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Kinetics of E. coli Topoisomerase I and Energetic Studies of DNA Supercoiling by Isothermal Titration Calorimetry

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

KINETICS OF *E. COLI* TOPOISOMERASE I AND ENERGETIC STUDIES OF DNA
SUPERCOILING BY ISOTHERMAL TITRATION CALORIMETRY

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

in

CHEMISTRY

by

Xiaozhou Xu

2010

To: Dean Kenneth Furton
College of Arts and Sciences

This thesis, written by Xiaozhou Xu, and entitled Kinetics of *E. coli* Topoisomerase I and Energetic Studies of DNA Supercoiling by Isothermal Titration Calorimetry, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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ABSTRACT OF THE THESIS

KINETICS OF *E. COLI* TOPOISOMERASE I AND ENERGETIC STUDIES OF DNA
SUPERCOILING BY ISOTHERMAL TITRATION CALORIMETRY

by

Xiaozhou Xu

Florida International University, 2010

Miami, Florida

Professor Fenfei Leng, Major Professor

In this thesis, on the basis of the asymmetrical charge distribution of *E. coli* topoisomerase I, I developed a new rapid procedure to purify *E. coli* DNA topoisomerase I in the milligram range. The new procedure includes using both cation- and anion-exchange columns, i.e., SP-sepharose FF and Q-sepharose FF columns. *E. coli* topoisomerase I purified here is free of nuclease contamination. The kinetic constants of the DNA relaxation reaction of *E. coli* DNA topoisomerase I were determined as well.

I also used isothermal titration calorimetry to investigate the energetics of DNA supercoiling by using the unwinding properties of DNA intercalators, ethidium and daunomycin. After comparing the enthalpy changes of these DNA intercalators binding to supercoiled and nicked or relaxed plasmid DNA pXXZ06, I determined the DNA supercoiling enthalpy is about 12 kcal/mol per turn of DNA supercoil, which is in good agreement with the previously published results.

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LIST OF ABBREVIATIONS

°C – centigrade

ΔH – change in enthalpy

μg – microgram

μL – microliter

3D – three dimensional

Asp – aspartic acid

Arg – arginine

Bij-58 – polyoxyethylene (20) cetyl ether

bp – base pair

Cd – cadmium

Cys – cysteine

DEAE – diethylaminoethyl cellulose

DNA – deoxyribonucleic acid

E. coli – *Escherichia coli*

EDTA – ethylenediaminetetraacetic acid

FF – fast flow

g – gram

Gly – glycine

Glu – glutamic acid

His – histidine

kcal – kilocalorie

KAc – potassium acetate

kJ – kilojoule

KCl – potassium chloride

L – liter

LB – lysogeny broth media

Lys – lysine

Met – methionine

mg – milligram

Mg – magnesium

Mg(Ac)₂ – magnesium acetate

min – minute

mL – milliliter

mM – millimolar

M – molar

mol – mole

(NH₄)₂SO₄ – ammonium sulfate

nm – nanometer

nM – nanomolar

NMR – nuclear magnetic resonance spectroscopy

OD₅₉₅ – optical density at 595 nm

polymin P – poly(ethyleneimine)

RNA – Ribonucleic acid

rpm – revolutions per minute

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

TAE – tris base, acetic acid and EDTA

Tyr – tyrosine

UV-Vis – ultraviolet-visible

V/V – volume/volume ratio

W – watt

Zn – zinc

Chapter I

1.0. Abstract

On the basis of the asymmetrical charge distribution of *E. coli* topoisomerase I, I developed a new rapid procedure to purify *E. coli* DNA topoisomerase I in the milligram range. The new procedure includes using both cation- and anion-exchange columns, i.e., SP-sepharose FF and Q-sepharose FF columns. The *E. coli* topoisomerase I purified here is free of nuclease contamination. The kinetic constants of the DNA relaxation reaction of *E. coli* DNA topoisomerase I were determined as well.

1.1. Introduction

1.1.1. Definition and classification of DNA topoisomerases

The DNA topoisomerases are essential enzymes for all biological organisms. They specifically change DNA topology by cleavage and religation of one or both strands of a DNA double helix (1-3). The DNA topoisomerases can be classified into two types according to the number of the DNA strands cleaved during catalysis. Type I DNA topoisomerase cleaves one DNA strand at a time, passing the other strand through the gap of the cleaved strand. Cleavage of DNA strand can be achieved by forming a phosphodiester bond between the tyrosine residue of the enzyme and either the 5' or 3' end of the cleaved single DNA strand. Type I DNA topoisomerase is further classified into IA and IB subgroups. The IA subgroup indicates a formation of phosphodiester bond to the 5' end of cleaved DNA strand, while subgroup IB to 3' end of cleaved DNA strand. Type II DNA topoisomerase, on the other hand, simultaneously cleaves both

strands of the DNA double helix (the G strand) and another DNA double helix (the T strand) is transferred through the generated gap. As a result of decades of dedicated efforts, the number of DNA topoisomerases has increased by the discovering of this specific enzyme(s) in many diverse organisms (2). As a consequence, subgroup topoisomerase IA contains eubacterial DNA topoisomerase I and III, yeast and mammalian DNA topoisomerase III and eubacterial archaeal reverse DNA gyrase. Subgroup topoisomerase IB contains eukaryotic DNA topoisomerase I, poxvirus DNA topoisomerase and hyperthermophilic eubacterial DNA topoisomerase V (1).

1.1.2. Biological role of DNA topoisomerases in Escherichia coli

Since more DNA topoisomerases have been discovered, scientists have paid a lot of attention to find new enzymes and elucidate the biological significance of them. As a result, the correlation between DNA topoisomerase activity and other important cellular activities, such as DNA transcription and replication, was discovered. *Escherichia coli* (*E. coli*) has been the most extensively studied bio-system for understanding the biological role of DNA topoisomerase. Thus far, four topoisomerases have been discovered from *E. coli*: DNA topoisomerase I, III, IV and DNA gyrase. Topoisomerase I, coded by the *topA* gene (4), and topoisomerase III, the product of the *topB* gene (5), belong to type I DNA topoisomerase; DNA gyrase, translated by the *gyrA* and *gyrB* genes (6-8), and topoisomerase IV, from the *parC* and *parE* genes (9), are type II DNA topoisomerases.

Among the four topoisomerases in *E. coli* cells, DNA topoisomerase I and gyrase are reported as being responsible for keeping the balance of the topological state of cellular DNA. Inhibition of DNA gyrase by novobiocin or oxolinic acid causes the accumulation

of positive supercoils in plasmids, which indicates that the DNA gyrase is the major contributor for relaxation positively supercoiled DNA in vivo (10, 11). The degree of negative supercoiling of plasmid DNA was observed to be highly enhanced in the *E. coli* topA mutant (12, 13). Also, it is observed when DNA gyrase is inhibited by norfloxacin in topA⁺ or topA⁻ *E. coli* stains, only plasmids from the topA⁺ stain show less negatively DNA supercoiling (14). Moreover, the negative supercoils generated by advancing transcription complex have also been demonstrated to be removed by DNA topoisomerase I (11, 15, 16). These results indicate negative supercoil in vivo is mainly relaxed by the activity of *E. coli* topoisomerase I. Therefore, a “twin-domain model” was developed by James C Wang in 1987 (17), which describes the plausible reasons for the generation of a highly supercoiled DNA domain during transcription and also how DNA topoisomerase I and gyrase work together to decrease DNA to a normal state. According to this model, rotation of RNA polymerase complex is supposed to be hindered by the relatively high rotation torque. So the advancing RNA polymerase complex may introduce positive supercoils in the DNA template ahead and negative supercoils behind. These accumulated DNA supercoils with reverse signs are then effectively resolved into a normal state by DNA topoisomerase I and gyrase. Notably, the formation of R-loops were observed from the hypernegatively supercoiled plasmid DNA isolated from *E. coli* topA mutant (18). The DNA topoisomerase I would be responsible for the suppression of R-loop formation during transcription (18). The formation of R-loop during transcription in the absence of DNA topoisomerase I is associated with failed of cell growth (19). This inhibitory effect, however, can be partially compensated by over-expression of RNase H. A coordinated action provided by DNA topoisomerase I and RNase H is suggested in

suppression of R-loop formation during the process of transcription (19).

Topoisomerases activity plays an essential role during the DNA replication in *E. coli*. Since both the chromosomes and plasmid are in a circular shape, free rotation of DNA molecules is forbidden without the aid of DNA topoisomerases. As a consequence, although positive DNA supercoiling can be partially compensated by instinct negative supercoiled DNA, it will be generated by unwinding of the parental DNA during replication process. This accumulation can result in a failure of DNA replication. Both in vivo (20) and in vitro (21) studies have shown the evidence that the topoisomerases activity is required to maintain the chain elongation during the early stage of replication. At the final stage of replication, the catenated daughter duplexes are also unlinked by DNA topoisomerases. Three of the four *E. coli* topoisomerases including DNA topoisomerase III, IV and DNA gyrase, can keep nascent chain elongation (22, 23). However, only two, DNA topoisomerase III and IV, have the capability of decatenating the replicated daughter DNA duplexes (23, 24, 25). It was suggested that during the θ -type replication, a positive supercoil of unreplicated parental DNA generated ahead of the advancing replication forks may be equilibrated with a precatenane formed between replicated daughter duplexes (26). DNA gyrase is more active on positively supercoiled DNA, while topoisomerase IV prefers multiply linked DNA dimers (26, 27). However, *E. coli* topoisomerase I exhibits no effect on the decatenation of daughter duplexes (24).

1.1.3. E. coli topoisomerase I — structure and mechanism

The first DNA topoisomerase was discovered in the extracts of *E. coli* cells in 1971 (28). This enzyme, which is named *E. coli* topoisomerase I, can change the DNA topology by relaxing the circular negatively supercoiled DNA. The *E. coli* topoisomerase I is a 97

kDa protein. It is coded by *topA* gene and contains 865 amino acids (29). Generally, three domains are included in the 865 amino acids sequence of *E. coli* topoisomerase I. The first domain contains 582 N-terminal amino acids with a tyrosine at position 319 as the active site. The second domain, with 162 amino acids, features a Zn (II) - binding domain with three tetracysteine motifs which can form zinc finger triplet (30). The 14 kDa C-terminus that enhances DNA binding forms the third domain of *E. coli* topoisomerase I (31).

The N-terminal fragment of *E. coli* topoisomerase I, containing 596 amino acids, was demonstrated to have a capability of cleaving single-stranded oligonucleotide. It cannot, however, relax negatively supercoiled DNA (32). In 1994, the three-dimensional structure of the 67 KDa N-terminal fragment of *E. coli* topoisomerase I was resolved via X-ray crystallography (33). The folding of the polypeptide chain was observed to generate a “large hole”, which is big enough to accommodate a B-form DNA. The mechanism proposed for DNA strand cleavage generally includes six stages (33): 1) A single-stranded DNA, or single-stranded region of DNA double helix, binds to the enzyme, and then was located into position of the active site; 2) The single stranded DNA is cleaved by a residue of tyrosine by forming a covalent bond between the 5'-phosphoryl group and hydroxyl group of tyrosine, leaving the 3' end of DNA strand bound to the enzyme via non-covalent interaction; 3) The other DNA strand passes through the generated gap by the cleaved DNA strand; 4) The structure of enzyme transforms into a closed state; 5) The cleaved DNA strand is religated; 6) Both DNA strands are released by the enzyme.

The active site, Tyr319, was found to interact with some highly conserved amino acids

through hydrogen bonds (33). Therefore, numbers of highly conserved residues near key functional region were investigated by the method of mutagenesis. The mutagenesis of Glu9, Asp111, Asp113, Glu115 and Arg321 were found to partially reduce the activity of *E. coli* topoisomerase I (34). The Asp111, Asp 113 and Glu115 were considered as potential binding sites for Mg (II) and induced the related conformational change required for the relaxation activity (35). Strictly conserved residue Gly194 demonstrated an essential function for cleavage and relaxation activity by changing the conformation of the flexible hinge region (36). Furthermore, His365 was observed to be involved in the DNA strand binding and also to be responsible for the optimal pH value for catalysis (37).

