conformational changes to increase the rate of a complex reaction.

The solution to this conundrum was revealed by Rybenkov et al. in 1997 (*84*). Nonsupercoiling topo II enzymes act to shift the equilibria of topoisomerase reactions in the direction of topology simplification. That is, they produce steady-state

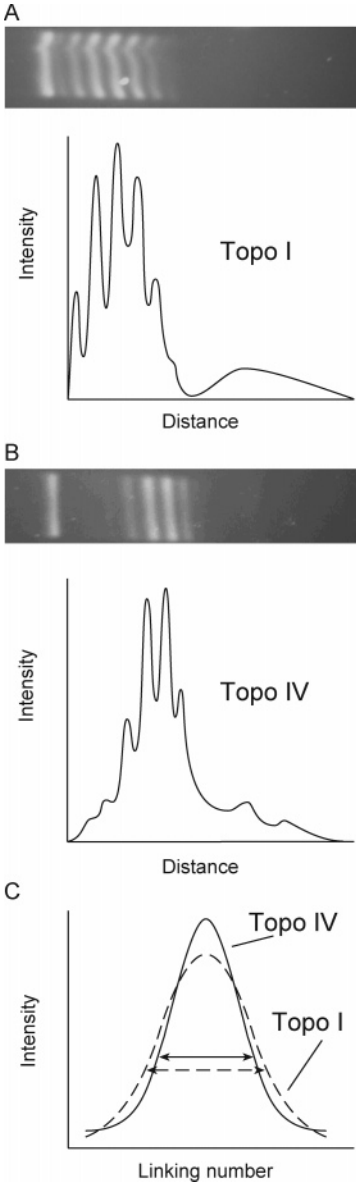

FIGURE 7: "Nonequilibrium" relaxed topoisomer distribution generated by *E. coli* topoisomerase IV. Topoisomer distributions generated by relaxation of supercoiled plasmid pBR322 DNA by wheat germ topo I (A) and *E. coli* topo IV (B); top panels show ethidium-stained agarose gel tracks, and the resulting densitometric scans are shown below. (C) Plotted topoisomer distributions and fitted Gaussians; topo IV produces a narrower (lower variance) distribution.

levels of supercoiling, catenation, and knotting lower than those occurring at equilibrium under the same conditions. For example, in Figure 7, topo IV produces a narrower, more relaxed topoisomer distribution (i.e., one of lower variance) than topo I, which generates equilibrium product distributions. At the time, this proposal excited some comment suggesting that the enzymes acted mysteriously and contrathermodynamically, as in Maxwell's demon (*85*). Of course, there is no mystery: since the enzymes hydrolyze ATP, they can couple the free energy produced to operate against an equilibrium, just as gyrase or any other coupled enzyme can. In terms of cellular physiology, this makes sense in that the enzymes are able to remove more supercoils, knots, and catenanes than would be possible via an equilibration process.

The outstanding question was the mechanistic basis of this energy coupling effect. The possibilities become clearer when we think about the more "obvious" gyrase effect. The negative supercoiling reaction of gyrase relies on its ability to select a T segment from the same DNA molecule with the correct orientation to ensure a decrease in linking number after strand passage. As we have seen, this selection is mediated by wrapping by the C-terminal domains of GyrA (*21*) and is very strong; decatenation is several hundred-fold less efficient as a reaction of gyrase than negative supercoiling (*71*). This large selection in terms of binding energy between alternative T segments ensures that a subsequent ATP-driven unidirectional strand-passage mechanism will result in efficient negative supercoiling. On the other hand, the efficiency of strand passage also seems to be independently mediated by the torsional strain present in the DNA, once T segment capture has taken place (*27*, *32*, *36*). We can envisage that these same steps may be the basis of the specificity of the other type II enzymes, although the effect is clearly more subtle. For example, in the case of catenated DNA at equilibrium, an enzyme must be selective (that is operate faster) for binding, capture, and strand passage of G and T segments from linked circles of a catenated DNA molecule, relative to its operation on unlinked circles. While the wrapping by gyrase is a straightforwardly local effect, the reactions of the other topo IIAs seem to depend on more global topological features of their substrate DNA molecules. The considerations are similar for knotted and supercoiled DNA, but we have used catenated DNA as the most easily visualized example. Note that although this notion seems, on the face of it, to be similar to the specificity for decatenation or relaxation of positive supercoils that we have already considered above, it is actually a rather harder problem. These nonequilibrium effects are apparent at very low levels of supercoiling and, indeed, have been demonstrated on nicked DNA up to 10 kb in length in the case of decatenation and unknotting, and hence, the geometries imposed by relatively high degrees of supercoiling are not available for the enzymes to use for selection.

