DNA topoisomerases—the 'relaxers' and 'unknotters' of the genome

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Living organisms must access, maintain, and duplicate the genetic information found in DNA. To utilize or copy the information in DNA, the strands of the double helix must be able to separate, but this process causes excessive twisting of the double helix known as supercoiling. Supercoiling must be carefully monitored and maintained for DNA metabolism to take place. Cells employ a family of enzymes known as DNA topoisomerases to maintain the topological state of DNA and to allow routine cellular processes like transcription and replication to occur. Topoisomerases reversibly cut one or both strands of the double helix in order to resolve supercoiling, knotting, and tangling in the DNA. Without topoisomerases, transcription and replication could not occur because of excessive positive supercoiling associated with separating the strands of DNA. Topoisomerases are also responsible for unlinking sister chromatids following replication so that the chromosomes can be segregated properly during mitosis. Further, topoisomerases do not conform to standard evolutionary phylogenies and require multiple independent origins (described as 'convergent evolution') to explain the various classes. Topoisomerases represent a fascinating family of enzymes that play a critical role in cellular life.

Biological life as we know it is dependent on instructions, most of which come in the form of genetic information contained in DNA. The human genome exists as 46 segments of DNA called chromosomes, which in most of our cells consists of 2 copies of chromosomes 1–22 plus either XX or XY. Human chromosomes vary in length and consist of around 100 to 200 million base pairs (bp, pairs of nucleotides, which are the monomers of nucleic acids) each and together add up to around 6 billion bp. If these chromosomes were stretched out from their compacted form and lined up, there would be around 2 m of DNA per human cell! This set of chromosomes is found in every body cell except those lacking a nucleus (e.g. mature red blood cells) or the reproductive cells, which contain one copy of each chromosome rather than two.

While the information encoded by the nucleotide bases exists as a linear sequence, it is compacted in the form of the double helix we know as the structure of DNA (figure 1A). The double helix is an elegant form with very precise structural constraints. From a cellular perspective, the picture is more complex, as the double helix exists in a nuclear matrix and is compacted by winding around histone proteins (in eukaryotes and some archaea) (figure 1B). The typical image of a chromosome 'spread' or karyotype shows the fully compacted forms of the chromosomes as they exist during cell division, which represent a compaction of around 10,000 fold. During other parts of the cell cycle, chromosomes exist in dynamic states of compaction depending on which regions of the genome are needed in a given cell type.

This leads us to some interesting questions: how does the cell access compacted regions of the genome for transcription and replication? What challenges are faced when dealing with the double helical nature of the DNA? Further, how does a cell deal with the enormous amount of genetic information in the nucleus? James Watson and Francis Crick understood that the double helix itself would pose certain challenges to the cell. However, their confidence in their structure left them to conclude: "we feel that these objections will not be insuperable."[1] Their understanding of nuclear processes was far less developed than our modern understanding, but their foresight proved to be accurate.

To envision the problem, imagine a double-stranded rope where the strands are wrapped around each other into a double helix (figure 2). Trying to separate these ropes by simply pulling the two strands apart is quickly met with resistance because they are wound around one another (assuming the duplex is extremely long or the opposite end is fixed in space and cannot untwist freely). As seen in figure 2, strand separation leads to the ropes becoming more tightly wound ahead of the separation. Overwinding causes supercoiling, which looks similar to an old-fashioned telephone cord that has become twisted (see figure 2). If there is no way to relieve this torsional strain, the separation of strands will be halted. The same challenges exist for our DNA when the strands of the double helix are separated.[2]

For example, during replication DNA helicases 'unzip' the strands of DNA in an ATP-dependent process in order for DNA polymerases to copy the DNA. However, the work of the helicases results in torsional strain and supercoiling within the DNA strands that must be relieved for replication

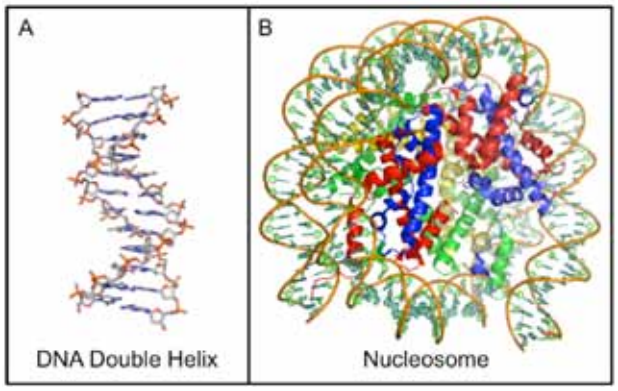

Figure 1. Panel A: Stick diagram of DNA double helix segment. Structure from PDB ID 1BNA. Panel B: Ribbon and line diagram of crystal structure of DNA wrapped around histone proteins to form a nucleosome. Structure from PDB ID 5AV5. Images generated using Pymol.

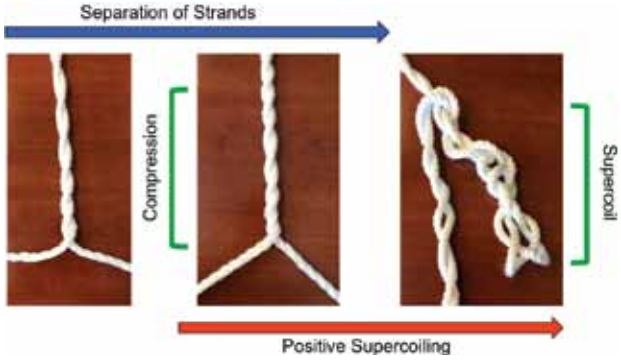

Figure 2. Supercoiling results from pulling apart coiled strands. As the ends of a double-stranded coil are pulled apart, the coils become compressed. If the compression is not relieved, it will result in supercoils. These are termed positive supercoils.

to continue.[2] Furthermore, transcription involves strand separation mediated by the RNA polymerase and faces positive supercoiling as seen with replication.[2] These are critical challenges in the cell. If separating the DNA strands for replication and transcription is necessary yet causes torsional strain, how could cells survive unless there was a mechanism to alleviate this problem?