The function of the remaining two domains of the *E. coli* topoisomerase I was also investigated. On the basis of cysteines arrangement in the amino acids sequence of *E. coli* topoisomerase I, fourteen cysteines were found in a region between 586 and 762 amino acids and can form three three-tetracysteine motifs (30). Each of these motifs contains a similar sequence of Cys-X₂-Cys-Gly-X₂-Met-X₁₂₋₁₃-Cys-X₄₋₁₀-Cys which form coordination sites for Zn (II) (30, 38). When the Zn (II) is removed from the *E. coli* topoisomerase I, the enzyme can not relax negatively supercoiled DNA although retains a reduced capability for cleaving single stranded DNA (39). Addition of Zn (II) or Cd (II) can reactivate the Zn (II)-removed enzyme (39). Moreover, the relaxation activity is recovered when the purified 30 kDa C-terminal domain is added to the 67 kDa N-terminal domain (40). These results indicate that the 30 kDa C-terminal domain plays an essential role in the DNA strand cleavage and passage reaction (39, 40). Other than Zn (II), Mg (II) acts as a cofactor for the relaxation activity of *E. coli* topoisomerase I,

although it is not required for the cleavage reaction (41, 42). In order to obtain detectable relaxation activity, one molecule of *E. coli* topoisomerase is supposed to bind with 1-2 Mg (II) (42). A tertiary structure conformational change of *E. coli* topoisomerase, which may be required for strand passage, religation or release, was induced by the binding of Mg (II) (42, 43).

In 1995, the structure of the C-terminal 14 kDa fragment of *E. coli* topoisomerase I was elucidated by means of NMR (44). The binding site which contains positively charged and aromatic amino acids has been shown to contribute to a higher binding capability with single-stranded DNA which influences relaxation activity of the whole enzyme (44, 45). Sequence and structure analysis reveals the evolutionary relationship between the C-terminal fragment and Zn (II)-binding domains. The C-terminal fragment contains two domains which are homologous to three Zn (II)-binding domains. The C-terminal fragment is supposed to have lost the cysteine residues in the process of evolution and therefore, can not bind with Zn (II) (46).

1.1.4. Type IA topoisomerase purification from different organisms

With the biological characteristics of forming a 5'-phosphodiester bond between tyrosine residue and the cleaved DNA strand, *E. coli* topoisomerase I and III are firstly termed as type IA DNA topoisomerase. The discovery and function elucidation of the EDRI gene product of yeast, led to an expansion of type IA DNA topoisomerase (47). Another kind of DNA topoisomerase was found in numbers of thermophilic archaea and bacteria (48, 49, 50, 51, 52). The enzyme, which was named as 'reverse gyrase', shares ATP-dependent characteristics of introducing positive supercoiling into relaxed DNA. Therefore, a number of purification protocols were set up in order to obtain pure or

partially purified DNA topoisomerases. Some operations such as streptomycin precipitation, ammonium sulfate precipitation and polymin P precipitation were widely used in the early stage of enzyme purification. Column chromatography is always utilized for final stages of the enzyme purification process. Different types of resin including DEAE (52, 53, 54), phosphocellulose (47, 48, 52, 53, 54, 55), hydroxyapatite (52, 55), single-stranded DNA-agarose (47, 56), phenyl-sepharose (49, 50) and heparin-sepharose (52, 54) were considered to separate the target enzyme from other components in the protein mixture.

1.2. Objectives

One important application of the recombinant DNA technique is the purification of over-expressed gene products. During the investigation of *E. coli* topoisomerase I, some biophysical methods such as X-ray crystallography and NMR play a dominating role for elucidating the relationship between enzyme function and intermediate structure. The former purification protocol, however, includes at least three consecutive stages of column chromatography for the purification. The difficulty of obtaining large quantities of the enzyme obstructs application of these effective research methods.

Therefore, a rapid protocol for purification of *E. coli* topoisomerase I is essential for the further study of this enzyme, and even to the entire type IA DNA topoisomerases. This thesis proposes a rapid protocol for purification of *E. coli* topoisomerase I by the use of two consecutive SP-Sepharose FF and Q-Sepharose FF column chromatography. The SDS-PAGE electrophoresis demonstrates the high purity of the final purification product without contamination of nuclease and nucleic acids.

1.3. Materials and methods

1.3.1. Materials

The Q-Sepharose FF, SP-Sepharose FF were purchased from GE Healthcare (Piscataway, NJ). Bij-58, polymin P, lysozyme, isopropyl-beta-D-thiogalactopyranoside (IPTG), dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich Company (St. Louis, MO). Bradford reagent was a product of Bio-Rad (Hercules, CA). Bovine serum albumin (BSA) was obtained from New England Biolabs (Beverly, MA).

1.3.2. Plasmids

Plasmid, pUNB was constructed by inserting the annealing product of 5'-CCGCTGAGGAG- 3' and 5'-ggcctcctcagcggacgt- 3' contains a nicking restriction enzyme Nb. BvcI site and a BglII site into the AatII and Eco0109 sites of pUC18.

Plasmid pXXZ06 was made by the insertion of a 1870 bp BamHI- HindIII fragment of pLUC3 into the BamHI-HindIII sites of pUNB.

The *E. coli* strain BL21(DE3)/pLysS containing plasmid pTI-1 to express *E. coli* topoisomerase I was kindly provided by Dr. K. J. Mariani (Memorial Sloan-Kettering Cancer Center, NY).

1.3.3. E. coli topoisomerase I purification

E. coli strain BL21(DE3)/pLysS which contains the plasmid pTI-1(pET3c+ topA) was grown in LB media containing 50 µg/mL of the antibiotic ampicillin overnight at 37 °C. The overnight cell culture was then transferred to 4 L of terrific broth media at a ratio of 100:1 (v/v). The cell growth at 37 °C was monitored at OD₅₉₅ using a Cary 50 Bio UV-

Vis spectrometer. When the OD₅₉₅ reached to ~0.6, IPTG was added to 1 mM to induce the expression. Cells were harvested after an additional 3 hours' incubation with centrifugation at 4,000 rpm at 4 °C for 30 min. The supernatant was disposed and the cells were re-suspended in a cold Tris-HCl buffer (50 mM Tris-HCl, pH 8.0, 0.4 M KCl, 1 mM DTT and 1 mM PMSF) at 1 mL per gram of cells. Then lysozyme was added to a final concentration of 1 mg/mL. The cells in the cold Tris-HCl buffer were incubated on ice for at 1 hour then frozen in liquid nitrogen, and stored in a -80 °C freezer. In the following day, the *E. coli* cells were thawed at room temperature and refrozen in liquid nitrogen. After three freezing and thawing cycles, the solution was sonicated on ice for 6 times at 30 W with 2 min interval between each sonication. The Brij-58 was added to the sonicated solution to achieve a final concentration of 0.5 %. After mixing, the tubes were incubated at 4 °C for 15 min and followed by centrifuging at 15,000 rpm at 4 °C for 30 min. After centrifugation, 10 % polymin P (pH 7.5) was added to the supernatant to a concentration of 0.7 %. The sample was centrifuged again at 15,000 rpm at 4°C for 10 min and the pellet was discarded. In the next step, solid (NH₄)₂SO₄ was added to the supernatant to 30%, 50% and 70% saturation in steps and the protein samples were centrifuged at 12,000 rpm at 4°C for 25 min for each step. The pellet precipitated from each (NH₄)₂SO₄ saturation stage were re-dissolved in Buffer I (20 mM potassium phosphate, pH 7.4, 10 % w/v glycerol, 1 mM EDTA) with the addition of 1 mM freshly prepared DTT and PMSF, respectively. The solution was dialyzed extensively against 2 L Buffer I plus 0.1 M KCl extensively twice at 4 °C. After the dialysis, the protein solution from the 50-70 % (NH₄)₂SO₄ saturation steps were combined and loaded onto a 30 mL

SP-Sepharose cation-exchange column pre-equilibrated with Buffer I plus 0.1 M KCl. The loaded column was washed with 150 mL Buffer I plus 0.2 M KCl. Then the protein was eluted with a 300 mL KCl gradient of 0.2- 0.5 M KCl in Buffer I. *E. coli* topoisomerase I was eluted approximately at 0.32 M of KCl. The relaxation activity assay was also used to monitor the activities of *E. coli* topoisomerase I. Fractions with high relaxation activities and low nuclease activities were pooled and dialyzed against 2 L Buffer I plus 20 mM KCl extensively twice at 4 °C. The dialyzed protein solution was loaded onto a 40 mL Q-Sepharose anion-exchange column pre-equilibrated with Buffer I plus 20 mM KCl. The loaded column was washed with 40 mL of Buffer I plus 20 mM KCl. Then the protein was eluted with a 300 mL KCl gradient of 20- 300 mM KCl in Buffer I. *E. coli* topoisomerase I was eluted approximately at 80 mM KCl. The relaxation activity assay and nuclease activity assay were used to monitor the quality of the *E. coli* topoisomerase I.

1.3.4. Bradford Assay and UV absorbance

The concentration of *E. coli* topoisomerase I was determined utilizing the method of Bradford assay. The OD₅₉₅ of protein sample prepared by mixing 30 µL of elution fraction with 1.5 mL Bradford reagent was detected by a Cary 50 Bio UV-Vis spectrometer. Ultraviolet absorbance of *E. coli* topoisomerase I at 280 nm was also measured and the concentration of *E. coli* topoisomerase I was calculated using molar extinction coefficient $\epsilon_{280} = 98,210 \text{ M}^{-1}\text{cm}^{-1}$.

1.3.5. The DNA relaxation activity assay

The DNA relaxation activity assay using supercoiled plasmid pXXZ06 was used to monitor the activities of *E. coli* topoisomerase I during the purification procedure.

Briefly, in a 25 μ L of reaction mixture containing 20 mM Tris-acetate, pH 7.9, 50 mM KAc, 6 mM Mg(Ac)₂, 1 mM DTT and 0.25 μ g/ μ L BSA, 1 μ g of supercoiled plasmid DNA pXXZ06 was relaxed by using certain amount of *E. coli* topoisomerase I at 37 °C for 15 min. The reaction was then stopped by direct extraction with an equal volume of phenol. The topological state of each DNA sample was analyzed by electrophoresis in 1 \times TAE buffer, pH 7.8, in a one- dimensional 1 % agarose gel. One unit of *E. coli* topoisomerase I was defined as the amount of enzyme that catalyzes the relaxation of 0.5 μ g of supercoiled plasmid DNA pXXZ06 in a 25 μ L of reaction mixture containing 20 mM Tris-acetate, 50 mM KAc, 6 mM Mg(Ac)₂, 1 mM DTT and 0.2 μ g/ μ L BSA at 37 °C for 15 min.

1.3.6. The nonspecific nuclease activity assay

The nonspecific nuclease activity was used to monitor the quality of *E. coli* topoisomerase I. In this assay, 1 μ g of the supercoiled plasmid DNA pXXZ06 was incubated with 1 μ g of *E. coli* topoisomerase I in a 25 μ L of Tris-acetate buffer containing 20 mM Tris-acetate, pH 7.9, 50 mM KAc, 6 mM Mg(Ac)₂, 1 mM DTT and 0.25 μ g/ml BSA for 2 hours at 37 °C. The reaction was then stopped by direct extraction with an equal volume of phenol. The DNA samples were analyzed by electrophoresis in 1 \times TAE buffer, pH 7.8, in a one- dimensional 1 % agarose gel in the presence or absence of 1 μ g/mL of chloroquine.

1.3.7. The determination of the initial relaxation velocity of E. coli DNA topoisomerase I

To determine the initial relaxation velocity of *E. coli* DNA topoisomerase I, different concentrations of the negatively supercoiled plasmid pXXZ06 were relaxed with 3.5 units of *E. coli* DNA topoisomerase I (8 nM) in a 200 μ L of reaction mixture containing 20

mM Tris-acetate, pH 7.9, 50 mM KAc, 6 mM Mg(Ac)₂, 1 mM DTT and 0.1 mg/mL BSA. The reactions were stopped by direct extraction with an equal volume of phenol. The topological state of each DNA sample was determined by electrophoresis in 1×TAE buffer, pH 7.8, in a one-dimensional, 1% agarose gel and analyzed by using KODAK 1D Image Analysis Software. The initial relaxation velocities (V_0) of *E. coli* DNA topoisomerase I were calculated by linear fitting the following equation:

$$V_0 = [\text{scDNA}] \cdot \frac{1-r}{t}$$

where [scDNA] is the initial concentration of supercoiled DNA pXXZ06, r is the ratio of the area of the supercoiled DNA band at time zero versus the area of the supercoiled DNA band at time t . The steady state kinetic constants K_M and V_{\max} were determined by the analysis of the Michaelis-Menten equation using the double-reciprocal or Lineweaver-Burk plot.

1.4. Results and discussion

1.4.1. Purification of *E. coli* topoisomerase I

The enzyme, *E. coli* DNA topoisomerase I is the first DNA topoisomerase discovered and purified in James C. Wang's laboratory about 30 years ago (28). It has 865 amino acid residues (Figure 1.1) and contains three domains, i.e., the N-terminal transesterification domain, the Zn(II) domain, and the C-terminal DNA-binding domain (3). The active Tyrosine residue (Tyr319) is located in the N-terminal transesterification domain (Figure 1.1). Recently, the accumulating evidence showed that bacterial topoisomerase I is a potential target for the discovery of antibacterial compounds (58).