A number of suggestions have been made about a possible mechanism for this energy coupling effect, although experimental evidence is relatively sparse and in some cases contradictory. The models may be divided into two categories, those that rely on the "straightforward" geometric selection of a preferred T segment (essentially analogous to the gyrase wrapping effect), and those that incorporate a further kinetic selection effect mediated by ATP hydrolysis.

*Geometric Selection Models***.** Vologodskii et al. (*70*) suggested that T segments might be selected geometrically if the topoisomerase were to bend the G segment in a curve toward the T segment clamp (Figure 8A). In principle, this would have the effect of orienting the enzyme on a circular DNA, with the clamp directed toward the "inside" of the circle, where binding of a T segment from a catenated partner DNA, or a knotted or supercoiled strand, might be facilitated, leading to selective topology simplification. Monte Carlo simulations of knotted DNA with a bent hairpin segment, as in Figure 8A, seem to suggest that this mechanism could account for the observed unknotting effect, although this was subsequently disputed by Yan et al. (*86*) who favor a more elaborate mechanism (see below). It is perhaps intuitive to understand how the bent G segment mechanism might work

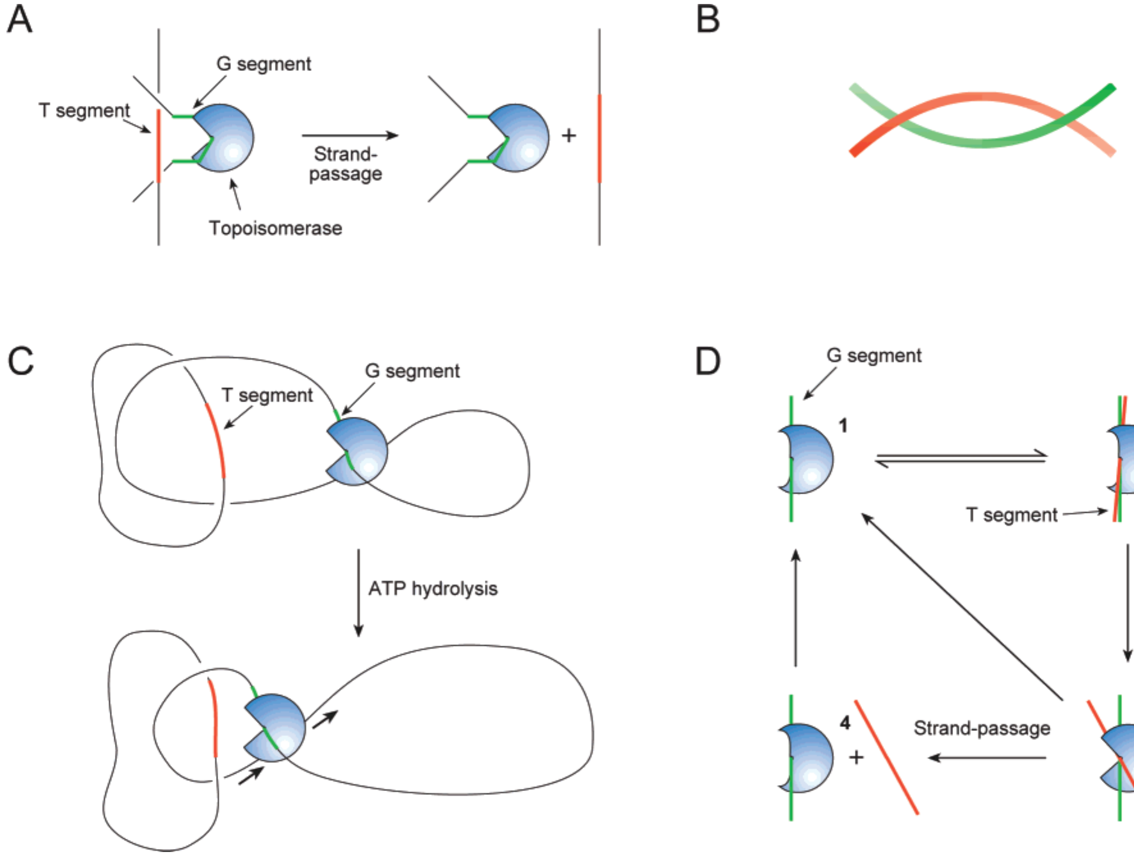

FIGURE 8: Models to explain the Rybenkov effect. (A) G segment bending (*70*). If the enzyme bends the G segment in a curve or hairpin toward the T segment clamp, then the enzyme will orient itself on a circular DNA such that a T segment from inside the DNA contour will be preferentially captured. (B) Hooked juxtapositions (*88*). Such juxtapositions may occur more frequently in supercoiled, catenated, and knotted DNA; if the enzyme acts preferentially at these sites, relaxation, decatenation, and unknotting will be favored. (C) Enzyme tracking (*84*). The enzyme binds a G segment and another site on the DNA. ATP-driven tracking of the enzyme along the DNA at the second site will lead to constraint of the supercoiled, catenated, and knotted potential T segment in a loop of decreasing size, facilitating its capture. (D) Kinetic proofreading (*92*). A low level of T segment selectivity, such as in panel A or B, can be amplified by an ATP-dependent process. For example, initial selection of a T segment (**1** → **2**) is followed by an effectively irreversible ATP-dependent step, changing the protein−DNA conformation (**3**). From **3**, there is a competition between loss of the T segment (**3** → **1**) and strand passage (**3** → **4**). As long as this competition is selective for transport of, for example, a catenated T segment, this step can amplify or proofread the initial selective T segment binding.

efficiently in small DNA circles, where the DNA will have a relatively clearly defined inside and outside. For larger DNA circles, it is much more difficult to see how this local bending effect could provide the required level of selection. At the very least, the prediction of this model ought to be that the effect would be strong in small circles and become weaker as the circle size increases. In fact, in experiments investigating the relaxation reaction of *S. cerevisiae* topo II, the effect decreases and is eliminated in small DNA circles but is independent of circle size with larger circles (*87*). In addition, although Volgodskii et al. demonstrate the bending of DNA by topo IV using cyclization probability experiments, and in electron micrographs (*70*), analogous experiments using yeast topo II suggest that no such bending takes place (*87*), implying that the mechanism cannot be a general one for all type II enzymes.

