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An Investigation into the Interaction of Various Salt Ions with DNA Topoisomerase IA during the Relaxation of Negatively-Supercoiled DNA.

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ABSTRACT

Topoisomerases are enzymes that change the topological state of DNA by catalyzing the cleavage, subsequent strand passage and religation of either one or both strands of DNA during DNA replication and transcription. This research thesis focused on *Escherichia coli* DNA topoisomerase IA, an enzyme largely responsible for the maintenance of supercoiling and the specific relaxation of negative supercoils in *E. coli* DNA. The various types of topoisomerases and their functions were discussed. As a basis for this paper, some of the structural properties not yet determined in topoisomerase IA were mentioned with respect to the catalytic mechanism. A description was given of the methods used for laboratory research, Monte Carlo simulations. The goals of this research, both short-term and long-term, were also discussed. Known background information about topoisomerase IA was given in detail with regard to the enzyme's structure and catalytic mechanism, followed by a discussion on the interaction of ions with the enzyme throughout its catalytic mechanism. The results of Monte Carlo simulation data were presented in the thesis, and a discussion on these results was given along with the future hopes of this important research.

INTRODUCTION

Over the last several years the mystery behind the structure and mechanism of action of topoisomerases has become of increasing interest to the scientific community. Topoisomerases are enzymes that change the topological state of DNA by catalyzing the cleavage, subsequent strand passage and religation of either one or both strands of DNA during DNA replication and transcription. Type I topoisomerases cleave single-stranded DNA (ssDNA), whereas type II enzymes cleave double-stranded DNA (dsDNA).

In recent years, as a result of their vital role in such cellular processes as reproduction and maintenance of homeostasis, topoisomerases have become the target of drug inhibitors, which have become widely used due to their efficacy as antimicrobial and antitumor agents [1]. *Escherichia coli* DNA topoisomerase I, belonging to the type IA subfamily found only in prokaryotes, is one such enzyme being targeted. This enzyme is responsible for the removal of excess negative supercoiling from chromosomal DNA [2].

Cellular DNA is coiled around itself along an axis in the form of a double helix. Supercoiling is a result of further coiling of the DNA along its axis. Negative supercoils are introduced in the strands by topoisomerases called gyrases and by other enzymes responsible for unwinding DNA. These enzymes catalyze the replication and transcription of DNA by separating the two complementary strands so that the information contained within the double helix may be read. Topoisomerases are able to alter a property of DNA called the linking number, which can be defined as the number of times one strand of DNA crosses the other. Negative supercoiling, which is the result of the underwinding of the DNA double helix, reduces the linking number of the relaxed DNA strand, while positive supercoiling, caused by the overwinding of the double helix, increases the linking number [3].

Since the topology and supercoiling of DNA play a pivotal role in virtually all cellular processes, the control of topoisomerases is of major pharmaceutical concern, and knowledge detailing their structure and mechanism of action is of great interest. This study will focus on the type I enzyme *Escherichia coli* DNA topoisomerase IA. In this area of research, some of the major questions involve the aspect of ion activity during the topoisomerase IA catalytic mechanism. For instance, there are various salt ions that actively contribute to the maintenance of the enzyme's stability throughout its mechanism of action. However, their positions and interaction with DNA topoisomerase IA during the relaxation of negatively-supercoiled DNA are not entirely clear. In addition, although no metal ions were found in the DNA topoisomerase IA crystal structure, Mg(II) is thought to be directly involved in the catalytic mechanism and its proposed location is somewhere near the acidic triad [1].

It is possible, through the use of computer simulations, to answer some of the mysteries surrounding the structure and mechanism of action of topoisomerase IA. Monte Carlo (MC) simulation software is a powerful and efficient tool used to generate sample configurations of the molecules in a system, which is subsequently used to calculate the bulk properties of the system by means of statistical mechanics. However the procedure for moving the constituent atoms is non-dynamical. The method employs small, random moves of the atoms in the system to generate sequences of atomic configurations. These simulations can generate millions of favorable configurations of an enzyme of interest and its complex with DNA during its mechanism of action. The software randomly places the ions within and around the enzyme such that the ions seek positions of lowest energy. If a configuration is favorable, the x,y,z coordinates for every ion and amino acid composing the structure are then stored on the computer's hard drive. If a configuration is not favorable, it is discarded and a new random configuration is

generated. This process is repeated over millions of cycles, efficiently sampling the possible configurations. Since the method is stochastic (i.e. non-deterministic) in nature it cannot be used to calculate time-dependent properties. It can however produce thermodynamic properties and details of structure such as radial distribution functions and x-ray diffraction patterns. The special advantages of Monte Carlo simulation software include the ability to make `unphysical' random moves, such as changing an atom's identity, and thus compute novel properties. Trends in the activity of salt ions (e.g., where ion coordination patterns around the enzyme shift at different stages of topoisomerase activity) may be noted by observing these simulations of the mechanism of action of DNA topoisomerase IA. In turn, observation of these trends may yield information about ion participation in topoisomerase structural stability and enzyme catalysis.