Historically, *E. coli* topoisomerase I has been over-expressed and purified in a few different laboratories (28, 30) and the purification procedure includes passing through a few columns, such as the cation-exchange columns, i.e., DEAE cellulose column, phosphocellulose column and ssDNA agarose column (28, 30). These procedures usually are quite time-consuming and sometimes cannot completely remove the contamination of different nucleases (my observation). In this study, we re-analyzed the primary structure of *E. coli* topoisomerase I and found that it contains 132 positively charged amino acid residues, i.e. Lys and Arg and 122 negatively charged residues, i.e., Glu and Asp. I also found that *E. coli* topoisomerase I has a region (residue 524 to 598, the region between the N-terminal transesterification domain and the Zn (II) domain) is highly negatively charged (18 negative charged amino acids versus 6 positively charged amino acids). I reasoned that *E. coli* topoisomerase I should bind to the anion-exchange column, i.e., Q-Sepharose FF ion-exchange column. Indeed, my results demonstrated that *E. coli* topoisomerase I binds to both cation- and anion-exchange columns, such as SP-Sepharose FF and Q-Sepharose FF columns. The Q-Sepharose is particularly important because it removed most of the nuclease activities in the purification procedure (see below for details). The basic purification steps include cell lysis, $(\text{NH}_4)_2\text{SO}_4$ precipitation, SP-Sepharose and Q-Sepharose ion-exchange chromatography. The purification can be completed in five days and produces 50 to 100 mg of *E. coli* topoisomerase I from 4 liters of cell culture.

(i) Cell growth and lysis. Here, I used BL21(DE3)/pLysS/pTI-1 for over-expressing the *E. coli* DNA topoisomerase I with IPTG induction. The addition of 1 mM of IPTG did not significantly affect the cell growth (data not shown). After an additional 3 hours of

incubation, the cells were harvested by centrifugation at 4 °C. In this procedure, I utilized

```

10      20      30      40      50      60
MGKALVIVES PAKAKTINKY LGSDYVVKSS VGHIRDLPST GSAAKKSADS TSTKTAKPKK

      70      80      90      100     110     120
KDERGALVNR MGVDPMHNWE AHYEVLPQKE KVVSELKQLA EKADHIYLAT DLDREGEAIA

      130     140     150     160     170     180
WHLREVIGGD DARYSRVVFN EITKNAIRQA FNKPGEINID RVNAQQARRF MDRVVGVMVS

      190     200     210     220     230     240
PLLWKKIARG LSAGRVQSVL VRLVVERERE IKAFVPEEFW EVDASTTTPS GEALALQVTH

      250     260     270     280     290     300
QNDKPFRRPVN KEQTQAAVSL LEKARYSVLE REDKPTTSKP GAPFITSTLQ QAASTRLGFG

      310           319     330     340     350     360
VKKTMMMAQR LYEAGYIT YM RTDSTNLSQD AVNMVRGYIS DNFGKKYLPE SPNQYASKEN

      370     380     390     400     410     420
SQEAHEAIRP SDVNVMAESL KDMEADAQKL YQLIWRQFVA CQMPAKYDS TTLTVGAGDF

      430     440     450     460     470     480
RLKARGRILR FDGWTKVMPA LRKGEDRIL PAVNKGDAIT LVELTPAQHF TKPPARFSEA

      490     500     510     520     530     540
SLVKELEKRG IGRPSTYASI ISTIQDRGYV RVENRRFYAE KMGEIIVTDRL EENFRELMNY

      550     560     570     580     590     600
DETAOMENSL DQVANHEAEM KAVLDHFESD ETQQLDKAEK DPEEGGMRPN QMVLTSIDCP

      610     620     630     640     650     660
TCGRKMGIRT ASTGVFLGCS GYALPPKERC KTTINLVPEN EVLMVLEGED AETNALRAKR

      670     680     690     700     710     720
RCPKCGTAMD SYLIDPKRKI HVCGNNPCTD GYEIEEGEFR IKGYDGPIVE CEKCGSEMHL

      730     740     750     760     770     780
KMGRFGKYMA CTNEECKNTR KILRNGEVAP PKEDPVPLPE LPCEKSDAYF VLRDGAAGVF

      790     800     810     820     830     840
LAANTFPKSR ETRAPLVEEL YRFRDLPEK LRYLADAPQQ DPEGNKTMVR FSRKTKQQYV

      850     860
SSEKDGKATG WSAFYVDGKW VEGKK

```

Figure 1.1 865 amino acid sequence of DNA topoisomerase I from *E. coli* substrain

MG1655 of strain K-12.

a combination of lysozyme digestion of the cell wall and the freezing-thawing cycle to break the cell membrane. After the freezing-thawing cycles, the cell lysate became very viscous and the sonication was used to reduce the viscosity. Cell debris was then removed by centrifugation at 15,000 rpm at 4 °C. This relatively mild lysis procedure ensure that I obtained highly active *E. coli* DNA topoisomerase I.

(ii) SP-Sepharose FF chromatography

After dialysis with Buffer I plus 0.1 M KCl, the protein sample was loaded on a SP-Sepharose FF column, a strong cation-exchange column. The column was equilibrated with the dialysis buffer to ensure an identical ionic environment. After loading the sample, I washed the column using 2 to 3 column volumes of buffer I plus 0.2 M KCl. From the SDS-PAGE gels, I found that a large portion of cellular proteins with none or very weak interaction with SP-Sepharose resin was removed by the washing buffer. No detectable *E. coli* DNA topoisomerase I was eluted at this washing step. The *E. coli* DNA topoisomerase I was eluted with a 0.2 to 0.5 M KCl gradient. Figure 1.2.a shows the elution profile where *E. coli* DNA topoisomerase I was eluted in buffer I from 275 to 375 mM of KCl. After this SP-Sepharose FF column, I performed a DNA relaxation assay for each fraction as described in the Materials and methods. The results are shown in Figure 1.3.a. Although I incubated the DNA samples only for 15 minutes, I still observed significant nuclease activity for all fractions of the *E. coli* DNA topoisomerase I, especially those fractions eluted with high salt concentrations (fractions 46 and up, Figure 1.3.a). These results suggest that the *E. coli* DNA topoisomerase I after this column cannot be used for certain experiments especially for those biophysical assays required a

large amount of the enzyme. Regardless, when I reduced the enzyme concentration to

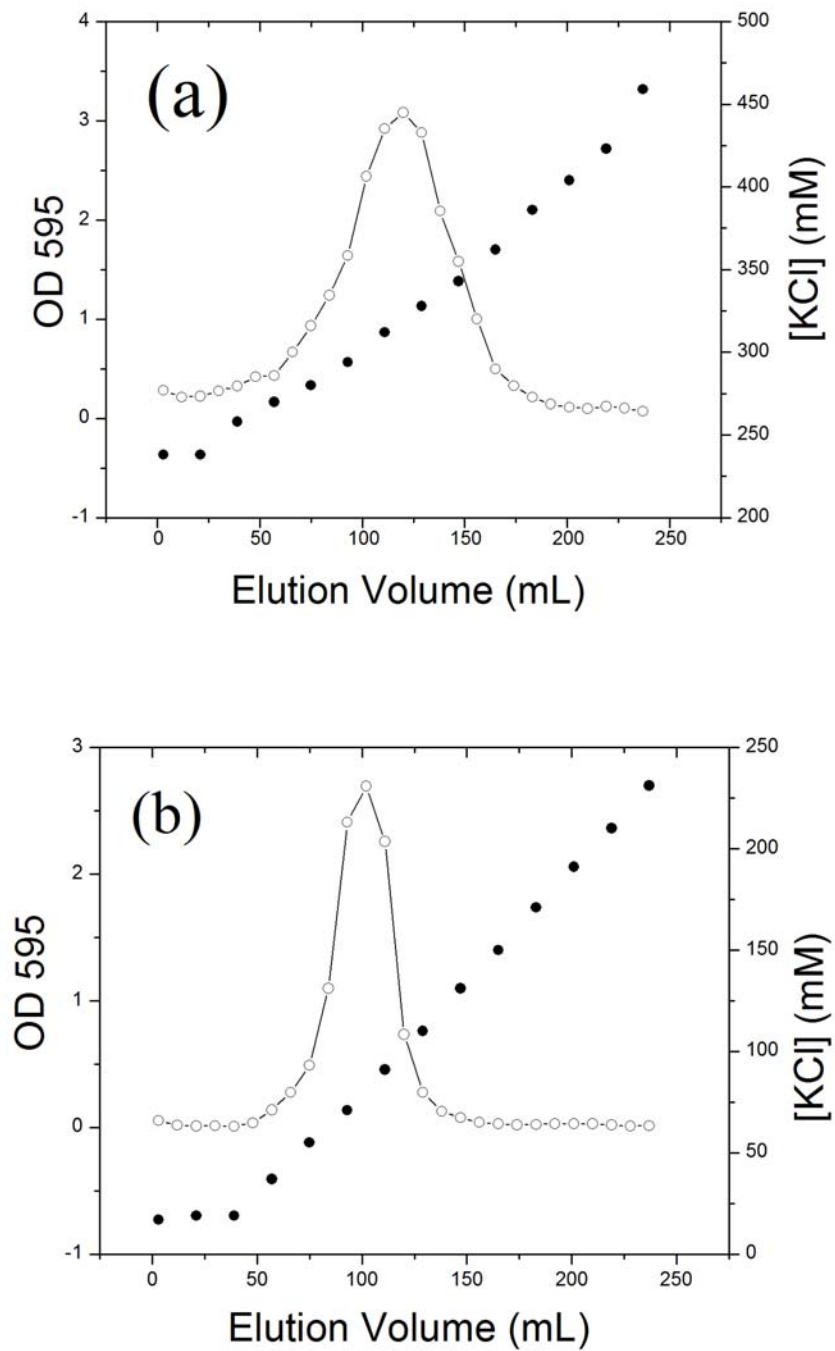


Figure 1.2 (a) Cation-exchange chromatography of *E. coli* topoisomerase I on SP-Sepharose FF column; (b) Anion-exchange chromatography of *E. coli* topoisomerase I on

Q- Sepharose FF column.

about 5 to 20 nM, I did not observe nonspecific nuclease activities, which allow me to determine the specific activities of the *E. coli* DNA topoisomerase I purified at this stage (Figure 1.3.b). Because of the nonspecific nuclease activities, I decided to pool those fractions with less nonspecific nuclease activities (fractions 25 to 42; from elution volume 70 mL to 125 mL and the total volume is about 55 mL) for the next step.