As it turns out, Watson and Crick were right—the challenges were not 'insuperable'! In fact, cells overcome these challenges by using a family of enzymes known as DNA topoisomerases. A topoisomerase is an enzyme that identifies the topology (topo-) of DNA and alters the three-dimensional conformation of it (-isomerase), without changing the chemical nature. The end result is that topoisomerases relieve strain and also remove knots and tangles.

Types of topoisomerases

Topoisomerases are classified based on the structures and mechanisms of the specific classes. There are two primary types: I and II. Within these types are several subclasses (i.e. IA, IB, IC) that differ in specific details (table 1).[2–4] Broadly speaking, type I enzymes cut one strand of the DNA double helix while type II enzymes cut both strands of the DNA (figure 3).[4] In both type I and II enzymes, this involves an active site tyrosine amino acid becoming covalently bonded to either the 3′ or 5′ end of the cleaved DNA. Conveniently, type I topoisomerases have been named with odd numbers (I, III, V) while type II topoisomerases have been named using even numbers (II, IV, VI, VIII).[3,4]

Table 1. Families of topoisomerases and representative examples. Domains where specific enzymes are found are denoted: A, archea; B, bacteria; E, eukaryote. Activity is classified by the ability to support relaxation (rel) or supercoiling (sup) and the direction of supercoiling is denoted: – for negative supercoils and + for positive supercoils. Representative species are denoted at right. It should also be noted that there are viral topoisomerases in the IA, IB, and IIA families that are not shown in the table above.

Family	Enzyme	Linkage	Mechanism	Cofactors	Activity	Rep. Species
IA	topoisomerase I (B) topoisomerase III(a,b) (E) topoisomerase III (A, B) reverse gyrase (A)	5′	enzyme-bridged strand passage	Mg^{2+}	rel -	*E. coli* *H. sapiens*
				Mg^{2+}, ATP	sup +	*E. coli* *Sulf. acidocaldarius*
IB	topoisomerase IB (E, B) topoisomerase IB mitochondrial (E)	3′	controlled rotation/swiveling	none	rel -+	*H. sapiens*
IC	topoisomerase V	3′	controlled rotation/swiveling	none	rel -+	*M. kandleri*
IIA	topoisomerase II (E) topoisomerase II(a,b) (E) topoisomerase IV (B) gyrase (A,B)	5′	double-strand passage 4-bp overhang	Mg^{2+}, ATP	rel -+	*S. cerevisiae* *H. sapiens*
					sup -	*E. coli* *E. coli*
IIB	topoisomerase VI (A, E)	5′	double-strand passage 2-bp overhang	Mg^{2+}, ATP	rel -+	*Sulf. shibatae*

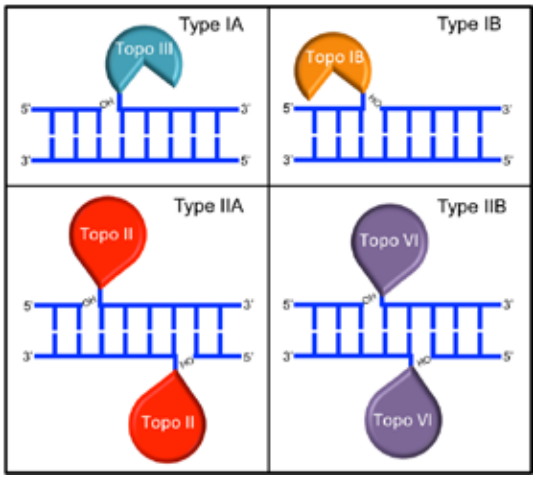

Figure 3. Various DNA cleavage mechanisms of topoisomerases. Topoisomerases cleave one or both strands of DNA by making a covalent link between a tyrosine on the enzyme and a phosphate of the DNA backbone. Type I topoisomerases cleave one strand of the duplex by linking to either the 5′ end (type IA) or 3′ end (type IB). Type II topoisomerases cleave both strands of the double helix with either a four base-pair (type IIA) or two base-pair (type IIB) stagger. It should be noted that bacterial topoisomerase I is a type IA, while mammalian topoisomerase IB is a type IB.

Topoisomerase function can also be described mathematically using the topology concept of linking number, which is discussed elsewhere.[2]

First, type I topoisomerases are generally monomeric enzymes (a single protein) that are able to: 1) bind to DNA, 2) cut one strand by forming a transient covalent bond with it, 3) relieve the torsional strain by either controlled rotation (IB/IC) or strand passing (IA), and 4) ligate (covalently reconnect) the cut DNA strand back together and release the DNA (figure 3).[4] The process facilitated by most type I topoisomerases is known as relaxation because it 'relaxes' supercoils in the DNA (figure 4). This is needed during both DNA replication and transcription of RNA. There are other roles for specific subclasses of type I topoisomerases, including the resolution of recombination intermediates.[3]