My laboratory research aims to generate hypotheses for the Mg(II) locations within the topoisomerase IA structure throughout the catalytic mechanism, learn favorable configurations the enzyme may hold in its complex with DNA during its mechanism of action, and understand the role the various other ions play in the relaxation of negatively-supercoiled DNA. This research will be combined with others' research in an attempt to more fully understand the specific details of the enzyme's structure throughout its catalytic mechanism. In the future, I hope that this research will lead to the synthesis of topoisomerase inhibitors that will inhibit bacterial cell function, leading to cell death and will thus act as antibiotics against *Escherichia coli* and perhaps other pathogens as well.

There are many aspects of the structure of topoisomerase IA that are understood. The tertiary structure of the enzyme consists of four domains (I-IV) with a hinge at the interface between domains II and III, and also between domains II and IV [4] (Figure 1). These hinges allow for domains I and III, which are normally in close contact, to temporarily separate so that

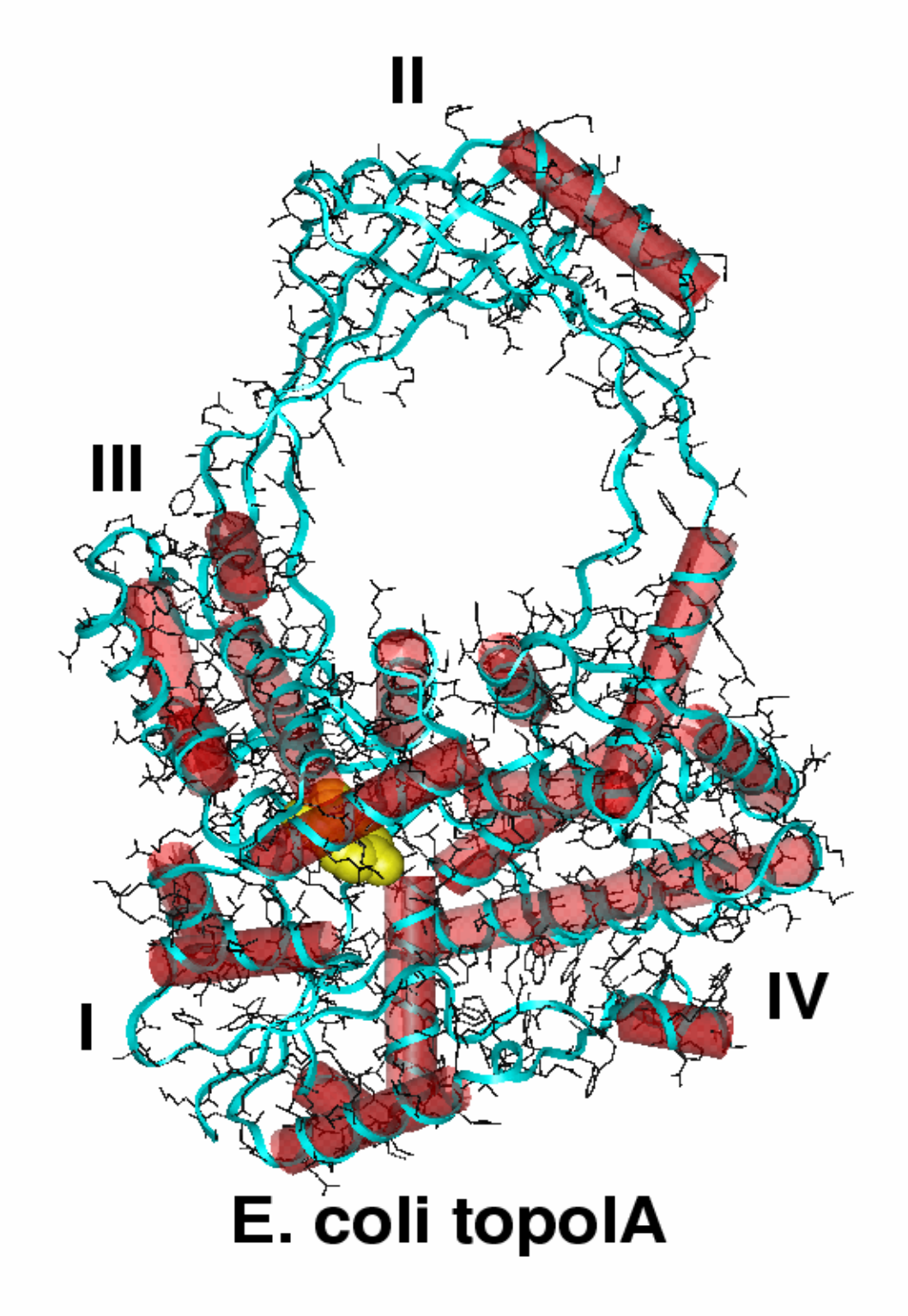

Figure 1. Crystal structure of *E. coli* topoisomerase IA. The toroidal structure of *E. coli* topoisomerase IA is shown looking directly through the hole created by the closed topological state. Domains I, II, III, and IV are labeled. The alpha-helices are represented as magenta cylinders. The active site tyrosine is colored yellow. (Figure courtesy of Dr. Daniel Strahs).

single-stranded DNA may move into the central cavity for strand cleavage, passage and subsequent religation. “Domain I contains a four-stranded, parallel β-sheet connected by several long α-helices. Domain II contains a small β-barrel, formed by the interfacing of two long, curved, three-stranded β-sheets. Domains III and IV are primarily α-helical and each contains a helix-turn-helix motif similar to those found in certain DNA-binding proteins such as the *E.* coli catabolite activating protein (CAP)” [1]. The polypeptide chain, beginning at the N-terminus, runs from domain I, through domain IV, domain II, and then through domain III, where it turns around and runs back through domain II, and ends at domain IV at the C-terminus. Domains I and III are on opposing ends of the enzyme, and the active site is located here.