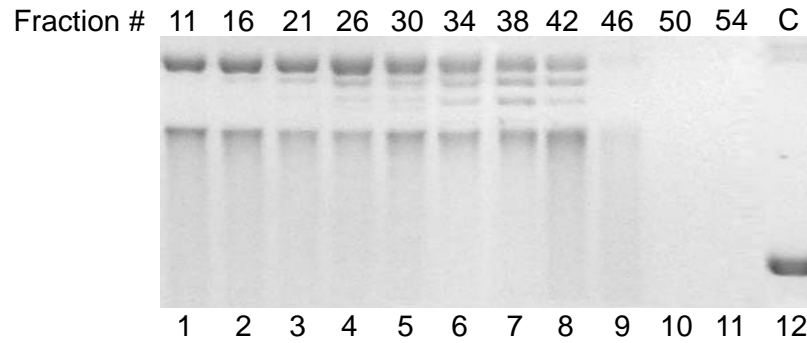
(iii) Q-Sepharose FF chromatography

The pooled fractions from SP-Sepharose FF column were dialyzed against Buffer I plus 20 mM KCl. The dialyzed protein sample was then loaded onto a 40 mL Q-Sepharose FF column equilibrated with Buffer I plus 20 mM KCl and washed with 40 mL of the same buffer. *E. coli* DNA topoisomerase I was not detected at this washing step, indicating that *E. coli* DNA topoisomerase I indeed binds to the Q-Sepharose FF column at the low salt concentration. The *E. coli* topoisomerase I was eluted in Buffer I plus a 20 to 300 mM KCl gradient. Figure 1.2.b shows the elution profile where *E. coli* DNA topoisomerase I was eluted in Buffer I from 50 to 100 mM KCl. I also performed a relaxation assay and a nonspecific nuclease activity assay for all fractions purified with the Q-Sepharose FF column. For the 15 min relaxation assay, the enzyme from all fractions relaxed the supercoiled DNA sample and no nuclease activities were detected (Figure 1.4.a). For the nonspecific nuclease activity assays (the incubation time is 2 hours), I observed some nonspecific nuclease activities for the early fraction eluted with very low salt concentrations (fractions 22 to 27; data not shown). For the most fractions, I did not observe any nuclease activities (Figure 1.4.b), indicating that the Q-Sepharose FF column is able to remove most nonspecific nuclease activities from the *E. coli* DNA

topoisomerase I samples. The *E. coli* DNA topoisomerase I samples are particularly

SP- sepharose FF

(a)



(b)

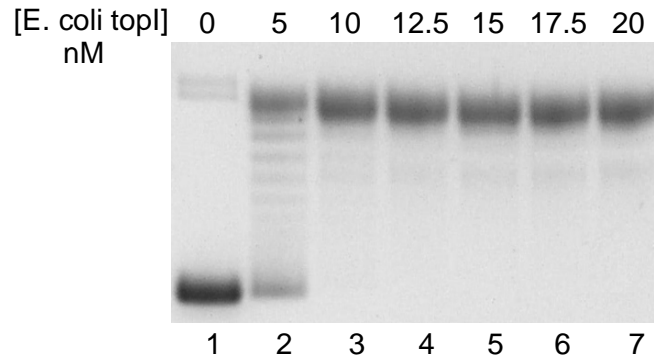


Figure 1.3 DNA relaxation activity assay (a) and DNA specific relaxation activity assay (b) of *E. coli* topoisomerase I from SP-Sepharose FF column. (a) 1 μ g of supercoiled plasmid DNA pXXZ06 was relaxed by 1 μ g of indicated fraction samples from SP-Sepharose FF column at 37 $^{\circ}$ C for 15 min. Lane 1-11: relaxation assay of indicated fraction samples; lane 12: control assay without adding *E. coli* topoisomerase I. of *E. coli* topoisomerase I from SP-Sepharose FF column. (b) 0.5 μ g of supercoiled plasmid DNA pXXZ06 was relaxed by *E. coli* topoisomerase I with indicated increasing concentration

from SP-Sepharose FF column at 37 °C for 15 min.

useful for the biochemical and biophysical assays that require a large amount of the enzyme and incubate for a long period of time. I also determined the specific activities of the enzyme after the Q-Sepharose FF column. After the Q-Sepharose FF column, the *E. coli* DNA topoisomerase I is essentially pure as judged from SDS-PAGE gels. Figure 1.5 is a 10% SDS-PAGE gel of the protein samples at different stages of the *E. coli* DNA topoisomerase I purification. Figure 1.6 and table 1.1 are a summary scheme and a summary table for the purification of *E. coli* DNA topoisomerase I, respectively.

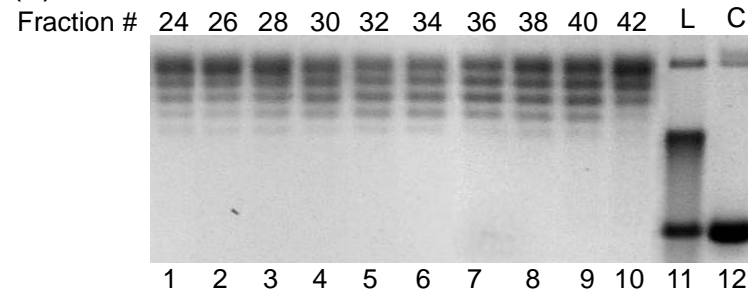
*1.4.2. The determination of V_{max} and K_M of the *E. coli* topoisomerase I for the relaxation of the supercoiled plasmid DNA template pXXZ06*

Previous studies from Tse-Dinh's laboratory demonstrated that Mg^{2+} is required for the relaxation of the negatively supercoiled DNA templates (41, 42) and the optimal Mg^{2+} concentration was determined to be ~ 6 mM (59). In this study, I also performed a similar study and my results are shown in Figure 1.7. The results clearly demonstrate that 6 mM of Mg^{2+} is the optimal concentration for the relaxation activities of *E. coli* DNA topoisomerase I (compare lanes 1 to 8 of Figure 1.7). Next, I carried out a time course for the relaxation of different concentrations of negatively supercoiled plasmid pXXZ06 by using 3.5 units of *E. coli* topoisomerase I. The results are shown in Figure 1.8. The velocities for these experiments are linear for the first two minutes. In this case, I may use them to calculate the initial velocities for the relaxation of the negatively supercoiled plasmid pXXZ06 (Table 1.2). These kinetic analysis results are displayed in a double-reciprocal or Lineweaver-Burk plot (Figure 1.9.b). The K_M and V_{max} were determined to be 7.52 ± 2.17 nM and 7.98 ± 0.95 nM/min, respectively (Table 1.3). The turnover number

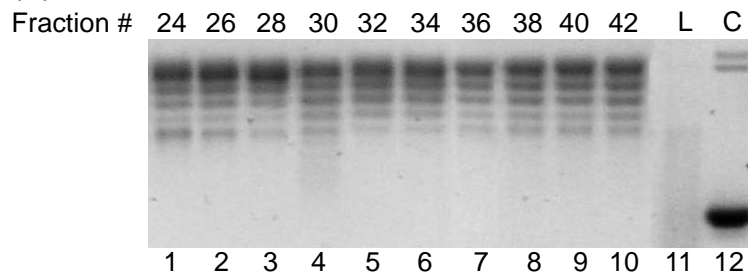
(k_{cat}) was calculated to be 1.20 ± 0.47 per min. These kinetic parameters can serve as an

Q- sepharose FF

(a)



(b)



(c)

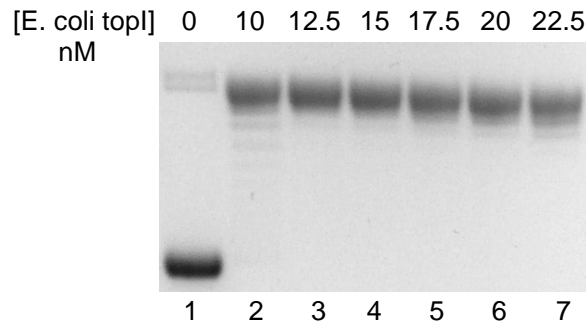


Figure 1.4 DNA relaxation activity assay (a), DNA nonspecific nuclease activity assay (b) and DNA specific relaxation activity assay (c) of *E. coli* topoisomerase I from Q-Sepharose FF column. (a) 1 μ g of supercoiled plasmid DNA pXXZ06 was relaxed by 1 μ g of indicated fraction samples from Q-Sepharose FF column at 37 $^{\circ}$ C for 15 min. Lane 1-10: relaxation assay of indicated fraction samples; lane 11: relaxation assay of eluted loading buffer; lane 12: control assay without adding *E. coli* topoisomerase I. (b) 1 μ g of

supercoiled plasmid DNA pXXZ06 was relaxed by 1 µg of indicated fraction samples from Q-Sepharose FF column at 37 °C for 2 hrs. Lane 1-10: relaxation assay indicated fraction samples. lane 11: relaxation assay of eluted loading buffer; lane 12: control assay without adding *E. coli* topoisomerase I. (c) 0.5 µg of supercoiled plasmid DNA pXXZ06 was relaxed by indicated concentration of *E. coli* topoisomerase I from Q-Sepharose FF column at 37 °C for 15 min.

Table 1.1 Quantification Chart of *E. coli* topoisomerase I purification

Step	Total protein ^a (mg)	Total activity ^b (unit)	Specific activity ^c (unit/mg)
Homogenate (After Bij-58)	1472	NA	NA
Polymin P	1174	NA	NA
(NH ₄) ₂ SO ₄ precipitation 50-70%	686	NA	NA
SP-Sepharose FF	155	4,555,450	29,390
Q-Sepharose FF	68	2,152,880	31,660

a. Quantity of total protein at different purification stages according to the Bradford assay result; b. Total relaxation activity of *E. coli* topoisomerase I. One unit of *E. coli* topoisomerase I was defined as the amount of enzyme that catalyzes the relaxation of 0.5 µg of supercoiled plasmid DNA pXXZ06 in a 25 µL of reaction mixture containing 20 mM Tris-acetate, 50 mM KAc, 6 mM Mg(Ac)₂, 1 mM DTT and 0.2 µg/µL BSA at 37 °C

for 15min; c. Specific relaxation activity of *E. coli* topoisomerase I.

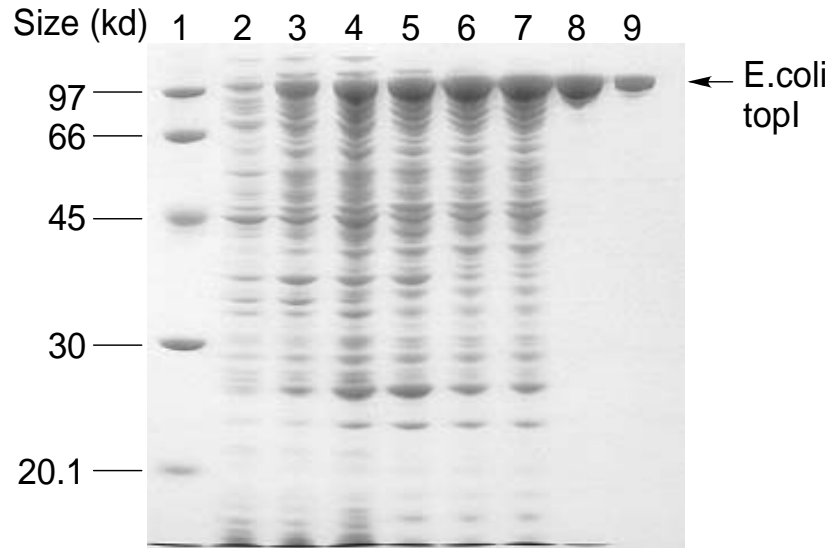


Figure 1.5 SDS-PAGE (10%) of protein samples from different purification stages.

Lane 1. LMW- SDS Marker (Amersham Biosciences). Lane 2. Cell extracts prepared from BL21/DE3/pLysS in the absence of IPTG. Lane 3. Cells extracts prepared from BL21/DE3/pLysS after 3 hour induction with 1mM IPTG. Lane 4. Homogenate prepared after applying Bij-58. Lane 5. Homogenate prepared after applying Polymin P. Lane 6. Homogenate prepared after $(\text{NH}_4)_2\text{SO}_4$ precipitation. Lane 7. Homogenate prepared prior to loading onto SP-Sepharose FF column. Lane 8. *E. coli* topoisomerase I prior to loading onto Q-Sepharose FF column. Lane 9. *E. coli* topoisomerase I after eluted from Q-sepharose FF column.