A somewhat related suggestion has been made by Buck and Zechiedrich (*88*). They suggest that “hooked juxtapositions” of DNA double strands (Figure 8B) will be overrepresented in knotted or catenated DNA and that, if the enzymes act preferentially at these sites, perhaps by preferential binding of a bent G segment as above, then this can explain the Rybenkov effect. This model has the same predictions of inverse length dependence and, perhaps, of G segment bending as the Vologodskii model described above. Buck and Zechiedrich (*88*) interpret the original data of Rybenkov et al. (*84*) to suggest a greater efficiency of unlinking at smaller circle size, consistent with their model. However, this seems to require the conflation of data from decatenation and unknotting reactions, which does not seem justified. As discussed above, Trigueros et al. (*87*) showed conversely that the Rybenkov effect for relaxation by yeast topo II is abolished with a small circle size. Recent Monte Carlo simulations of catenated (*89*) and knotted (*90*) DNA have suggested that enzyme action at hooked juxtapositions has the potential to form the basis of the effect, although an analysis of the electrostatics of such juxtapositions seems to cast doubt on their likely appearance under relevant conditions (*91*). As with the bent G segment, the effectiveness of this model depends on the ability of the enzyme to discriminate between alternative DNAs, particularly in large circles.

Roca and co-workers (*87*) have suggested that their three-site binding model (see above) might provide an explanation for the nonequilibrium effects. They point out that if the enzyme binds two pT segments before transporting one of them as the true T segment, then the first pT binding will divide a DNA circle into two smaller domains. Since smaller circles relax to give a narrower topoisomer distribution [since the free energy differences between topoisomers are larger in smaller circles (*2*)], subsequent relaxation of the small domains via the second pT segment can explain the nonequilibrium effect. In experiments with DNA relaxation by yeast topo II, the narrowing of the topoisomer distribution is accompanied by a shift of the center of the distribution to the positive supercoiled side (*87*). The authors believe that this bias can be explained by the different consequences of the binding of a positive or negative node as the first pT segment. Both the topoisomer narrowing and the shift in the distribution decrease together as the circle size decreases below ∼3 kb, suggesting that they are both mediated by the same process. This is consistent with three-site binding, in that binding of a third site might be expected to become more difficult as DNA bending becomes more unfavorable in a smaller circle. This is intriguing, but there are some difficulties. Division of the DNA into two domains by binding two DNA segments does not in itself lead to a nonequilibrium relaxation of topoisomers, as can be seen with a thought experiment. Imagine relaxation, by a non-ATP-dependent type I enzyme, of a normal plasmid and one divided into two domains by, for example, a Lac repressor tetramer looped complex. Would the latter give a more relaxed product distribution than the former? No, barring any minor perturbation due to the repressor binding, the result would be the same, since the low variances of the distributions in the two more domains would be added together when the repressor was removed.[2] Furthermore, the shift in the topoisomer distribution seen by Trigueros et al. (*87*), which seems highly correlated with the narrowing effect, was not mentioned by Rybenkov et al. (*84*), nor is it seen in more recent unpublished experiments (T. Stuchinskaya, A. D. Bates, and A. Maxwell, unpublished observations). In summary, the three-site model does not seem to fulfill the requirements for a geometrical selection of supercoiled or catenated T segments simply by virtue of forming a smaller DNA domain, although it may in principle combine with some other form of selection, such as G segment bending or action at hooked juxtapositions.