The catalytic tyrosine (Tyr-319) that attacks and cleaves DNA is located within domain III. This residue sits in the active site of the enzyme formed by domains I, III, and IV [1]. The highly conserved active domain of the enzyme named TOPRIM is, in large part, responsible for the catalytic cleavage and religation of negatively-supercoiled DNA. Within this domain, near Tyr-319, are three acidic residues, Asp-111, Asp-113 and Glu-115, forming the acidic triad, which resemble the “three acidic residues known to coordinate two divalent ions in Klenow fragment” [1, 5]. The literature states that relaxation activity for double and triple mutants lacking two or more of these acidic residues requires higher concentrations of Mg(II) for maximal activity, supporting the hypothesis that the carboxylates in these three conserved acidic residues are involved in coordinating at least one of the two Mg(II) required for relaxation activity [5]. Moreover, it was determined that a single amino acid substitution in the TOPRIM domain changing a strictly conserved glycine residue (Gly-116) to serine in topoisomerase I can result in a mutant enzyme that has SOS-inducing and cell-killing properties [6]. This implicates the importance of Gly-116 in the binding of Mg(II) necessary for functional relaxation activity of

E. coli topoisomerase I. This mutation, in addition to affecting the Mg(II) binding affinity, is likely to also alter the active site structure inhibiting DNA religation, so that while DNA cleavage could take place with the addition of Mg(II), relaxation activity was still not observed at Mg(II) concentrations up to 26mM. Gly-116 is thought to influence the coordination of Mg(II) by the acidic triad. The alteration of the relative positioning of the 3'-hydroxyl group and the phosphotyrosine linkage would have an effect on DNA religation, and could account for the stabilization of the covalent cleaved complex and loss of relaxation activity [6]. In addition, there are other various ions, Na^+, K^+, Zn^{2+} and Cl^-, which interact with the residues of topoisomerase IA, helping to maintain the enzyme's structural stability throughout its catalytic mechanism. Also, these ions contribute to the enzyme's binding of DNA both in the active site pocket and, along with a positively-charged β-sheet, in the central cavity.

There are many aspects of the mechanism of action of topoisomerase IA that are understood as well. The structure of topoisomerase IA is said to resemble a padlock or ring clamp, with a "thickened base (domains I and IV) attached to a mobile arch (domain II)" [1] (Figure 2). This arch is hinged at domains III and IV, and permits domain III to be separated from domain I to allow DNA strands access to the active site. This opening and closing of the active site interface regulates the cleavage, strand passage and religation of single-stranded DNA. In the closed state, domain III is in close proximity to domains I and IV. Domain III is then pulled away from the interface, allowing a single strand of DNA to enter and be non-covalently bound to the active site. The O-4 oxygen of the tyrosine side chain is thought to then act as a nucleophile and attacks a specific position on the phosphodiester backbone bound at the active site [2, 4]. Following the cleavage of the DNA strand, the side chain of Tyr-319 remains covalently linked to the 5'-phosphate of one end of the cleaved DNA in a phosphotyrosine