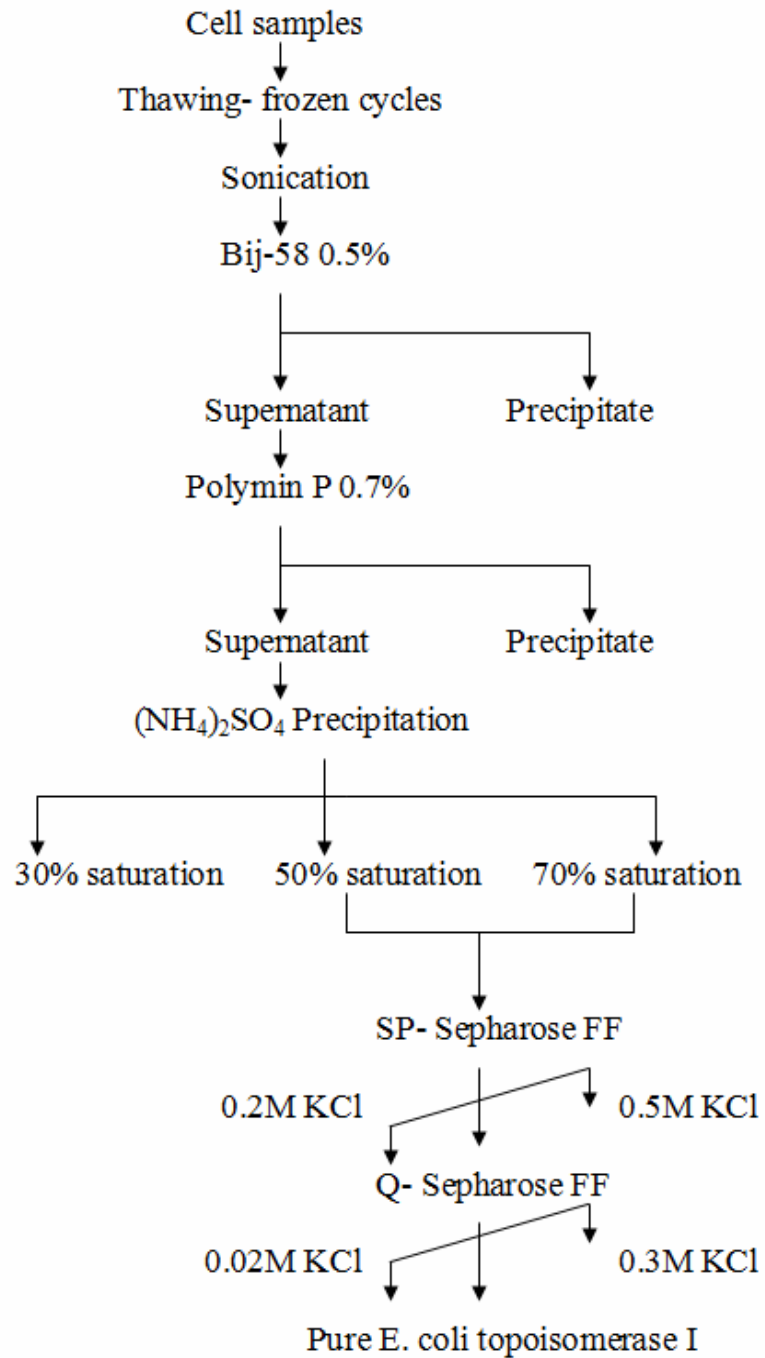


Figure 1.6 Flow chart of purification of *E. coli* topoisomerase I

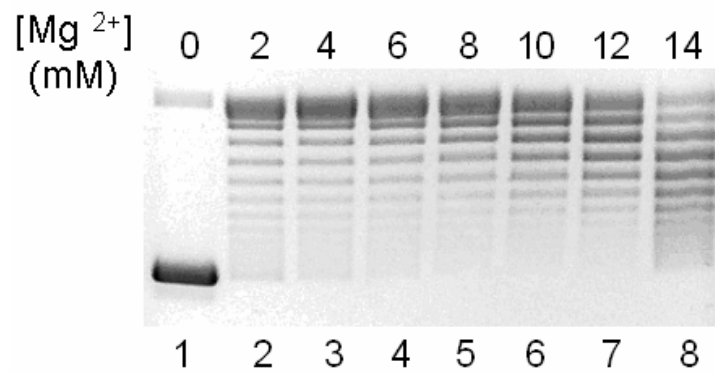


Figure 1.7 Influence of concentration of Mg^{2+} to the relaxation activity of *E. coli* topoisomerase I. 1 μ g of plasmid DNA pXXZ06 was relaxed by 10 nM *E. coli* topoisomerase I at indicated concentration of Mg^{2+} at 37 °C for 15 min.

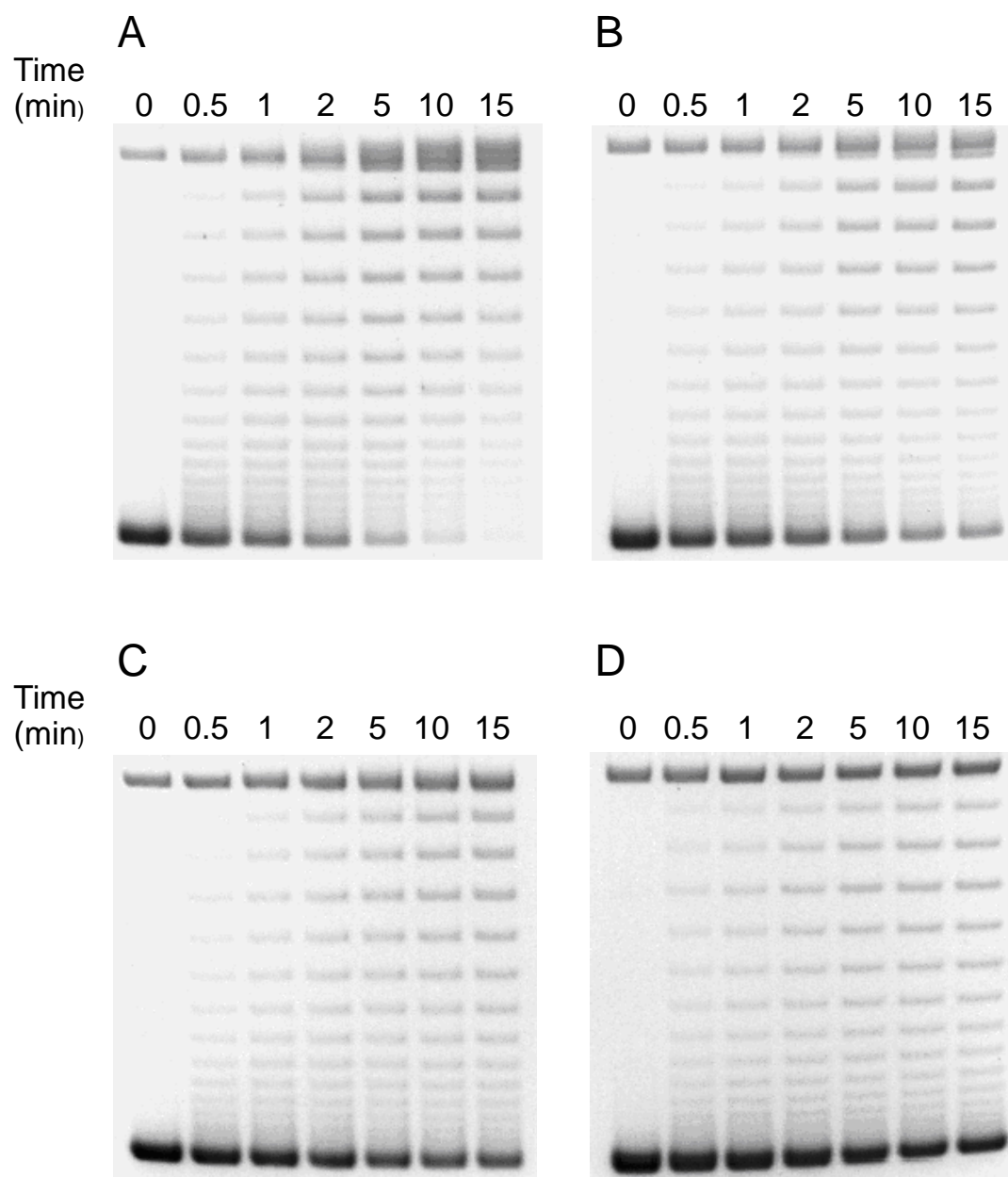


Figure 1.8 Time course DNA relaxation assay by *E. coli* topoisomerase I. Different quantity of plasmid DNA (A) 3.5 µg (B) 5.3 µg (C) 7.0 µg (D) 10.5 µg pXXZ06 was relaxed by 3.5 units of *E. coli* topoisomerase I. The reaction condition is described in “Materials and methods”.

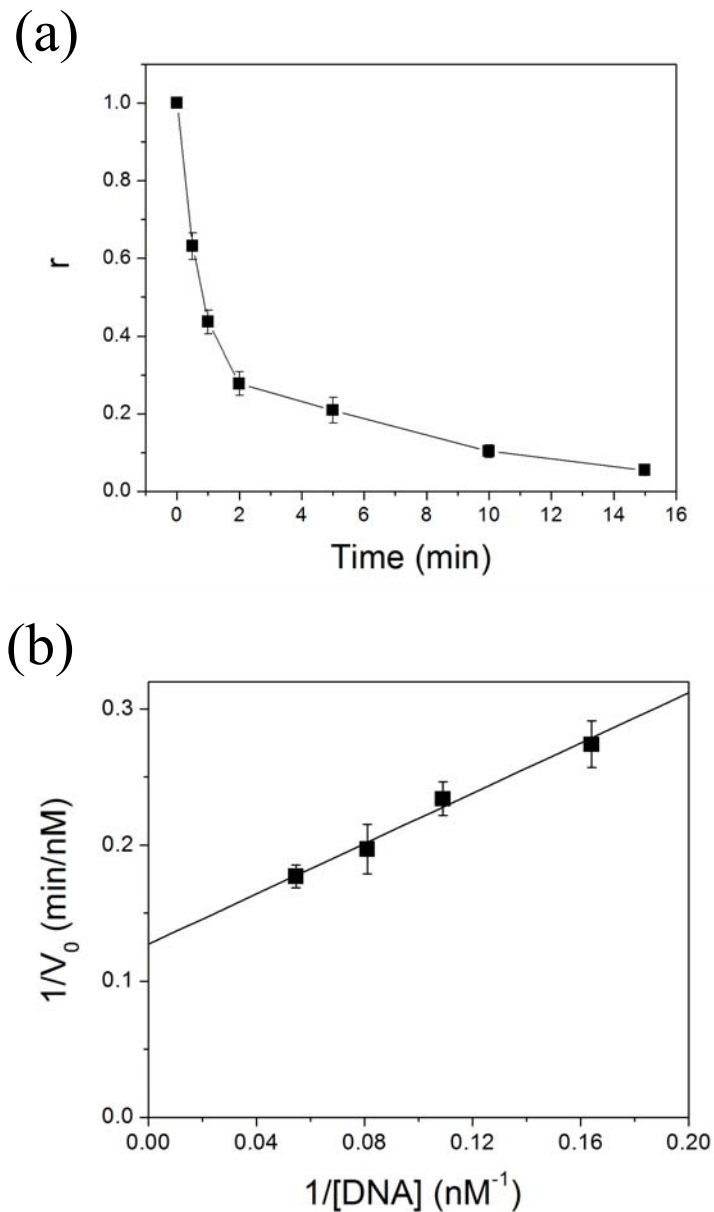


Figure 1.9 Kinetic data analysis of time course DNA relaxation assay. (a) Relaxation profile with initial concentration of supercoiled plasmid DNA at 6.1 nM. R represents the ratio of the area of the supercoiled DNA band at time zero versus the area of the supercoiled DNA band at time t. (b) Kinetic data analysis by Michaelis-Menten equation using the Lineweaver-Burk plot. Assay condition is described in “Materials and methods”. Kinetic data and analysis are listed in Table 1.2 and 1.3, respectively.

Table 1.2 Kinetic study of *E. coli* topoisomerase I

[scDNA] ^a nM	V ₀ ^b nM/min	1/[scDNA] nM ⁻¹	1/V ₀ min/nM
6.1	3.65±0.24	0.164	0.274±0.017
9.2	4.27±0.23	0.109	0.234±0.012
12.2	5.08±0.49	0.082	0.197±0.018
18.3	5.64±0.27	0.055	0.177±0.009

a. Concentration of supercoiled plasmid DNA pXXZ06. The concentration of supercoiled DNA is adjusted by removing concentration of nicked DNA from total concentration of DNA which is determined by UV spectrum.

b. Initial velocity of relaxation reaction between *E. coli* topoisomerase I and pXXZ06 supercoiled plasmid DNA.

Table 1.3 Kinetic parameters of *E. coli* topoisomerase I

K_M nM	V_{max} nM/min	k_{cat} min ⁻¹
7.52±2.17	7.98±0.95	1.20±0.47

* K_M , V_{max} and k_{cat} were determined according to Figure 1.9.b.

initial point for a comprehensive kinetic study and for drug discovery.

1.5. Conclusion:

In this thesis, I developed a rapid procedure to purify *E. coli* DNA topoisomerase I on a large scale for biochemical and biophysical studies that require a large amount of the enzyme, such as isothermal titration calorimetry. The purification procedure is on the basis of the analysis in which I found that the positive and negative charges are asymmetrical distributed over the primary structure of the enzyme. In this case, the enzyme binds to both cation- and anion-exchange columns. Using the combination for the SP-Sepharose FF and Q-Sepharose FF columns, I can purify more than 50 mg of *E. coli* DNA topoisomerase I in five days from 4 liters of *E. coli* cell culture. More importantly, this preparation of *E. coli* DNA topoisomerase I is free of nuclease and

nucleic acids contamination. I also performed a preliminary kinetic analysis of *E. coli* DNA topoisomerase I, which may be useful for the drug discovery studies.

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Chapter II

2.0. Abstract

I used isothermal titration calorimetry to investigate the energetics of DNA supercoiling by using the unwinding properties of DNA intercalators, ethidium and daunomycin. After comparing the enthalpy changes of these DNA intercalators binding to supercoiled and nicked or relaxed plasmid DNA pXXZ06, I determined the DNA supercoiling enthalpy is about 12 kcal/mol per turn of DNA supercoil, which is in good agreement with the previously published results.