Type I topoisomerases are present in all known life forms and are referred to by several specific names. For example, bacterial topoisomerase I, known as the ω protein (lower-case omega), is the first topoisomerase ever discovered.[5] Jim Wang of Harvard University discovered the ω protein while searching for factors that seemed to change the supercoiling of bacterial DNA, which turned out to be a type IA topoisomerase in bacteria.[5,6] Humans have two type IA topoisomerases known as topoisomerase IIIα and IIIβ, which are present in the nucleus.[3] Interestingly, there are also two type IB enzymes in humans known as topoisomerase IB and mitochondrial topoisomerase IB (Top1mt).[7,8] These enzymes primarily differ in the N-terminal domain, where the nuclear form has a nuclear localization sequence and Top1mt has a mitochondrial targeting sequence (and lacks about 150 amino acids found in the nuclear form N-terminus).[9]

Second, the type II topoisomerases involve a related but more complex mechanism.[2,10,11] These enzymes are generally homodimers or heterotetramers (A_2B_2), utilize ATP, and often require a metal cofactor (Mg^{2+}).[2,10,11] Type II topoisomerases 'recognize' helix-helix crossovers (see catenanes/links depicted in figure 4). As seen in figure 5, the enzyme works by: 1) binding at a crossover, 2) cleaving both strands of one double helix (the Gate- or G-segment), forming a covalent bond with the 5′ ends, 3) separating the ends of the broken segment of DNA (DNA gate) and passing the intact double helix, known as the

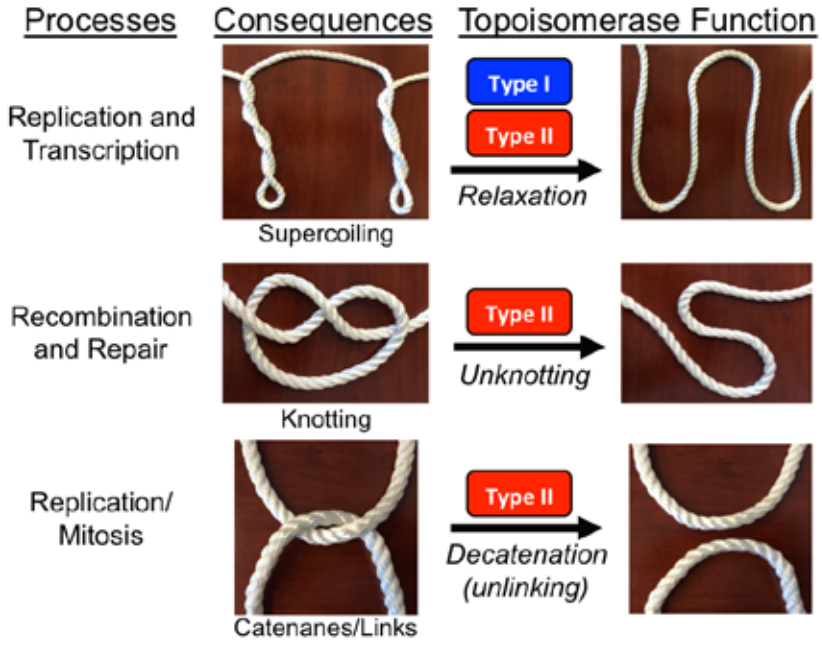

Figure 4. Topological consequences of biological processes and the alleviation of these challenges by topoisomerases. The figure above depicts a summary of some of the topological challenges addressed by topoisomerases. Note that in this figure, a single strand of the rope represents the double helix. Replication and transcription both cause positive supercoiling of the DNA, and this can be alleviated by type I and type II topoisomerases in a process called relaxation. Recombination and DNA repair may involve knots that can be removed by type II topoisomerases. Replication results in chromosomes that are catenated or linked. Type II topoisomerases can relieve these links so that mitosis can occur and chromosomes can segregate.

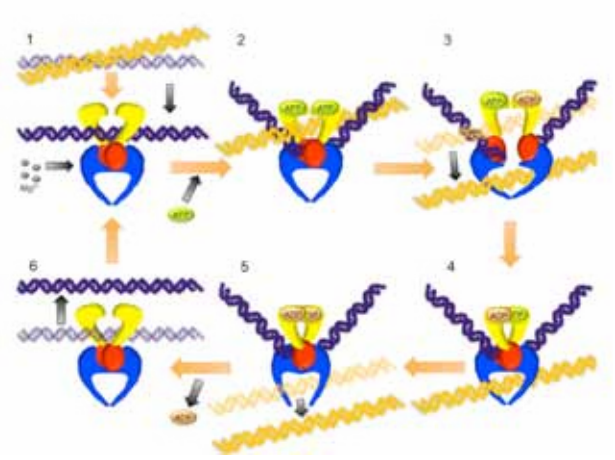

Figure 5. Catalytic cycle of type IIA topoisomerases. Topoisomerase II is depicted with distinct domains in different colours (yellow: N-terminal ATPase domain, red: central TOPRIM domain, and blue: C-terminal domain). Some details are omitted for clarity. 1) The enzyme binds to a helix-helix crossover (DNA in gold and purple). DNA cleavage is dependent upon metal ions (silver). 2) The gate segment (G-segment) in purple is bent and cleaved. The transport segment (T-segment) is captured by the N-terminal clamp in the presence of ATP (green). 3) The G-segment is opened and hydrolysis of one molecule of ATP induces strand passage of the T-segment. 4) After strand passage, the G-segment closes. 5) The C-terminal gate is opened to release the T-segment. ATP is also hydrolyzed in this step. 6) The enzyme releases the G-segment and is set for another round of catalysis.

Transport- or T-segment, through the break in an ATP-dependent manner, 4) closing the 'DNA gate' and ligating the G-segment, 5) releasing the T-segment, and 6) releasing the G-segment. The enzyme then is set to catalyze the reaction again as needed.