Kinetic Selection Models. In fact, a three-site binding model was also suggested by Rybenkov et al., in the original paper describing the nonequilibrium effect (*84*). This model was not as well-developed as that in Trigueros et al. (*87*) and is fundamentally different, as it posits an active role for ATP in the selection of the T segment. In this model, it is proposed that the type IIA enzyme can bind to a G segment, and to a second site (Figure 8C). ATP binding and hydrolysis then drive the enzyme unidirectionally along the DNA at the second site, leading to the confining of, for example, a catenated T segment in a loop of decreasing size, where it would be captured at a higher rate. No such increase in rate would occur for the capture of a T segment from an unlinked molecule, so decatenation would be favored over catenation. Again, similar considerations apply to knotted and supercoiled molecules. Although this proposal seems as if it would work, it is hard to reconcile with our knowledge of the effect of ATP binding and hydrolysis. As we have seen, there is good evidence that the effect of ATP binding is to close the N-terminal clamp, leading to the trapping of a DNA segment, and that hydrolysis leads to the subsequent opening of the clamp. There is nothing to suggest that this clamp closure has the effect of translocating the enzyme along the DNA, and in any case, such a captured T segment would be likely to undergo a strand-passage reaction. In the case of relaxation and decatenation reactions by yeast topo II, a significant proportion of the ATP binding events are thought to lead to strand passage, as judged by experiments using a nonhydrolyzable analogue (*72*). However, the large size and complexity of type II topoisomerases mean that we should at least keep open the possibility that these enzymes can perform another ATP-dependent reaction in addition to strand passage.

Even if it lacks plausibility, this suggested mechanism does raise the possibility that ATP hydrolysis may act directly to increase the specificity of the enzyme reaction, rather than simply providing a unidirectional strand-passage step. One well-established method for enhancing specificity is kinetic proofreading, which has been suggested as the basis of the topo IIA specificity by Yan et al. (*86*, *92*). Kinetic proofreading is a general kinetic scheme, rather than a specific mechanism, in which an energy-requiring step may be used to enhance the selectivity of an enzyme for a specific substrate (*93*). One of the classic examples is in the charging of tRNAs with the correct amino acid by aminoacyl tRNA synthetases. The direct selection by the enzyme of the "correct" amino acid substrate relative to an "incorrect" one is in many cases insufficient to explain the observed low error rate of charging. However, the first step of the reaction is the essentially irreversible ATP-dependent formation of an activated aminoacyl-AMP, with the elimination of pyrophosphate. This gives an opportunity for a further selective step, in which the "wrong" aminoacyl-AMP may dissociate at a higher rate than the correct one, before the next step of the reaction, coupling to tRNA. Provided that these two selective steps are separated by an irreversible reaction as above, their individual levels of selection can in principle be multiplied to give an overall much higher value; in other words, the second selective step amplifies or "proofreads" the first.