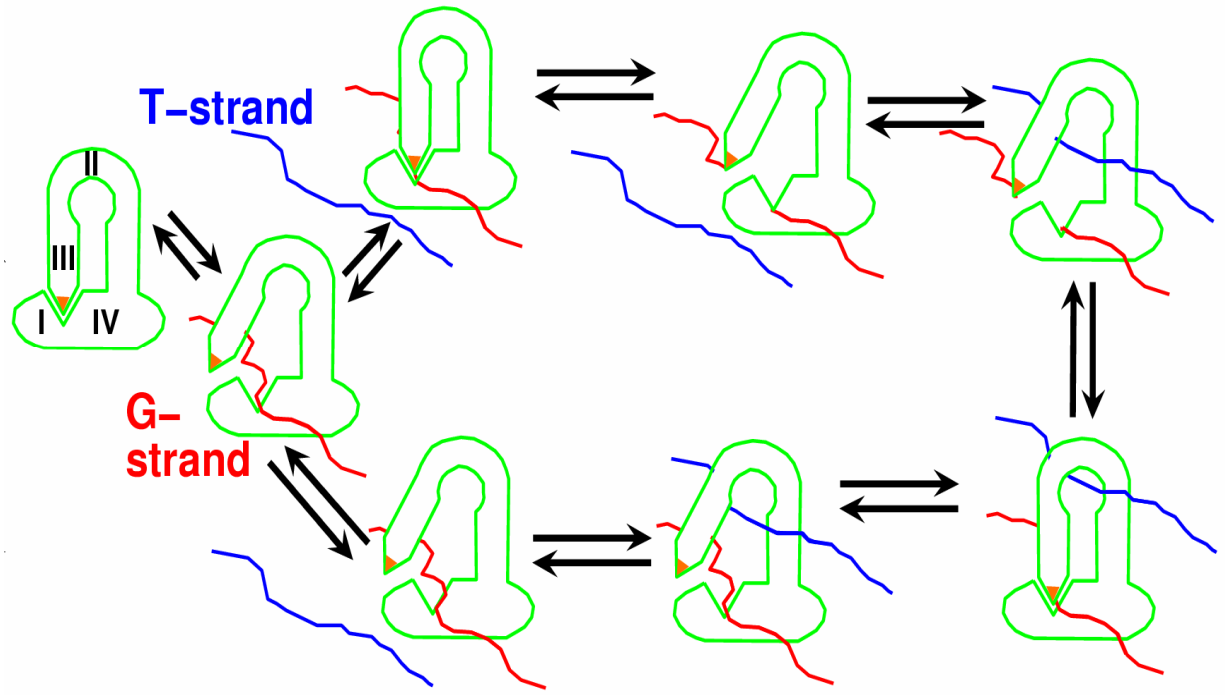

Figure 2. Schematic of the DNA cleavage/passage mechanism of *E. coli* topoisomerase I. As the orientation of the C-terminal 30-kDa region (residues 590-865) is not known, only the 67-kDa N-terminal domain is represented. From the closed state observed in the structure (step 1), a conformational change involving the separation of domain III from domains I and IV must occur to allow DNA to bind to the active-site pocket (step 2). The hinge region for this motion is proposed to reside between domains II and IV. Cleavage and religation of the bound ssDNA can only occur when domain III is reassociated with domains I and IV (step 3). Following cleavage, the 5' end of the bound ssDNA becomes attached to the active-site tyrosine while the other end remains non-covalently associated with the core; this allows the enzyme to open and 'bridge' the two ssDNA segments (step 4). The C-terminal 30-kDa region may assist in binding this DNA as well. The transported DNA (either duplex or single-stranded) is thought to temporarily reside in the central hole of the 67-kDa fragment during the cycle (step 6). Note that the molecule can effectively transport a DNA through a second, bound ssDNA segment by proceeding around the cycle in either direction. It is possible that the presence or absence of DNA in the central hole may affect the decision to open the clamp with or without having cleaved the bound ssDNA (step 4 vs. 8). Such a bias could provide directionality to the cycle [1]. (Figure courtesy of Dr. Daniel Strahs).

enzyme/DNA adduct while the free 3'-hydroxyl end occupies a nucleotide binding site within the domain I/IV cleft (the active site pocket) [1-2, 4]. Positively-charged ions may assist in the stabilization of the association of the cleft with the 3' end of the cleaved DNA. After cleavage, domain III is lifted away from domain I while still remaining attached to the 5' end of the broken strand, thus creating a space through which the complementary unbroken strand may pass into the central cavity. Domain III is then thought to close so the cleaved strand may be religated. The 3'-hydroxy group then acts as a nucleophile and attacks the 5'-phosphotyrosine linkage, thus religating the broken strand [4]. The enzyme must open and close once again to release the passed strand in order to complete the cycle. The linking number of DNA increases by one as a result of each cycle of enzyme action [2].

Physiological conditions predict that over two hundred ions interact with topoisomerase IA and DNA throughout the enzyme's mechanism. These ions specifically interact electrostatically with the side chains of the enzyme's amino acid residues and the DNA bound to the enzyme. It was previously mentioned that positively-charged ions may help stabilize the domain I/IV cleft with the 3' end of the cleaved DNA. This could be confirmed by the observation of positive ions in and around the cleft while the 3' of the cleaved DNA is docked there. Furthermore, the movement of ions in the near vicinity of the enzyme may exert strong local forces and may influence the conformational changes necessary for topoisomerase IA to interact with DNA.

Recently, the role of Mg(II) in the catalytic mechanism has become quite interesting. Recent studies have shown that although Mg(II) is not required for the cleavage of DNA and the formation of the covalent phosphotyrosine enzyme/DNA intermediate, it is required for the religation step [4, 7]. In addition, although it is not absolutely required for DNA cleavage, Mg(II)

has been shown to increase the rate of oligonucleotide cleavage [Dr. Yuk-Ching Tse-Dinh; private communication]. It is believed that Mg(II) plays both a direct and indirect role in catalysis. In a direct role, it is thought that "either one or more Mg(II) bound at the active site by the acidic triad may activate the Tyr-319 hydroxyl nucleophile, thus stabilizing the DNA 3'OH-leaving group during the cleavage step, and/or they may activate the DNA 3'OH as the attacking nucleophile and stabilize the Tyr-319 hydroxyl-leaving group during the religation step" [7]. In a more indirect role, it is thought that "Mg(II) coordination may place the DNA phosphates and enzyme catalytic groups in the positions required for catalysis" [7]. The discovery of the exact location of the Mg(II) may help determine how the Mg(II) interacts with the DNA scissile phosphate and tyrosine hydroxyl group during DNA cleavage as well as the phosphotyrosine intermediate and 3'OH of cleaved DNA during DNA religation.