Bacteria generally have two type II topoisomerases: DNA gyrase and topoisomerase IV. Gyrase works in a distinct manner from that of many other topoisomerases since these enzymes negatively supercoil DNA while other type II enzymes can only convert a molecule from supercoiled to relaxed. We will not go in to the mechanism in detail here, but reviews are available in the literature.[12]

In mammals, we find two versions of the type II enzyme: topoisomerase IIα and topoisomerase IIβ.[10] From a broad perspective, there are many similarities in these enzymes so they are often generically referred to as topoisomerase II or Top2. However, they differ in size (by around 100 amino acids), are expressed from distinct genes on separate chromosomes, and only share around 70% amino acid identity.[10] Further, there are some functional differences. For example, topoisomerase IIα is generally considered to be active during replication and mitosis while topoisomerase IIβ is active during transcription.[13–16] Topoisomerase IIα expression levels rise and fall during the cell cycle while topoisomerase IIβ remains more constant.[13,17–21] Topoisomerase IIβ is considered a housekeeping gene since all nucleated cells express it to support transcription.[22–24] Interestingly, the main difference between the sequences of α and β is in the C-terminal domain (less than 35% identity). This region is responsible for the differences in functional capabilities and influences the selection of substrate.[15,25]

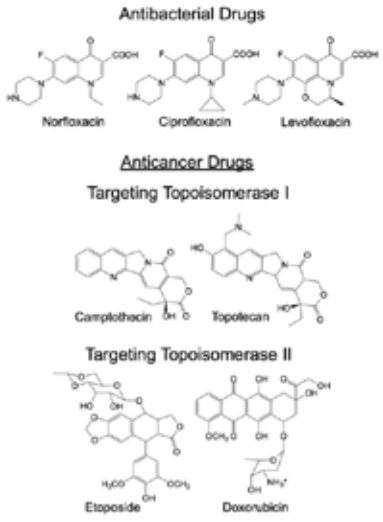

Figure 6. Topoisomerases are targeted for therapeutic purposes. Bacterial topoisomerases are targeted by agents such as norfloxacin, ciprofloxacin, and levofloxacin. Human topoisomerases can be targeted by anticancer agents targeting topoisomerase I (camptothecin and topotecan) or topoisomerase II (etoposide and doxorubicin).

Cellular roles and essential functions

What roles do these enzymes play in cells? In order to understand the functions, it is important to consider how the topology of DNA is altered by routine cellular processes. In biological systems, DNA exists in a supercoiled state.[2] As noted earlier, supercoiling is essentially what happens to a coiled telephone cord that becomes excessively twisted over time (see figure 2). Coiled cords tend to become supercoiled until at some point they are unwound (usually out of frustration!). DNA generally exists in a state that is slightly negatively supercoiled, which means that the double helix is slightly opened compared to a fully relaxed DNA segment.[2] During the processes of transcription and replication, DNA strands are separated and become positively supercoiled to compensate for the strand separation. If left unresolved, excessive supercoiling will inhibit replication and transcription. Both type I and type II topoisomerases are able to relieve supercoiling that results from these processes by working ahead of replication forks and transcription bubbles (figure 4).[26] It has been found that type I enzymes are essential in a number of different organisms, probably because of the role of type I topoisomerases in relaxation, though other roles also exist.[8]

Additionally, the type II enzymes are also involved in unknotting and unlinking DNA molecules (figure 4).[10] Replication is the process by which

chromosomes are copied and results in the sister chromatids (the products of replication of a single chromosome) being wound around each other or catenated.[2,10] Decatenation is the technical term used to describe the process of separating linked chromosomes (figure 4). Only type II topoisomerases can decatenate and this is considered to be the essential function of type II topoisomerases.[2,4,10] Evidence suggests that the progress of decatenation can serve as a cell cycle checkpoint during mitosis and promotes genomic stability by ensuring the proper separation of sister chromatids.[27] Thus, all known living organisms have at least one isoform of the type II enzyme.[3,8] Further, topoisomerase IIα is essential in mammalian cells as it serves the role of decatenating linked chromosomes.[10] Cellular DNA in eukaryotes and some other life forms exists as a complex of protein and DNA known as chromatin. Chromatin is condensed and decondensed depending on epigenetic regulation and the phase of the cell cycle, which again relies on type II topoisomerases.[10] Aside from these roles, there is a topoisomerase-like protein known as Spo11, which is involved in creating strand breaks associated with meiosis, but this is beyond the scope of the current discussion.[28–30]

Therapeutic targeting

As a result of the universal nature of these enzymes and their essential functions in living organisms, they are also used as therapeutic targets (figure 6).[31] In particular, bacterial versions are targeted by antibacterials like levofloxacin (targeting a bacterial topoisomerase II).[31] In addition, mammalian topoisomerases can be targeted by a number of anticancer drugs such as camptothecin and topotecan, which target topoisomerase I, and doxorubicin and etoposide, which target topoisomerase II.[11,31] These agents generally