Marko and co-workers (*86*, *92*) proposed that kinetic proofreading might form the basis of the specificity for topology simplification of the nonsupercoiling type II topos. This model relies, as in the aminoacyl tRNA synthetase example, on amplification of an underlying selection for a catenated, knotted, or supercoiled T segment by an irreversible ATP-dependent step. Yan et al. suggest that the initial formation of a G and T complex triggered by ATP binding leads to an activated state of the enzyme in which the T segment has been lost. A second selective capture by the

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[2] In fact, it may be possible to contrive a version of this experiment that would produce a narrowed topoisomer distribution. If a DNA domain of <∼2 kb were constrained by the repressor [a size below which the energetic cost per unit length of supercoiling increases (*2*)], then relaxation by topo I followed by removal of the repressor could produce a lower variance than at equilibrium. This would require stoichiometric levels of repressor, with the required energy input coming from repressor binding. For a type II topo to operate in this way would require the energy of ATP hydrolysis to be used either to produce the smaller DNA domain (see next section), or to drive the ultimate release of the constrained loop, neither of which are consistent with the accepted role of ATP binding and hydrolysis in trapping and transporting a T segment.

activated enzyme leads to productive T segment capture and strand passage. Thus, an initial low level of selection of a T segment on some geometrical basis is amplified through this two-step process. Kinetically, this scheme is entirely plausible, but the specific mechanism that is proposed is less so. Our understanding of type IIA mechanism as outlined here is that ATP binding is intimately associated with clamp closure, and it is hard to reconcile how loss of the initial T segment could lead to an activated state that is still able to capture a T segment a second time. If the clamp were already closed, for example, then recapture could not occur. Furthermore, the loss of the T segment at the irreversible ATP-dependent step, as described by Yan et al., would seem to eliminate the selective effect of the initial step.

One possibility for two selective steps is the following, elaborated from the discussion of Yan et al. (Figure 8D). An initial, low-efficiency selective binding of a potential T segment by the enzyme (perhaps by the C-terminal DNA-binding domains) is independent of ATP. When ATP subsequently binds, the transition to the clamp-closed, T segment captured state must involve a conformational change in the T segment (bending or angular change) that is sensitive to its topology such that capture is also more probable for a catenated, knotted, or supercoiled T segment. Thus, the initial binding selectivity is proofread by the ATP-dependent topology-sensitive transition to clamp closure, whereupon strand passage takes place.

However, there is an alternative possibility that is suggested by our knowledge of uncoupled reactions in DNA gyrase. As recounted above, the DNA-stimulated ATPase activity of gyrase, which is believed to be dependent on the trapping of the T segment by the N-terminal clamp, may be uncoupled from the strand-passage reaction (*27*, *35*, *36*). This has been developed into a model in which obligatory capture of a T segment upon ATP binding sets up an on-enzyme equilibrium across the DNA gate. ATP hydrolysis and clamp opening can lead to either completed strand passage or abortive release of the T segment from the clamp without strand passage (Figure 4). In gyrase, the relative probability of these alternatives is proposed to depend on the supercoiling torsion in the DNA (*27*, *36*). This scheme has some of the features of kinetic proofreading. If a similar situation occurs in the nonsupercoiling type IIA enzymes, the position of the strand-passage equilibrium could itself be sensitive to some geometrical aspect of the T segment state (as suggested above, it may involve bending or relative angular motion of the G and T segments, for example). The relative probabilities of strand passage or abortive release for alternative T segments would then also form an ATP-dependent proofreading step, to amplify an initial selection of T segment binding.

It is apparent that the exact mechanism of product simplification by type II topoisomerases is at present unclear and that further experimental work aimed at exploring aspects of the models described above will be required to elucidate this mechanism.

CONCLUSIONS

The type II DNA topoisomerases are extraordinary molecular machines that utilize the free energy of ATP hydrolysis to drive energetically unfavorable reactions involving the passage of one DNA double helix through another. In the case of DNA gyrase, ATP hydrolysis drives the introduction of negative supercoils into DNA. The steps involved in this process are now clearly defined; however, exactly how the chemical energy of ATP is transduced to transfer the T segment through the DNA gate and the G segment is not clear, and the precise roles played by the hydrolysis of two molecules of ATP are not yet known. Detailed elucidation of this process will provide valuable insight into the general principles of ATP-driven mechanical processes in biology.

In the case of the nonsupercoiling type II enzymes (topo II and topo IV), the principles governing the selectivity of their reactions (e.g., decatenation vs relaxation) remain to be established. The role of ATP hydrolysis, which was previously obscure, is now established as the generation of nonequilibrium product distributions. However, the geometrical and/or kinetic mechanisms underlying the coupling of ATP hydrolysis to these reactions remain unclear, and a full description of this process is likely to reveal fundamental features of this and other energy coupling systems.

ACKNOWLEDGMENT

We dedicate this review to the memory of Nick Cozzarelli, in recognition of his outstanding work in this area. We thank James Berger, Lionel Costenaro, Sylvain Mitelheiser, Andrzej Stasiak, and Tanya Stuchinskaya for their comments on the manuscript.

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BI700789G