Binding of Mg(II) by the acidic triad may also induce the conformational changes necessary for the mechanism of action to take place. In fact, one study suggests that the occupation of two Mg(II) binding sites is required for the relaxation of negatively-supercoiled DNA [7]. This same study demonstrates that, even in the absence of any DNA substrate, and hence DNA phosphates, Mg(II) could bind to sites on the enzyme and induce catalytic conformational changes [7]. Another study suggests that two of the residues in the acidic triad, Asp-111 and Asp-113, may have overlapping roles in Mg(II) binding, but demonstrates that the "elimination of both functional groups would greatly diminish Mg(II) binding" [5]. This indicates the reliance of Mg(II) binding by the three residues Asp-111, Asp-113, and Glu-115 in the TOPRIM domain.

Furthermore, the residue Glu-9 may interact with the acidic triad and the magnesium ion possibly coordinated at this site. It has been reported that forcing Glu-9 into a geometry where it

may be unable to interact within the acidic triad and Mg(II) may disrupt the catalytic mechanism [2]. In addition, it has also been reported that "changing Glu-9 to alanine abolished cleavage whereas a change to glutamine at this position had little effect" [4]. This result implicates the involvement of the side chain of Glu-9 in the catalytic mechanism, perhaps acting as a general base through an interaction with the 3' bridging oxygen of the scissile phosphate [4]. Further details state that "increased Mg(II) concentration had only a slight effect on the relaxation activity. This supports the interpretation that even though lower Mg(II) binding affinity may contribute to the decreased activity seen for the double mutants involving Glu-9, the major reason for activity deficiency is likely to be due to the effect of the Glu-9 mutation related to DNA cleavage/religation" [5].

EXPERIMENTAL PROCEDURES

Materials—Monte Carlo (MMC) simulation soft ware was used to create a large series of configurations of the topoisomerase enzyme and the ions it interacts with. The Visual Molecular Dynamics (VMD) program was used to display, animate, and analyze the data generated by the MMC software using 3-D graphics and built-in scripting. These programs were both installed and run using several custom-built Dell computers in the research laboratory of Dr. Daniel Strahs of the Biology and Health Sciences Department.

Organization of Raw Data—The coordinates for each favorable enzyme configuration generated by the Monte Carlo simulation software were grouped into several small chunks of 2500 frames each (one frame consists of a single set of x, y, z coordinates and each frame equals one .pdb format file) for archival purposes. The .pdb files were then converted to a binary coordinate format so they could be read and interpreted by the VMD program later on. The .pdb files were then deleted to release disk space for additional data analysis.

Observation of Data using VMD—Three chunks, on average, were loaded into the VMD program for observation. Once loaded, the simulation was graphically altered using the Graphical Representations option in the VMD program. To observe ion activity, the first representation created used the following selection criteria: 1. Selected atoms: ions. 2. Coloring method: name. 3. Drawing method: VDW. To observe enzyme structure without the interference of water molecules, the following criteria were used in creating the second representation: 1. Selected atoms: (not hydrogen and not water). 2. Coloring method: name. 3. Drawing method: lines. Each piece of the simulation (approximately 7500 frames) was observed for key points of interest including ion distribution in and around the active site and the enzyme/DNA complex, and ion movement in and around the enzyme's structure. Most important to this particular research, the exact location and movement of Mg (II) throughout the simulation was closely monitored.

RESULTS

Results of Ion Movement for the First 50,000 Frames—K^+ and Cl^- wobbling was observed in the enzyme's central pocket. This "wobbling" was noted as a shifting in ion position back and forth along an axis. Most of the wobbling in domains I and IV was caused by Cl^- while most of the wobbling in domains II and III was caused by K^+. The majority of the K^+ are located on the "right" side of the enzyme, whereas the majority of the Cl^- are located on the "left" side of the enzyme. The K^+ wobbling in domain II appeared to be at the hinge located at the domain II/III interface. The four K^+ exhibiting this motion are POT19 near residue GLU218, POT18 near residues ASP223, GLN237 and HSD467, POT 10 near residues GLN237, ARG247, VAL238, VAL222 and GLU221, and POT8 near residues GLU270, ARG271 and GLU272. Whether or not this is the location of the domain II/III interface hinge has yet to be verified. CLA97 has

moved away from the Mg(II) (MG598) at the active site, moving from a distance of 19.42Å to 29.72Å. On the other hand, CLA108 has moved toward MG598 at the active site, moving from a distance of 22.81Å to 16.10Å along with CLA119 moving from a distance of 24.88Å to 17.81Å. No Mg(II) movement was observed, indicating the ion's stability within the acidic triad.

Results of Ion Movement for the Next 60,000 Frames—The simulation was altered so that the ions were positioned away from the enzyme and allowed to move into electrostatically favorable positions within and around the enzyme's structure as the simulation progressed. Consequently, the number of frames was reset at a starting value of zero. For the entirety of this portion of the simulation, MG598 exhibited no movement and remained in a single position on the right side of domain IV near residues ASP518, GLN522 and ASN516. At the start of the simulation (frame 0), three of the four sodium ions (SOD600-602) were located at the back of domain IV, while SOD599 was found at the front of domain I (Figures 3 and 4). By frame 10,000, SOD600 and 601 had moved far from the enzyme's structure, moving closest to the right side of domain III. In addition, at the start of the simulation the vast majority of chloride ions were found to be located on the right side of the enzyme while the majority of potassium ions were located on the left side of the enzyme. As the simulation progressed, however, K^+ and Cl^- began migrating toward the opposite sides of the enzyme. Some K^+ and Cl^- were clumped together on the right side, but began moving apart from each other before migration across the enzyme took place. Overall, the majority of K^+ seem to favor a few positions including the right side of the enzyme near the central pocket, the left side surrounding the DNA molecule, and the rear of the enzyme near domain IV. By frame 20,000 SOD 602 was still at the back of domain IV near residue ARG41, but SOD599 had moved to the underside of domain I near residue ASP107. By the end of the simulation, SOD600 and 601 had begun clumping together with