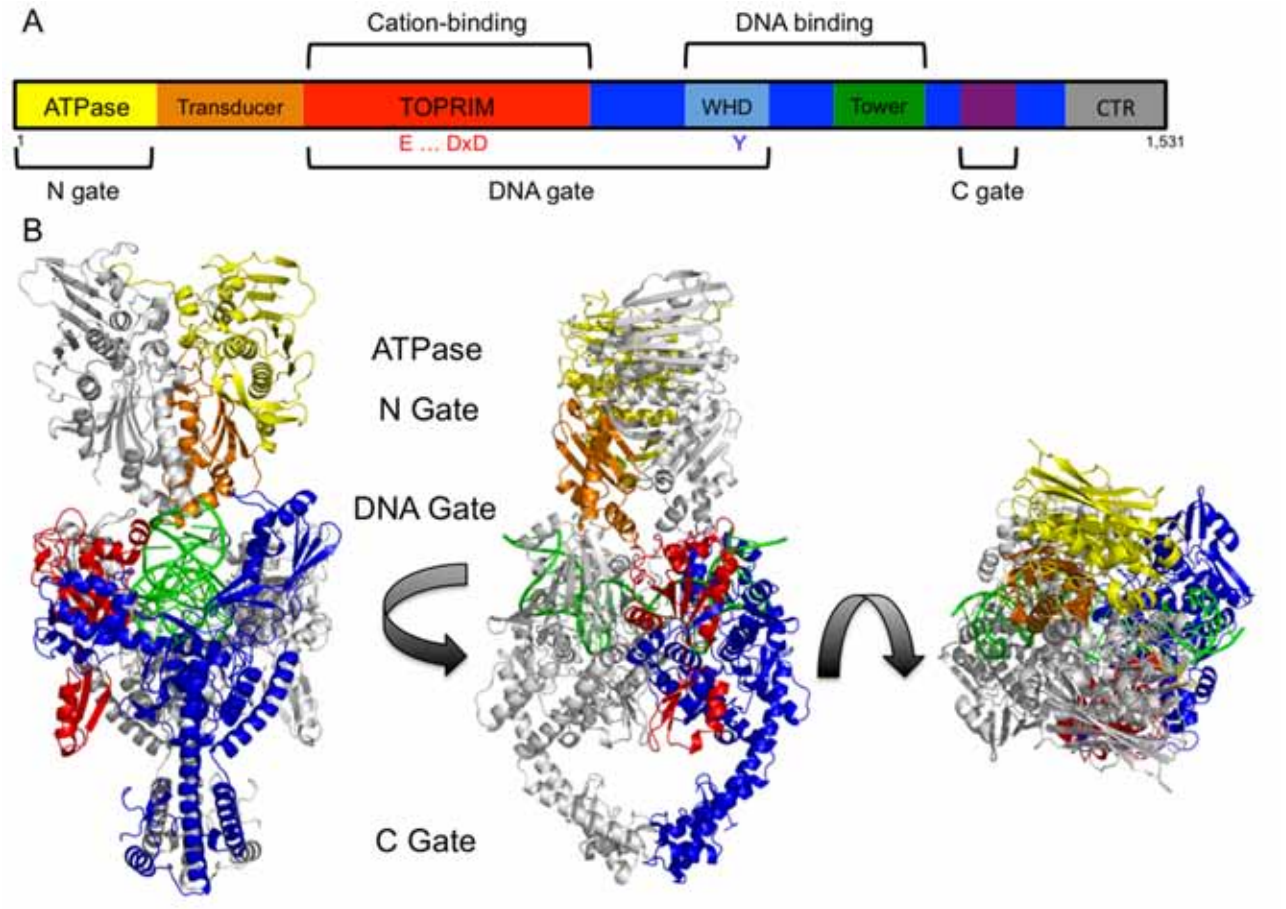

Figure 7. Domain structure diagram and crystal structure images of a type IIA topoisomerase. A) The general domain structure of a type IIA topoisomerase. Size and spacing of segments is approximate. Amino acid numbers correspond to human topoisomerase IIa. Location of acidic amino acids in the TOPRIM (E, D) and the active site tyrosine (Y) are denoted. The variable C-terminal region is also depicted (CTR). B) Ribbon diagram images generated from a crystal structure of Saccharomyces cerevisiae topoisomerase II (PDB ID 4GFH; residues 1–1177). Images show a 'side', 'front', and 'top' view, respectively. One protomer is shown in grey. The other protomer is colour coded: ATPase in yellow; transducer in orange; TOPRIM in red; DNA binding and C-gate in blue. It should be noted that this structure does not include the C-terminal region. Images generated using Pymol.

work by preventing the enzyme from completing the catalytic cycle, which causes an accumulation of single- and double-stranded DNA breaks.[11,31] Therefore, they are often called topoisomerase poisons.[11,31]

In contrast, a separate class of compounds, known as catalytic inhibitors, interrupt enzyme function by preventing ATP hydrolysis.[31] What is unique about these compounds is that cell death is not promoted by overwhelming the cell with DNA damage. These compounds block catalytic activity, which prevents topoisomerase II from participating in transcription- and replication-associated topology regulation.[2,32] Consequently, cells will get 'stuck' and will be unable to complete cell division. While theoretically this class of compounds should be useful for specific purposes, there are only a few examples of clinically relevant catalytic inhibitors.[31] Ongoing research is continuing to explore new options for this pathway.[33]

Type IIA topoisomerases—a more detailed look

Let us focus on the second class of enzymes and examine in more detail the intricate design of this molecular machine. Specifically, we will examine the eukaryotic topoisomerase II enzymes, which are homodimeric (two copies of the same protein), multi-domain proteins.[10,31] Each protomer is ~1500–1600 amino acids depending on the specific isoform, which makes this a rather large dimer (over 300 kDa). The general primary sequence includes: 1) an N-terminal ATPase domain; 2) a centrally located cleavage-and-ligation domain, and 3) a C-terminal variable domain (figure 7). This simplification leaves out the fact that several key features in this protein only exist in the three-dimensional structure.