2.1. Introduction

2.1.1. Definition and biological significance of DNA supercoiling

The structure of the DNA double helix was elucidated by James Watson and Francis Crick in 1953 (1). This great discovery led to a revolution in the realm of biological research. After that, great effort and enthusiasm contributed to a rapid development of life science in fields such as in molecular biology, where the structure of DNA was elucidated in more detail. Besides some general properties of the DNA double helix including base pair interactions or transformation among the B, A and Z DNA conformations, supercoiling is also considered as one of the main instinctive properties of the DNA double helix. DNA supercoiling can be defined as a higher-ordered coiling of the DNA double helix. For closed circular DNA without breakage on the DNA strand, the DNA supercoiling can save the elastic tension (energy) generated from the conformation change of the DNA helix. Meanwhile, the overall shape of the DNA molecule becomes

much more compact than the nicked form, where one DNA strand is cleaved. There are two theoretical geometric conformations, referred to as plectonemic and toroidal conformations, suggested for the circular closed supercoiled DNA. For the plectonemic conformation, the DNA helix is coiled around the other portion of the same DNA molecule. As an alternative, the toroidal conformation is formed when the DNA molecule wraps around an imaginary cylindrical surface. The plectonemic conformation occupies a dominant position in DNA supercoiling in vivo. However, the toroidal conformation can be formed and is considered as the model of DNA supercoiling when DNA is bound to some protein molecules.

Observations from various organisms are indicative of DNA existing in the negatively supercoiled form in vivo. The degree of DNA supercoiling can be altered by binding to proteins, including DNA topoisomerases, as described in Chapter I. In *E. coli* cells, other than when interacting with the four kinds of topoisomerases, the geometry of the circular chromosomal DNA changes by binding to many nucleoid proteins, such as HU (2) and H1 (3) protein. Moreover, DNA supercoiling can also facilitate DNA conformational changes, such as cruciform formation or Z-DNA transformation from B-DNA, and dominantly influence other important biological activities, such as DNA replication (4, 5, 6) and transcription (7, 8).

2.1.2. Intercalating reagent and their unwinding effect to DNA molecule

There are a numbers of chemical compounds referred as DNA intercalators, which have a poly-aromatic ring structure, can insert into the spaces between DNA base pairs. The interacting effect has been characterized by X-ray crystallography (9, 10). Intercalation into the DNA causes an unwinding effect to the DNA double helix, and the negative

DNA supercoiling becomes less negative than relaxed state, and even positive supercoiled state when more intercalator molecules are inserted. Ethidium bromide is the most commonly recognizable DNA intercalator and is also utilized as a DNA staining reagent. One intercalating behavior of the ethidium bromide leads to a 26° unwinding of adjacent base pairs (11, 12).

Other than intercalators binding between the DNA base pairs, another group of compounds can interact with the minor groove of the DNA helix (14, 15). Compared with DNA intercalators, netropsin (16) can induce a diverse effect of winding the DNA helix by binding to the minor groove of AT-rich region.

2.1.3. Energetic study of DNA supercoiling

As a result of energetically unfavorable conformation change, the circular supercoiled DNA helix conserves more free energy than the relaxed or nicked ones. This thermodynamic difference increases as more supercoillings are introduced into the DNA molecule. Among various research methods for investigating the interaction between intercalator and DNA, isothermal titration calorimetry (ITC) provides a useful method for studying the biological macromolecule interaction with their substrate. In the ITC instrument, two cells (one is called “reference cell” and the other is called “sample cell”) are kept at the same temperature by the heat controlling circuit in an adiabatic shell. When an injection occurs, by injecting solution into the sample cell, there will be heat released or absorbed from the reaction. The resulting temperature change of the solution in the sample cell can be detected and compensated by the heat controlling circuit system. During this procedure, the amount of compensating heat can be integrated and calculated in order to reflect the amount of heat from the reaction itself.

Utilizing the ITC technique, Seidl and Hinz (17) investigated the energy of DNA supercoiling formation by detecting the enthalpy change associated with relaxation of negatively supercoiled DNA by *E. coli* topoisomerase I. The value was reported at 34.9 kJ/ (mol of superhelical turn), which is equal to 8.34 kcal/(mol of superhelical turn). This enthalpy change value for DNA supercoiling unwinding is comparable to that reported by Lee (12.2 ± 0.4 kcal/ mol superhelical turn) in 1981 (18).

At the same time, a number of energetic investigations were carried out to determine the enthalpy and entropy contributions to the free energy of the intercalating effects (19, 20). The thermodynamic data show a general exothermic enthalpy change for the intercalating interaction between intercalators and the DNA helix. Enthalpy change of the intercalating reaction varies greatly when DNA substrates with different sequences are used for the ITC titrations. The differences of the free energy of intercalation, however, become much smaller than differences of the enthalpy change by the “compensating effect” from entropy contribution.

The length of the DNA has been observed to be influential on the energy of supercoiling, especially when the length is less than 1000 base pairs (21, 22). That is the result of high elastic tension in the constrained circular DNA. The concentration of counterions in the solution also changes the energy of the DNA supercoiling (23). When the counterion concentration increases, the repulsive force between negatively charged DNA helices decreases and facilitates the formation of a more stable DNA helix conformation.

2.2. Objectives

Utilizing the ITC system, enthalpy change of interaction between *E. coli* topoisomerase I and supercoiled plasmid DNA and interaction between DNA intercalator and supercoiled/

relaxed/ nicked plasmid DNA can be measured.

As a consequence, two kinds of buffers (Buffer I and II) are prepared for the ITC titrations between *E. coli* topoisomerase I and negatively supercoiled plasmid DNA pXXZ06. Due to the different relaxation activities observed with *E. coli* topoisomerase I in different buffers, the enthalpy changes associated with the relaxation of DNA supercoiling are compared with the one without relaxation. Since the different unwinding angles can be introduced by different intercalators, the interaction between DNA intercalator and supercoiled/ relaxed/ nicked plasmid DNA may give another possibility to estimate the enthalpy change associated with the formation of DNA supercoiling.

2.3. Materials and methods

2.3.1. Materials

Ethidium bromide, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, potassium acetate and dithiothreitol (DTT) were purchased from Sigma-Aldrich Company (St. Louis, MO). Daunomycin hydrochloride was obtained from Waterstone Technology (Carmel, IN). Magnesium acetate was a product of EM science (Gibbstown, NJ). The EDTA disodium salt was obtained from Fisher Scientific (Pittsburgh, PA). The concentrations of ethidium bromide, and daunomycin were calculated according to their extinction coefficients: ethidium bromide $\epsilon_{480\text{nm}} = 5,600 \text{ M}^{-1}\text{cm}^{-1}$ and daunomycin $\epsilon_{480\text{nm}} = 11,500 \text{ M}^{-1}\text{cm}^{-1}$, respectively. Nt. BbvCI, BamHI, HindIII and bovine serum albumin (BSA) were purchased from New England Biolabs (Beverly, MA). The negatively supercoiled plasmid DNA pXXZ06 was purified using QIAGEN Plasmid Giga Kits. *E. coli* topoisomerase I was purified according to the purification protocol as described in

Chapter I. The concentration of *E. coli* topoisomerase I was determined using the extinction coefficient at the wavelength of 280 nm of $98,210 \text{ M}^{-1}\text{cm}^{-1}$.

2.3.2. *The preparation of the relaxed and nicked plasmid DNA pXXZ06*

The relaxed plasmid DNA pXXZ06 was prepared in the relaxation buffer containing 20 mM Tris-acetate (pH 7.9), 50 mM KAc, 6 mM $\text{Mg}(\text{Ac})_2$, 1 mM DTT, $0.1 \mu\text{g}/\mu\text{l}$ BSA, 0.125 mg/mL negatively supercoiled plasmid pXXZ06, and 140 nM of *E. coli* topoisomerase I. After incubation at 37°C for 30 min, the relaxation reaction was stopped by extraction with an equal volume of phenol. For the preparation of 0.15 mM relaxed plasmid DNA pXXZ06, the DNA sample after phenol extraction was dialyzed extensively against $1\times\text{BPES}$ ($6 \text{ mM Na}_2\text{HPO}_4$, $2 \text{ mM NaH}_2\text{PO}_4$, 1 mM EDTA , 185 mM NaCl) at 4°C and used for the ITC experiments. To prepare 1 mM relaxed plasmid DNA pXXZ06, the DNA sample after phenol extraction was firstly dialyzed against $1\times\text{BPE}$ ($6 \text{ mM Na}_2\text{HPO}_4$, $2 \text{ mM NaH}_2\text{PO}_4$, 1 mM EDTA) at 4°C , and then dried in a lyophilizer. The freeze-dried DNA sample was re-dissolved in $1\times\text{BPES}$ and dialyzed against $1\times\text{BPES}$ at 4°C for the following ITC experiments.

The nicked plasmid DNA pXXZ06 was prepared in the buffer containing 20 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl_2 , 1 mM DTT, $0.1 \mu\text{g}/\mu\text{l}$ BSA, 0.1 mg/mL negatively supercoiled plasmid pXXZ06, and 150 units/mL Nt. BbvCI. After incubation at 37°C for 2 hours, the reaction was stopped by extraction with an equal volume of phenol. For the preparation of 0.15 mM relaxed plasmid DNA pXXZ06, the DNA sample after phenol extraction was dialyzed extensively against $1\times\text{BPES}$ ($6 \text{ mM Na}_2\text{HPO}_4$, $2 \text{ mM NaH}_2\text{PO}_4$, 1 mM EDTA , 185 mM NaCl) at 4°C and used for the ITC experiments. To prepare 1 mM relaxed plasmid DNA pXXZ06, the DNA sample after phenol

extraction was firstly dialyzed against 1×BPE (6 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1 mM EDTA) at 4°C, and then dried in a lyophilizer. The freeze-dried DNA sample was re-dissolved in 1× BPES and dialyzed against 1× BPES at 4 °C for the following ITC experiments. The DNA concentration of plasmid pXXZ06 was estimated using the following formula: 1 OD₂₆₀ = 0.15 mM (bp).

2.3.3. *The determination of supercoiling enthalpy by isothermal titration calorimetry*

In this study, two methods were used to determine the supercoiling enthalpy by using a VP-ITC titration calorimeter. In the first method, *E. coli* DNA topoisomerase I was used to relax the supercoiled plasmid pXXZ06. First, samples were extensively dialyzed against Buffer I (20 mM Tris-acetate, pH 7.9, 50 mM KAc, 6 mM Mg(Ac)₂, 10% w/v glycerol and 0.1 mM DTT) or Buffer II (20 mM Tris-acetate, pH 7.9, 50 mM KAc, 25 mM EDTA, 10% w/v glycerol, and 0.1 mM DTT). For a typical ITC experiment, a single injection of 100 µL of 1.5 mM (in bp) pXXZ06 supercoiled DNA into sample cell containing 0.1 µM of *E. coli* topoisomerase I was carried out. After the injection (15 min), the relaxation reaction was stopped by adding EDTA to a final concentration of 25 mM. The topological status of the DNA sample after the ITC experiment was determined by electrophoresis in 1×TAE buffer, pH 7.8, in a one-dimensional, 1% agarose gel. The heat liberated or absorbed is observed as a peak corresponding to the power required to keep the sample and reference cells at identical temperatures. The peak produced during the injection is converted to heat output by integration and corrected for cell volume and sample concentration. Control experiments were also carried out to determine the contribution of the heats of dilution arising from (1) *E. coli* topoisomerase I into buffer and (2) buffer into DNA. The net enthalpy for the relaxation reaction was determined by

subtraction of the component heats of dilution.

In the second method, I utilized several DNA intercalating agents to determine the enthalpy change of the relaxation of plasmid pXXZ06. I measured the DNA-binding enthalpy for the intercalators binding to three different types of DNA templates, i.e., the supercoiled, relaxed, and nicked plasmid pXXZ06, and then calculated the difference of the DNA binding enthalpy between the supercoiled and relaxed plasmid or the supercoiled and nicked plasmid. Since the unwinding angles of these DNA intercalators are known, I can derive the supercoiling enthalpy from the difference of the DNA binding enthalpy between the supercoiled and relaxed plasmid or the supercoiled and nicked plasmid. For the ITC experiments, the supercoiled, relaxed, and nicked plasmid pXXZ06 were extensively dialyzed against 1×BPES at 4 degree. The DNA intercalator solutions were prepared by directly dissolving the intercalating reagents into the 1×BPES that was used for the dialysis of the plasmid DNA pXXZ06. All ITC experiments were performed in a VP-ITC calorimeter in the laboratory. For a typical ITC experiment, the titration was set up so that 10 μ L of a 1 mM intercalating reagent was injected every 300 seconds, up to a total of 29 injections, into a DNA sample (1.44 mL of 150 μ M(bp)) in the sample cell or 5 μ L of 1 mM intercalating reagent was injected every 300 seconds, up to a total of 25 injections, into a DNA sample (1.44 mL of 1 mM(bp)) in the sample cell. The heat liberated or absorbed is observed as a peak corresponding to the power required to keep the sample and reference cells at identical temperatures. The peak produced during the injection is converted to heat output by integration and corrected for cell volume and sample concentration. Control experiments were also carried out to determine the contribution of the heats of dilution arising from (1) the DNA intercalators

into buffer and (2) buffer into DNA. The net enthalpy for the titration reaction was determined by subtraction of the component heats of dilution.