CLA681 far from the enzyme's structure (Figure 5). CLA681 was 36.94Å away from its closest residue, GLU336. Also, there was no significant movement observed with most of the ions; wobbling was the only trend observed with the ions by the end of the simulation. Only POT636 exhibited any movement, migrating significantly closer toward GLU229 in domain II moving from a distance of 20.49Å at the beginning of the simulation to a distance of only 4.45Å by the simulation's end.

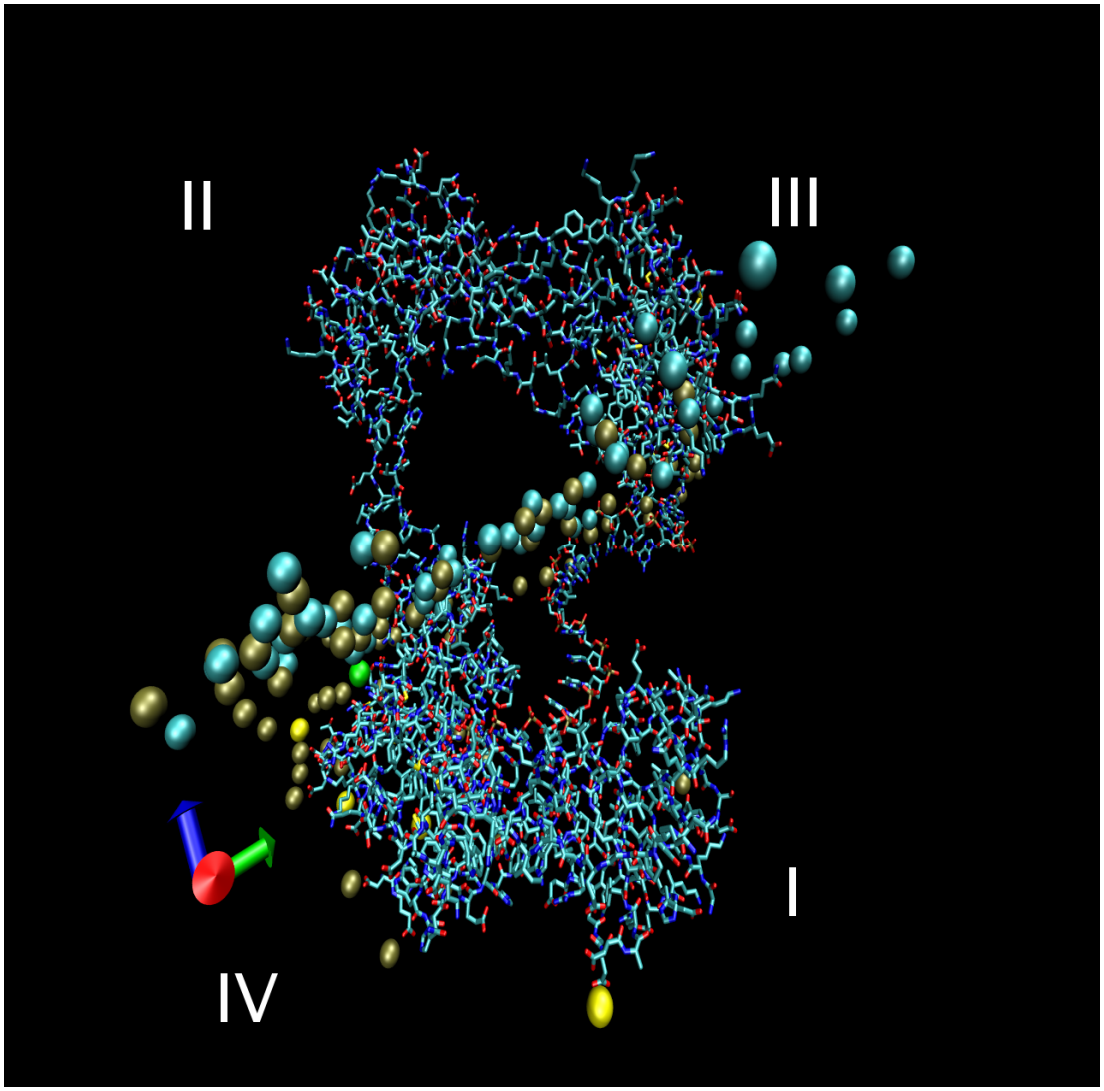

Figure 3. Simulation image of ion distribution around topoisomerase IA with the enzyme's backbone and DNA molecule present. Domains I-IV are labeled. Each ion is color-coded: MG (green), CLA (blue), POT (brown), SOD (yellow). This view is of the front "right" side of the enzyme.