First, topoisomerase II is a homodimeric enzyme with twofold axis of symmetry (figure 7). An amazing feature of the symmetrical nature of the enzyme is that the protein makes multiple points of contact between the protomers that are able to be alternately separated and rejoined. The full structure has three 'gates': an N-terminal upper gate, a C-terminal lower gate, and a centrally-located 'DNA gate'. Further, the separation of these gates is coordinated such that two points of contact are maintained during catalysis (figure 5).[34] For example, if the N-terminal gate is open, the DNA gate and lower gate are closed. To open the DNA gate, the N-terminal gate closes. Once the strand passage is completed, the DNA gate closes and allows the C-terminal gate to open. This coordination is amazing considering the machine involved is a protein made of amino acids. This type of coordination requires the enzyme to be able to cycle through various states of opening/closing in a concerted manner. These conformational changes in the protein are regulated by various factors including the presence of DNA,

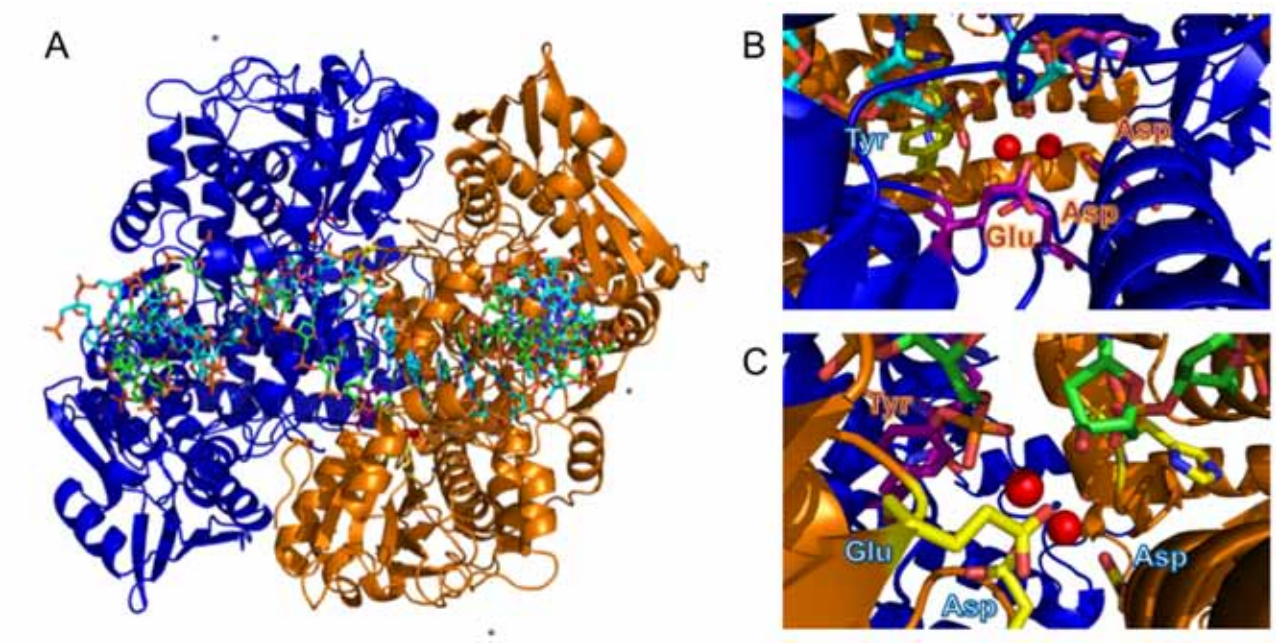

Figure 8: A crystal structure of a covalent topoisomerase II:DNA complex shows cooperation between the two protomers. A crystalized core of truncated yeast topoisomerase II (missing portions of the N- and C-terminus) is shown in complex with a segment of DNA. A) Top view showing protomers in orange and blue with DNA through the central cavity. B and C) A close-up view of the two active sites. Critical residues from each half cooperate in cleavage and ligation including the active site tyrosine (Tyr) and glutamic (Glu) and aspartic acid (Asp). Residues from the orange protomer are highlighted in yellow while those from the blue protomer are highlighted in purple. The red spheres represent two metal ions. The active site tyrosine is covalently linked with the 5' end of the cleaved DNA. Images were generated from PDB ID 3L4K using Pymol.

the topology of DNA, and the availability of ATP and metal ion cofactors.

Second, the axis of symmetry also sets up a very interesting scenario. For topoisomerase II to cleave DNA, there is a specific arrangement of amino acids in the active site (figure 8). This includes the TOPRIM (topoisomerase-primase) domain where several acidic amino acids form a characteristic motif (E … DxD) that coordinates Mg^{2+} ions.[35] Also, there are other amino acids that appear to play a role in the process such as an arginine and a histidine.[36] Further, the active site amino acid is tyrosine. Tyrosine has a hydroxyl (–OH) that is able to attack the backbone of DNA at a phosphate group in concert with the adjacent Mg^{2+} ions coordinated by the TOPRIM domain. The phosphate backbone is broken and the 5′-phosphate group is bound to the tyrosine. This leaves a free 3′-OH group on the other side of the break. Intriguingly, both active sites require amino acids from each half of the protein. The metal-ion coordinating residues of the TOPRIM domain come from one protomer and the active site residue and some others come from the other protomer.[36] So, the active site only exists as a whole when the DNA gate is closed (figure 8). Once the cleavage/ligation domain separates (opening the DNA gate), the active site residues are no longer in proximity, which maintains the DNA/enzyme linkage. Amazingly, the metal-ion-binding amino acids are in the correct place within the primary sequence so that in the folded, quaternary structure they are appropriately located adjacent to the opposing protomer's active site tyrosine prior to opening of the DNA gate (figure 8).