2.4. Results and discussion

Previously, Sedl and Hinz (17) used calorimetry to demonstrate that the unfavorable supercoiling free energy exclusively comes from the positive enthalpy associated with the formation of the negatively supercoiled plasmids. For the 11 kb ColE1 amp RSF2124 plasmid DNA with a superhelical density of -0.057, the supercoiling enthalpy was determined to be 2260 ± 409 kJ/mol of plasmid (541 ± 98 kcal/mol of plasmid). Since the plasmid used in their study has 63 superhelical turns, the enthalpy change per superhelical turn is 34.9 kJ/mole of superhelical turns (8.3 kcal/mol of superhelical turns). Since calorimetry technology has been improved significantly, I decided to use the VP-ITC to determine the enthalpy change associated with DNA supercoiling. The first effort was to utilize the property of the DNA intercalators that unwind the DNA templates upon binding to plasmid DNA. Since each ethidium unwinds the DNA for about 26 degrees (11, 12) (the standard deviation of the unwinding angle was estimated to be 2.6 degree (11)), I should be able to obtain the supercoiling enthalpy from the difference between the DNA binding enthalpy to the supercoiled DNA and the nicked or relaxed DNA. The first DNA intercalator used here is ethidium bromide. Figure 2.1 shows results from the ITC experiments in which ethidium were titrated into a large excess (1 mM(bp)) of either the supercoiled (Figure 2.1.a) or the nicked plasmid pXXZ06 (Figure 2.1.b). In this case, all added ethidium is bound to DNA after each injection. The enthalpy changes for ethidium binding to the supercoiled and nicked plasmid DNA

pXXZ06 are -7945 ± 42 cal/mol and -7120 ± 52 cal/mol, respectively. The enthalpy difference between ethidium binding to the supercoiled and the nicked DNA was calculated to be -825 ± 94 cal/mol. The supercoiling enthalpy for each turn of DNA supercoil should equal to $825 \times (360/26) = 11423$ cal/mol of turn of the supercoil (~ 10 bp) if I assume no additional enthalpy change is associated with the difference between ethidium binding to the supercoiled DNA and the nicked DNA molecules. I also estimated the DNA supercoiling enthalpy change by comparing the difference between ethidium binding to supercoiled and relaxed plasmid DNA pXXZ06 (Figure 2.2). The enthalpy changes for ethidium binding to the supercoiled and relaxed plasmid DNA pXXZ06 are -7945 ± 42 cal/mol and -7049 ± 52 cal/mol, respectively. The enthalpy difference between ethidium binding to the supercoiled and the relaxed DNA was calculated to be -898 ± 253 cal/mol that is almost equivalent to enthalpy difference between ethidium binding to the supercoiled and the nicked DNA. In this case, the supercoiling enthalpy is calculated to be $898 \times (360/26) = 12433$ cal/mol per turn of DNA supercoil. The DNA supercoiling enthalpy determined here is consistent with the results obtained from a previous study on the unwinding of dsDNA by dehydration (18) where the enthalpy value for unwinding a turn of DNA double helix (10 bp) was estimated to be 12.2 kcal/mol. However, my DNA supercoiling enthalpy is slightly larger than the one estimated from the calorimetric measurement of the DNA relaxation by *E. coli* topoisomerase I (17). A possible reason for the difference is that the relaxation reaction of *E. coli* topoisomerase I is a relatively slow process (see below for details) and it is difficult to obtain a good baseline for integration of the enthalpy. Therefore, it will result

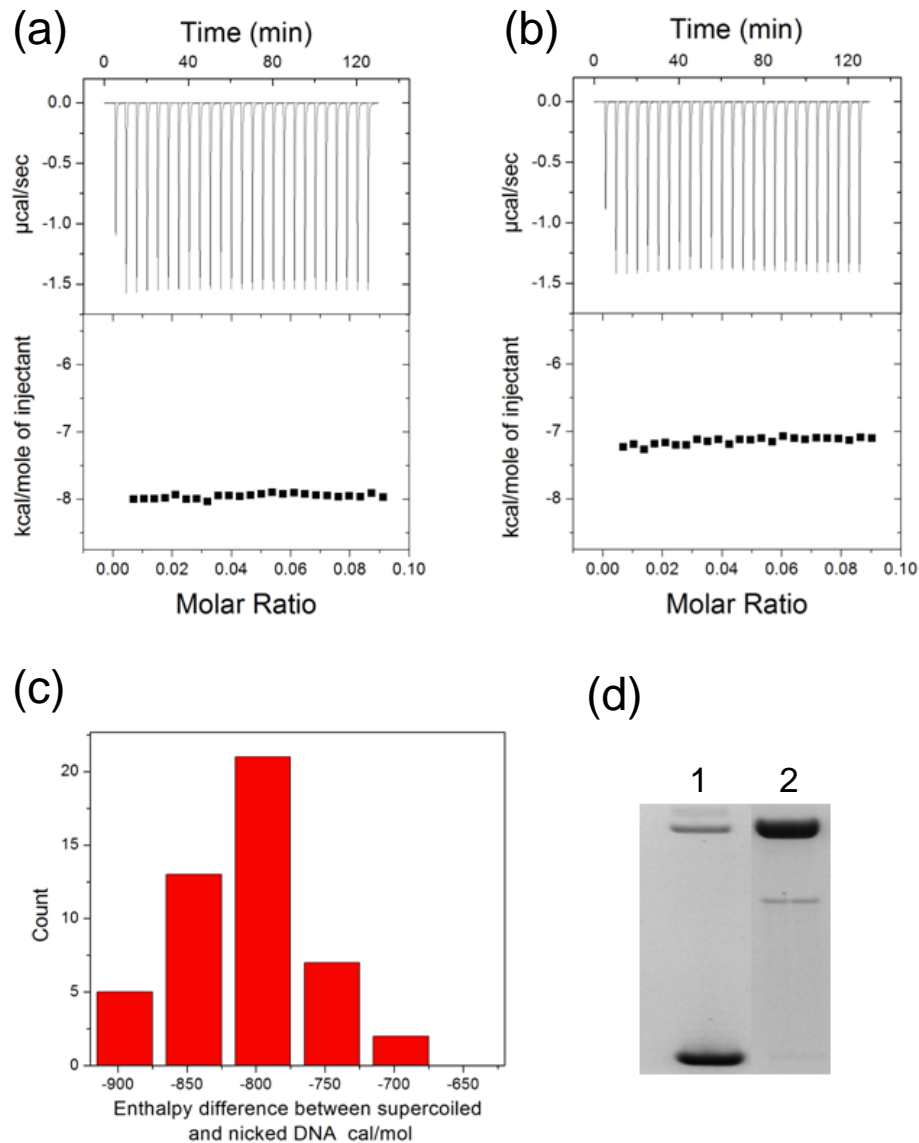


Figure 2.1 Sample raw ITC data for the titration of ethidium bromide into plasmid pXXZ06 (a) supercoiled and (b) nicked DNA at 25 °C in 1×BPES buffer. Top, each peak corresponds to the heat generated by injection of an aliquot of 5 μL of ethidium bromide (1mM) into DNA solution (1.7 mL of 1.0 mM (bp)). Bottom, binding isotherm by integration with respect to time; (c) Enthalpy difference between supercoiled and nicked DNA (d) 1% agarose electrophoresis analysis of DNA sample (1) pXXZ06 supercoiled

DNA; (2) pXXZ06 nicked DNA.

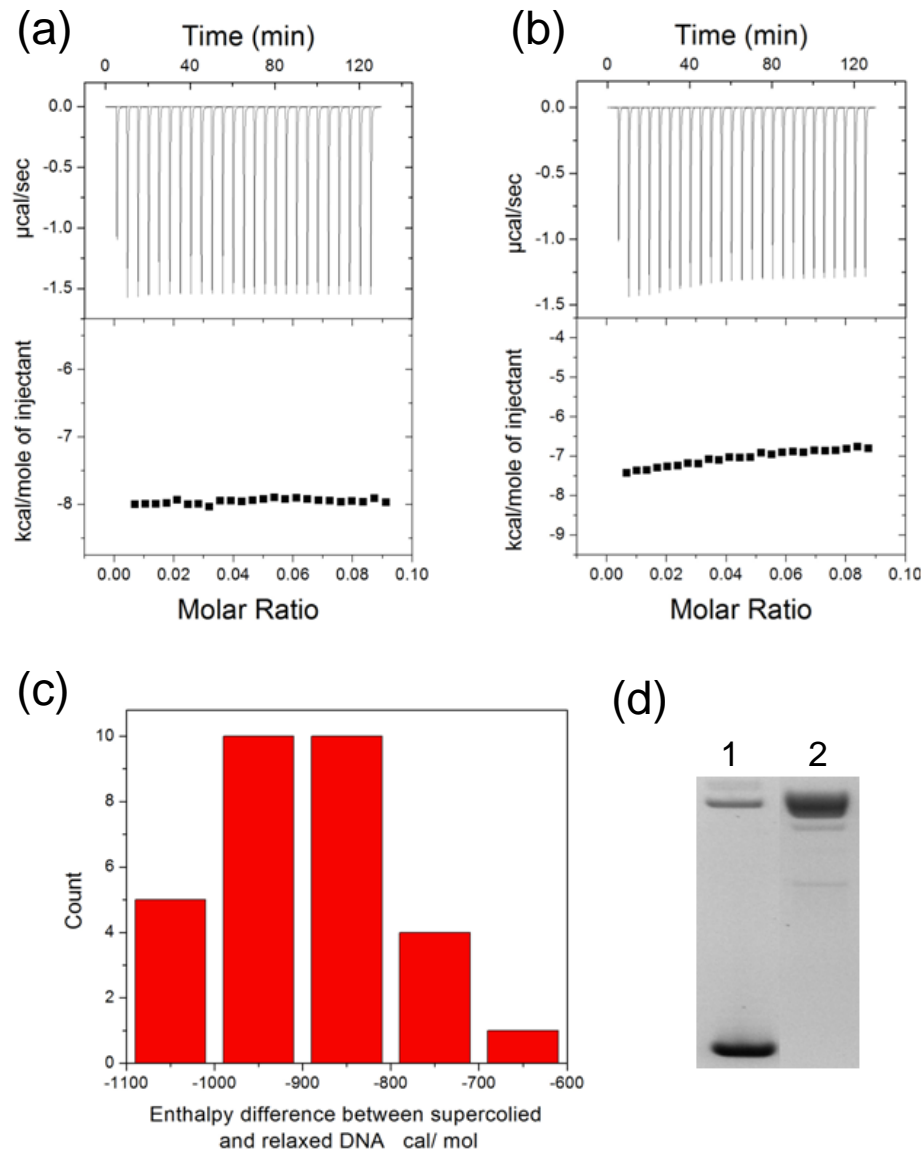


Figure 2.2 Sample raw ITC data for the titration of ethidium bromide into plasmid pXXZ06 (a) supercoiled and (b) relaxed DNA at 25 °C in 1×BPES buffer. Top, each peak corresponds to the heat generated by injection of an aliquot of 5 μL of ethidium bromide (1mM) into DNA solution (1.7 mL of 1.0 mM (bp)). Bottom, binding isotherm by integration with respect to time; (c) Enthalpy difference between supercoiled and relaxed DNA (d) 1% agarose electrophoresis analysis of DNA sample (1) pXXZ06

supercoiled DNA; (2) pXXZ06 relaxed DNA.

in a large error in the supercoiling enthalpy. Another possibility is that the enthalpy obtained from the relaxation reaction also contains a contribution from *E. coli* topoisomerase I binding to the supercoiled DNA template. In this case, the enthalpy obtained from the calorimetric measurement of the DNA relaxation reaction by *E. coli* topoisomerase I also has a contribution from *E. coli* topoisomerase I binding the supercoiled DNA template, which cause the supercoiling enthalpy deviated from the values obtained by using other techniques. Regardless, the enthalpy values determined by using these different methods are comparable.