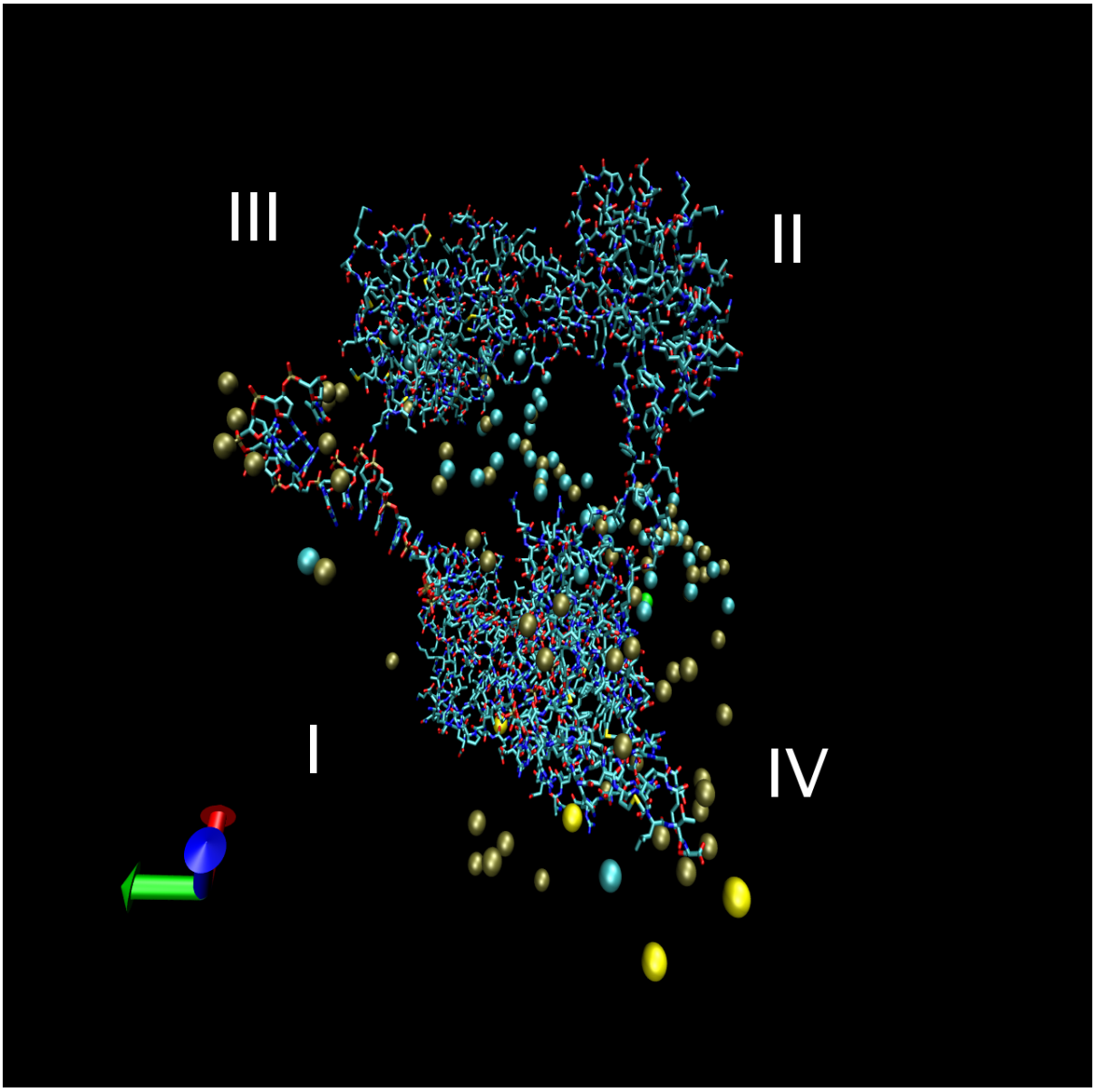

Figure 4. Simulation image of ion distribution around topoisomerase IA. This view is of the back "left" side of the enzyme. The DNA molecule is visibly sticking out of the central pocket and is being held by the active site located in domain I. SOD 600-602 are located at the back of domain IV. Several K^+ can be seen interacting with the DNA molecule while the majority of Cl^- are observed on the right side of the enzyme.