This design feature ensures that the DNA is brought back together, allowing the ends to line up, before the enzyme catalyzes the ligation reaction and releases the DNA.[37,38] Further, the mechanism is balanced in a way that the enzyme preferentially ligates rather than cleaves—keeping these enzymes from simply 'chewing up' the genome.[39] The transient nature of the interaction helps avoid situations where enzyme-DNA complexes exist as 'road blocks' to the transcription or replication machinery. It is precisely this interaction that is hijacked by several of the therapeutic agents mentioned above. The stabilization of this complex on DNA leads to DNA strand breaks and DNA damage.[11]

Third, the enzyme contains a DNA binding groove, complete with positive charges, attracting the DNA backbone that is negative at normal cellular pH, and two 'clamps' large enough to accommodate a double helix. Together, this demonstrates that topoisomerase II is well-suited to handle and manipulate DNA. The DNA binding groove again involves proper alignment of the axis of symmetry of the protomers to allow for the G-segment to bind in the active site for cleavage. Further, the upper and lower clamps accommodate the transport segment before and after strand passage, respectively. According to structural evidence, the active site tyrosine actually shifts after DNA cleavage, which moves 'levers' in the enzyme and closes the C-gate.[36] Again, the key arrangement of nucleotides in three-dimensional space is critical for this structure to be able to bind and manipulate DNA. Further, the enzyme actually induces an extreme bend in the G-segment supported by specific amino acids in the binding groove.[36,40] DNA cleavage occurs in a stepwise manner with the first strand break allowing for the second strand break to occur more rapidly.[39,41]

Fourth, the utilization of ATP has been an enigma for some forms of topoisomerases. The relaxation and decatenation reactions catalyzed by many topoisomerases are considered to be thermodynamically favourable. Since ATP is not needed to 'power' strand passage, why then is ATP required? The answer appears to be that the ATP helps maintain the contact between the two halves of the N-gate during strand passage and release in order to prevent dimer dissociation.[34,42] Structural evidence indicates that the ATPase domains twist or wrap around each other in the presence of ATP (figures 5 and 7).[42] In other words, the ATP appears to keep the dimer from falling apart while performing a unidirectional strand passage! Interestingly, evidence suggests that the ATP is hydrolyzed sequentially during the latter portion of the catalytic cycle (strand passage/ligation/strand release).[43] Although, the exact significance of the sequential hydrolysis is unknown. The ATPase sequence is followed by the transducer domain (figure 7). This domain is involved in 'communicating' to the cleavage/ligation domain when ATP is bound and likely helps coordinate the conformational changes occurring during catalysis.[10,44]

Implications of topoisomerases

First, DNA topoisomerases are clearly valuable and important enzymes for life since all identified living organisms depend on DNA. Since regulation of DNA topology is fundamental and essential to life, it is not a stretch to argue that topoisomerases are required to support life as we know it. In fact, DNA topoisomerases are present in living organisms from the 'simplest' to the most complex and even in some viral genomes.[3,8,45]

This brings up an interesting quandary. Topoisomerases are encoded on the DNA that is their substrate, which raises the question of which came first: DNA or the topoisomerases responsible for alleviating torsional strain in DNA? Without topoisomerases, cells lose the ability to regulate DNA topology, which quickly halts replication and transcription. Without DNA, there would be no means for encoding the information needed to build the enzyme.

Whatever evolutionary process may be posited, it is important to point out that DNA topoisomerases would have to be present very early, as even the simplest DNA genome would have a need for the regulation of DNA topology (assuming that this genome needs somewhere close to the number of genes required for living organisms today). The same could be argued for DNA polymerases, helicases, and a host of other enzymes that function on DNA. The essential function of a number of common enzymes must be in place for this system to work. The biochemical network that 'runs' cells is extraordinarily interdependent—proteins, DNA, RNA, carbohydrates, lipids—all of these biomolecules rely on one another at some level! In addition, this is not a matter of natural selection driving the process since natural selection really describes differential reproduction, which requires at least two self-reproducing entities. Topoisomerases are essential for this reproduction in DNA-based organisms. Thus, differential reproduction may help us understand some of the diversity of topoisomerases, but it cannot explain the *origin* of the topoisomerases.

Second, as described earlier, DNA replication results in sister chromatid pairs that are interlinked or catenated. This problem is alleviated by type II topoisomerases, though evidence shows that not all isoforms of the type II enzymes can fulfil this function. Decatenation by type II topoisomerases must occur in order for cells to segregate chromosomes into separate daughter cells. In other words, cell division would be impossible without the decatenation activity of type II topoisomerases.[10] In mammals, this involves topoisomerase IIα and not topoisomerase IIβ.[15] For this reason, loss of topoisomerase IIα cannot be compensated for by topoisomerase IIβ.[10] Further, whether chromosomes are circular or linear, the problem remains because any linear chromosomes large enough to encode the complement of genes required for the simplest living organisms (e.g. 580 kbp in *Mycoplasma genitalium*[46]) would be too large to decatenate on their own. Decatenation by a type II topoisomerase would be needed even for genomes much smaller than 500 kbp and is required to separate even small circles of DNA (plasmids).

Third, the simplest known type II topoisomerases contain approximately 800 amino acids split between two segments. Each half of the eukaryotic enzyme is actually a separate protein in bacterial versions. Thus, the quaternary protein structure in bacterial topoisomerase II is an A_2B_2 tetramer, which means there are two copies of the 'A' subunit and two copies of the 'B' subunit. From an evolutionary perspective, the genes for these subunits must fuse and expand over time to become what is found in eukaryotic cells (unless the eukaryotic enzymes had a distinct origin). An alternative explanation suggests that one gene (gene A, for example) gradually added features until reaching the point of no longer needing the other gene (gene B, in this case). These scenarios would rely on natural selection along with random mutation in order to explain the consolidation of functions that were already operating in an apparently effective manner since bacterial type II topoisomerases are tetrameric (with the exception of topoisomerase VIII, a recently discovered topoisomerase that is dimeric and distinct from type IIA and IIB topoisomerases[47]). Both the fusion and the consolidation scenarios are speculative at best. In fact, phylogenetic analysis, as discussed below, points to independent origins for the type IIA topoisomerases in bacteria and eukaryotes.

Fourth, examination of the family tree yields no clear evolutionary pathway for the development of topoisomerase genes over time. For example, consider the following assessment by topoisomerase researchers regarding the evolutionary origins of these molecular machines:

> "The phylogenetic distribution of DNA topoisomerases is thus *quite puzzling and clearly does not agree with the classical universal tree of life: neither with any of the alternative models such as the bacteria-first model nor with the ring of life model.* Another puzzling problem is the phylogenetic position of viral DNA topoisomerases. Whereas in some cases their placement can be explained by HGT [Horizontal Gene Transfer] from a cellular host (e.g. mimivirus Topo IA and IB), in other cases (e.g. Poxvirus Topo IB, T4 Topo IIA) the viral DNA topoisomerases form well-defined subfamilies that are only distantly related to their cellular counterparts [emphasis added]."[3]

Additionally, the recent discovery of the dimeric topoisomerase VIII in archaea and bacteria provides an additional example that does not fit well into evolutionary phylogenetics.

> "This is therefore another example of a topoisomerase with an *unusual phylogenomic distribution and complex evolutionary trajectory* … . Notably, bona fide topoisomerase VI enzymes present in bacteria cannot be distinguished from their archaeal homologs and branch with archaeal DNA topoisomerase VI enzymes in phylogenetic analyses; in contrast, topoisomerase VI and VIII enzymes are so divergent that their amino-acid sequences cannot be reliably aligned for phylogenetic analyses. It is *difficult to explain* why the fusion protein of the two topoisomerase VI-like subunits (i.e. the ancestor of topoisomerase VIII) would have diverged so rapidly in one particular bacterial lineage but remained conserved during its dispersion in various bacterial lineages [emphasis added]."[47]

In fact, the phylogenies are so incongruous that not only are there at least five proposed origins representing each of the classes (IA, IB, IC, IIA, IIB), but many of the members of the classes are proposed to have had independent

origins, particularly within the domains of life.[3,8,45] As noted above, each of these classes have highly similar mechanisms and protein structures. The implications of the similarities between the amino acid sequences are that these mechanisms and structures represent common designs used to solve the challenges associated with DNA topology. However, evolutionists conveniently rely on 'convergent evolution' to explain how 'nature' arrived at the same solution multiple times independently!

Further, one literature review on the evolution of topoisomerases suggests: "An intelligent designer would have probably invented only one ubiquitous Topo I and one ubiquitous Topo II to facilitate the task of future biochemists. The reality turned out to be quite different, and more interesting."[8] What an interesting (and bold) claim: an intelligent designer would want to 'facilitate the task of future biochemists' and simplify the understanding of topoisomerases by only designing one of each type. This seems to miss the point that the various classes of topoisomerases that exist appear to be playing unique and distinct roles (though at times there is redundancy of function). In addition, multiple versions of these enzymes allow for more complex regulation of function. Perhaps it would be simpler from an evolutionary perspective if there were only two but the fact that there are multiple classes and subclasses suggests that organisms are far more complex than evolutionists want to admit. The sophistication and interdependence of cellular biochemistry provides excellent evidence for how "fearfully and wonderfully made" we truly are.

To explain the alleged evolution of these enzymes, evolutionists have relied on the unprovable assumption of (multiple) horizontal gene transfer events and the alleged existence of hypothetical ancestral versions of the enzyme that no longer exist.[8] Further, even if horizontal gene transfer did occur to spread some of these genes across organisms (which is likely in some of the viral and plasmid-encoded forms), it has no power to explain the *origin* of the first topoisomerase genes.

One of the most recent proposed scenarios for the evolution of topoisomerases involves a complex transition from an RNA genome to a DNA genome with topoisomerases evolving along the way.[8] This scenario again is purely hypothetical and lacks evidence. Further, it glosses over another major problem in evolutionary phylogenetics. Topoisomerases are not the only enzymes that do not fit neatly into 'tree of life' phylogenies. In fact, topoisomerases are joined by DNA polymerases and several other classes of enzymes and proteins that do not fit the phylogenies and have significantly altered the 'tree of life' concept.[48,49] In summary, topoisomerases represent a significant challenge to evolutionary explanations of life, including abiogenesis and common ancestry.

Summary

DNA topoisomerases are complex molecular machines with multiple interacting domains and coordinated mechanisms. These enzymes resolve topological challenges in our genomes on an ongoing basis throughout the cell cycle and even in non-dividing cells. Both replication and transcription cause topological strain that must be alleviated for the cell to survive. Further, chromosomes become interlinked during replication and must be decatenated for cells to survive. Topoisomerases resolve these problems using a transient, enzyme-linked single- or double-stranded DNA break. The double-strand break mechanism of the type II enzymes allows for these enzymes to cleave the DNA and pass an intact double helix through the break while protecting the cleaved ends from forming a permanent double-strand break.

Evolutionary explanations for the chance development of topoisomerases are severely lacking and fail to account for the critical nature of these enzymes in living systems. Further, the symmetry, domain organization, and overall coordination of topoisomerases support the concept that these enzymes are very well designed and are perfectly suited to carry out the tasks of maintaining the topological state of the genome. Further, while the temporary strand breaks generated by topoisomerases are potential threats to the genome, the enzyme protects the genome by its preferential ligation of cleaved DNA. What an amazing class of molecular machines these enzymes are! Topoisomerases truly are the intricate molecular scissors and 'relaxers' of the genome!

Acknowledgements

I would like to express my appreciation to the numerous colleauges who have read and provided feedback on this manuscript. I also would like to thank my college and university for their support of my work.

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