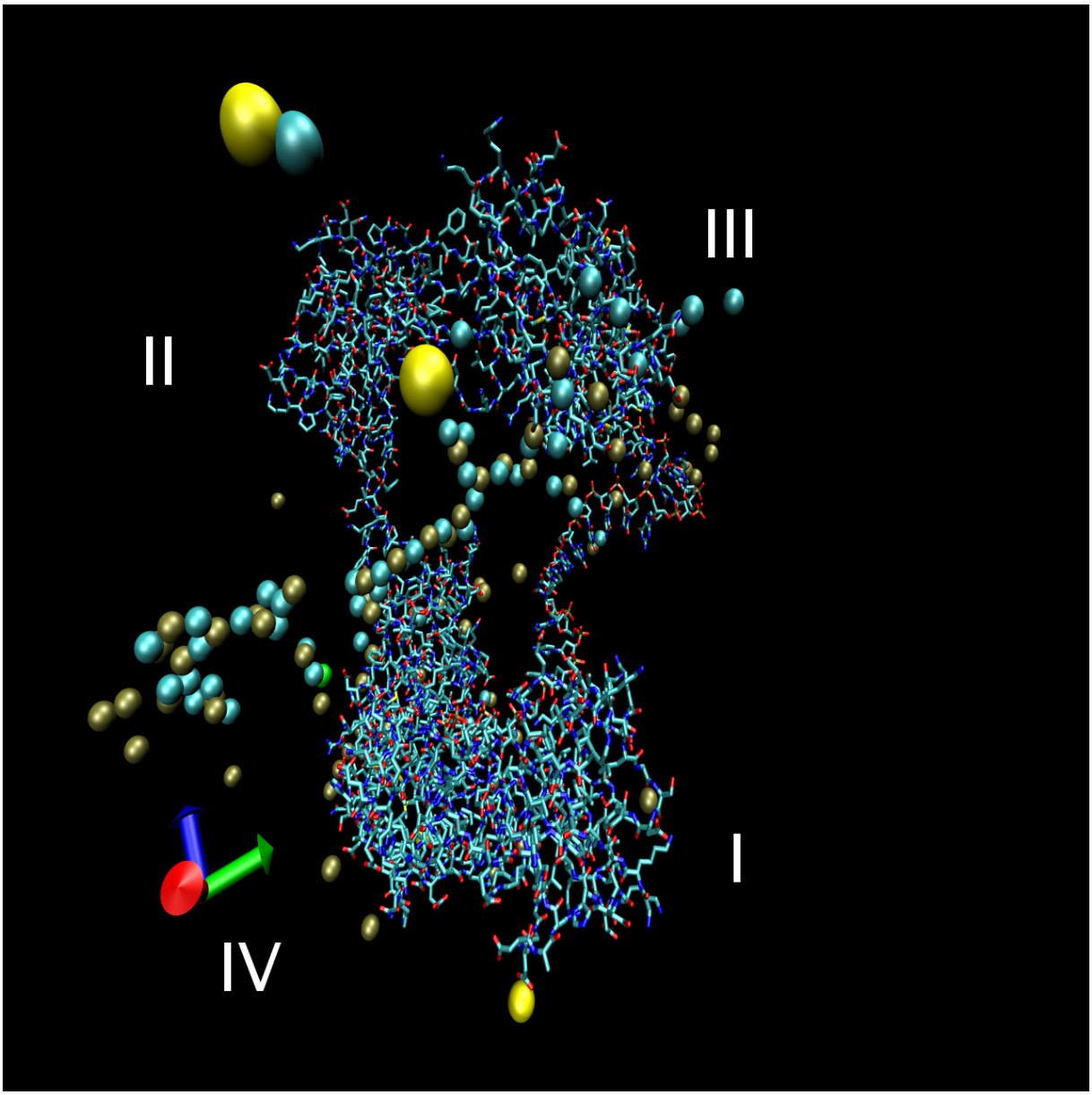

Figure 5. Simulation image of ion distribution around topoisomerase IA by the end of the simulation. SOD600 and 601 are visibly interacting with CLA681, while SOD599 is in an electrostatically favorable position at the bottom of domain I. Many K^+ and Cl^- are clearly interacting with each other.

DISCUSSION

Escherichia coli DNA topoisomerase IA requires the incorporation and interaction of various salt ions with its amino acid residues in order to carry out its catalytic mechanism. These ions have been shown to interact electrostatically with certain residues throughout the enzyme's mechanism of action. Interestingly, trends in ion activity were observed throughout the mechanism that were previously unknown to the scientific community. Two plausible locations of the magnesium ions have been deduced, one being near the acidic triad and the other near acidic residues in domain IV. Previous studies have predicted the magnesium ion's importance in the catalytic mechanism through its interaction with the acidic triad, and this study has validated that prediction.

In addition, this study shows the importance of other ions in maintaining the structural integrity of topoisomerase IA throughout its mechanism of action. Four sodium ions have been observed mainly interacting with domains I and IV, while various potassium and chloride ions have been observed interacting with all four of the enzyme's domains. It is apparent that potassium ions play a direct role in the direction and interaction of the DNA molecule with topoisomerase IA through the catalytic mechanism. They may also play an integral role in the movement of the domain II/III hinge. Many chloride ions were observed interacting with the central pocket, indicating their importance in maintaining its structural integrity. Moreover, these ions preferred to interact with the right side of the enzyme, which indicates a partial positive charge present on this side. Conversely, the majority of potassium ions preferred to interact with the left side of the enzyme, which indicates a partial negative charge present on this side. This is also probably the reason why the DNA molecule was seen only present on the left side of the enzyme.

This research study has provided valuable information on the further understanding of DNA topoisomerase IA and its mechanism of action. We have now validated the exact location of the magnesium ion as it interacts with the acidic triad and the DNA molecule. Furthermore, the locations of many other ions as they interact with the enzyme throughout its mechanism have been determined. This information, in conjunction with data collected from other similar studies, will help scientists deduce the step-by-step process of DNA cleavage, strand passage and subsequent religation as it relates to DNA topoisomerase IA. Consequently, inhibitors of this enzyme and its mechanism of action will be synthesized, which will block DNA replication and thus lead to bacterial cell death.

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