In this study, I carried out another type of ITC experiments in which a series 10 μ L of 1 mM ethidium bromide were titrated into 0.15 mM (bp) of the supercoiled, nicked and relaxed plasmid DNA solution. Under this condition, all DNA binding sites on DNA lattice will be titrated up by ethidium bromide. Figures 2.3 and 2.4 show the results of these experiments. The DNA binding enthalpy for ethidium binding to the supercoiled, nicked, and relaxed plasmid DNA pXXZ06 were determined to be -7439 ± 51 , -6670 ± 66 , and -6601 ± 45 cal/mol, respectively (Table 2.2). These values are consistent with those determined in Figures 2.1 and 2.2 (Table 2.1). Interestingly, the enthalpy differences between the supercoiled and nicked plasmid DNA are quite dramatic over the entire course of the titration experiment. They are initially negative, then positive, and finally close to zero (Figure 2.3.c). I believe that this ITC binding curve is a result of ethidium unwinding the DNA double helix upon binding to the negatively supercoiled DNA. In this case, the twist is reduced, the writhe is increased, and therefore the DNA templates become more relaxed. Further addition of ethidium leads to the production of positively

supercoiled plasmid DNA. These results suggest that a positive enthalpy is associated

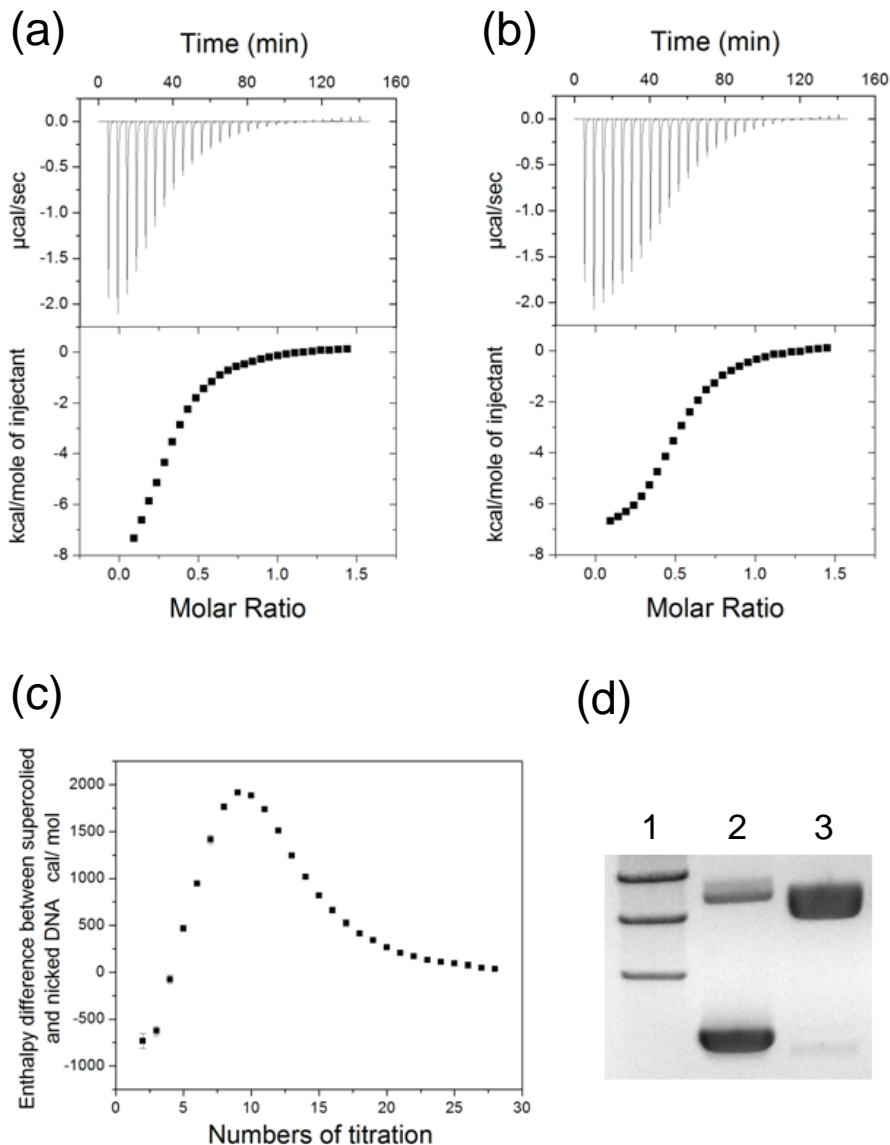


Figure 2.3 Sample raw ITC data for the titration of ethidium bromide into plasmid pXXZ06 (a) supercoiled and (b) nicked DNA at 25 °C in 1× BPES buffer. Top, each peak corresponds to the heat generated by injection of an aliquot of 10 μL of ethidium bromide (1mM) into DNA solution (1.7 mL of 0.15 mM (bp)). Bottom, binding isotherm by integration with respect to time; (c) Enthalpy difference between supercoiled and nicked DNA (d) 1% agarose electrophoresis analysis of DNA sample (1) Marker, λ DNA

Hind III digested; (2) pXXZ06 supercoiled DNA; (3) pXXZ06 nicked DNA.

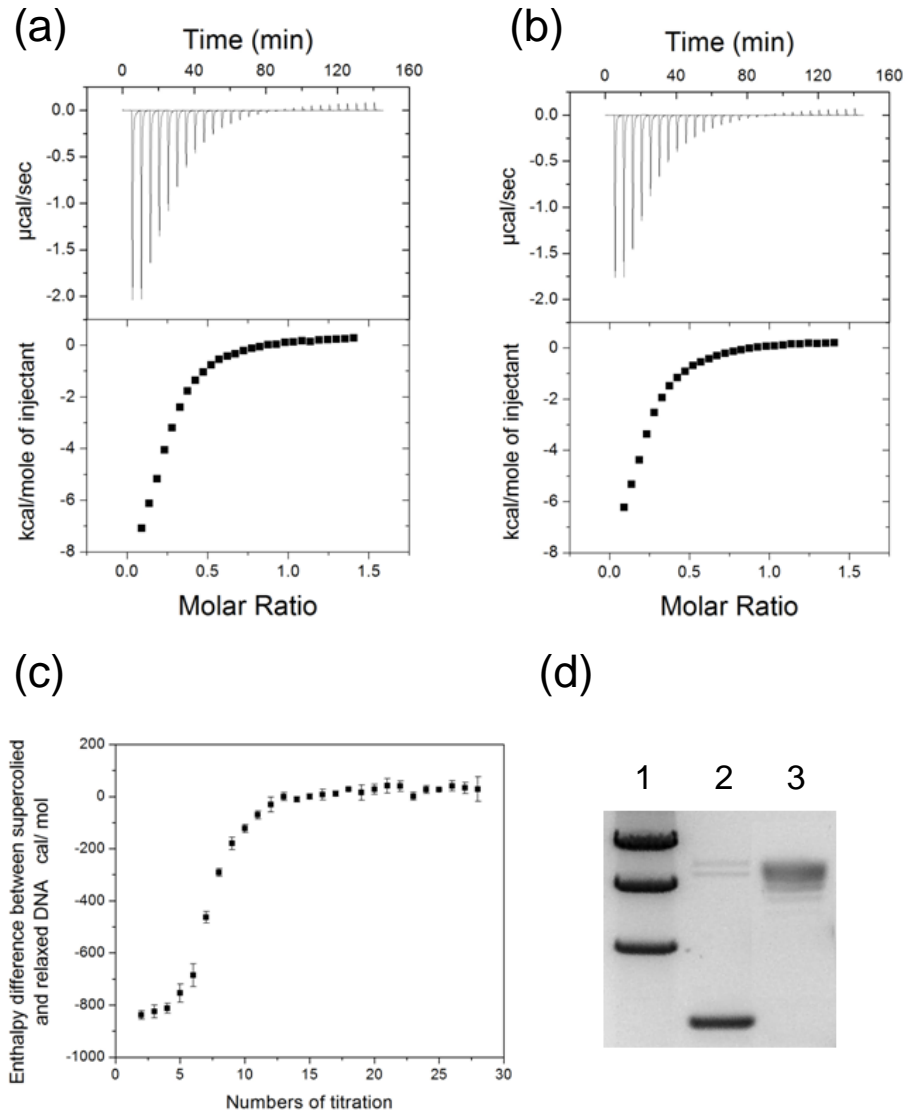


Figure 2.4 Sample raw ITC data for the titration of ethidium bromide into plasmid pXXZ06 (a) supercoiled and (b) relaxed DNA at 25 °C in 1× BPES buffer. Top, each peak corresponds to the heat generated by injection of an aliquot of 10 μL of ethidium bromide (1mM) into DNA solution (1.7 mL of 0.15 mM (bp)). Bottom, binding isotherm by integration with respect to time; (c) Enthalpy difference between supercoiled and relaxed DNA (d) 1% agarose electrophoresis analysis of DNA sample (1) Marker, λ

DNA Hind III digested; (2) pXXZ06 supercoiled DNA; (3) pXXZ06 relaxed DNA.

with the formation of the negative superhelical turns and a negative enthalpy is involved in the generation of the positive superhelical turns. For the enthalpy differences between ethidium binding to negatively supercoiled and relaxed DNA templates, I only observed a “normal” titration curve (Figure 2.4.c) where no positive enthalpy was involved. The main reason is that the titration of ethidium into the relaxed plasmid DNA templates also leads to the production of the positively supercoiled DNA. Regardless, the DNA supercoiling enthalpy was calculated to be 10648 ± 1620 or 11603 ± 1329 cal/mol, which is consistent with the values determined above.

Next I used another DNA intercalator, daunomycin to study the DNA supercoiling enthalpy by isothermal titration calorimetry. Since the DNA unwinding angle of daunomycin is 11 degrees (13) (the unwinding angle of daunomycin binding to DNA was estimated to be 8-12 degrees in a few different studies (24, 25) and no standard deviation was given in these estimates. Regardless, in a more recent paper, the DNA unwinding angles of daunomycin was determined to be 12.1 ± 1.2 degrees (26), which is consistent with the previous results). The enthalpy difference between daunomycin binding to the negatively supercoiled and the nicked or the relaxed DNA templates should be half of the value of the enthalpy difference for ethidium as the DNA intercalator. The results are shown in Figures 2.5, 2.6, and 2.7. For the ITC titration experiments using a large excess (1 mM(bp)) of either the supercoiled (Figure 2.5a) or the nicked (Figure 2.5b) or the relaxed pXXZ06 DNA (Figure 2.6b), the enthalpy changes for daunomycin binding to the supercoiled, nicked and relaxed plasmid DNA pXXZ06 are -8538 ± 85 , -8030 ± 52 , and 8086 ± 92 cal/mol, respectively. The enthalpy difference between daunomycin binding to

the supercoiled and the nicked or the relaxed DNA was calculated to be -452 ± 134 and -508 ± 177 cal/mol, respectively (Table 2.1). The supercoiling enthalpy is calculated to be $452 \times (360/11) = 14792 \pm 4386$ or $508 \times (360/11) = 16625 \pm 5793$ cal/mol per turn of DNA supercoil. Considering the standard deviations of these measurements, these values are consistent with the values I determined by using ethidium as the DNA intercalator. For the ITC titration experiments using 0.15 mM (bp) of the supercoiled and relaxed plasmid DNA solution, the DNA supercoiling enthalpy was calculated to be 15643 ± 2749 cal/mol, which is also in the same range of the DNA supercoiling enthalpy determined above and in the previous studies (17, 18).

In this study, I also tried to use the relaxation reaction of *E. coli* topoisomerase I to determine the supercoiling enthalpy in the isothermal titration calorimeter. For this ITC experiment, a single injection of 100 μ L of 1.5 mM (in bp) pXXZ06 supercoiled DNA was titrated into the sample cell containing 0.1 μ M of *E. coli* topoisomerase I. After the injection (~ 10 min), the relaxation reaction was stopped by adding EDTA to a final concentration of 25 mM. The topological status of the DNA sample after the ITC experiment was shown in Figure 2.8. These results demonstrate that the negatively supercoiled plasmid DNA pXXZ06 was fully relaxed by *E. coli* topoisomerase I. The raw ITC curve for this relaxation reaction is shown in Figure 2.9, demonstrating that the relaxation reaction of *E. coli* topoisomerase I is a very slow and complex process. In this case, it is difficult to determine when the reaction is stopped and where the baseline is for the integration to obtain a reasonable enthalpy change for the relaxation reaction. Nevertheless, I tried to integrate the titration peak to estimate the enthalpy change associated with the relaxation reaction and the results are shown in Figure 2.8. The

relaxation enthalpy was estimated to be ~ 1017 kcal/mol. The supercoiling density of

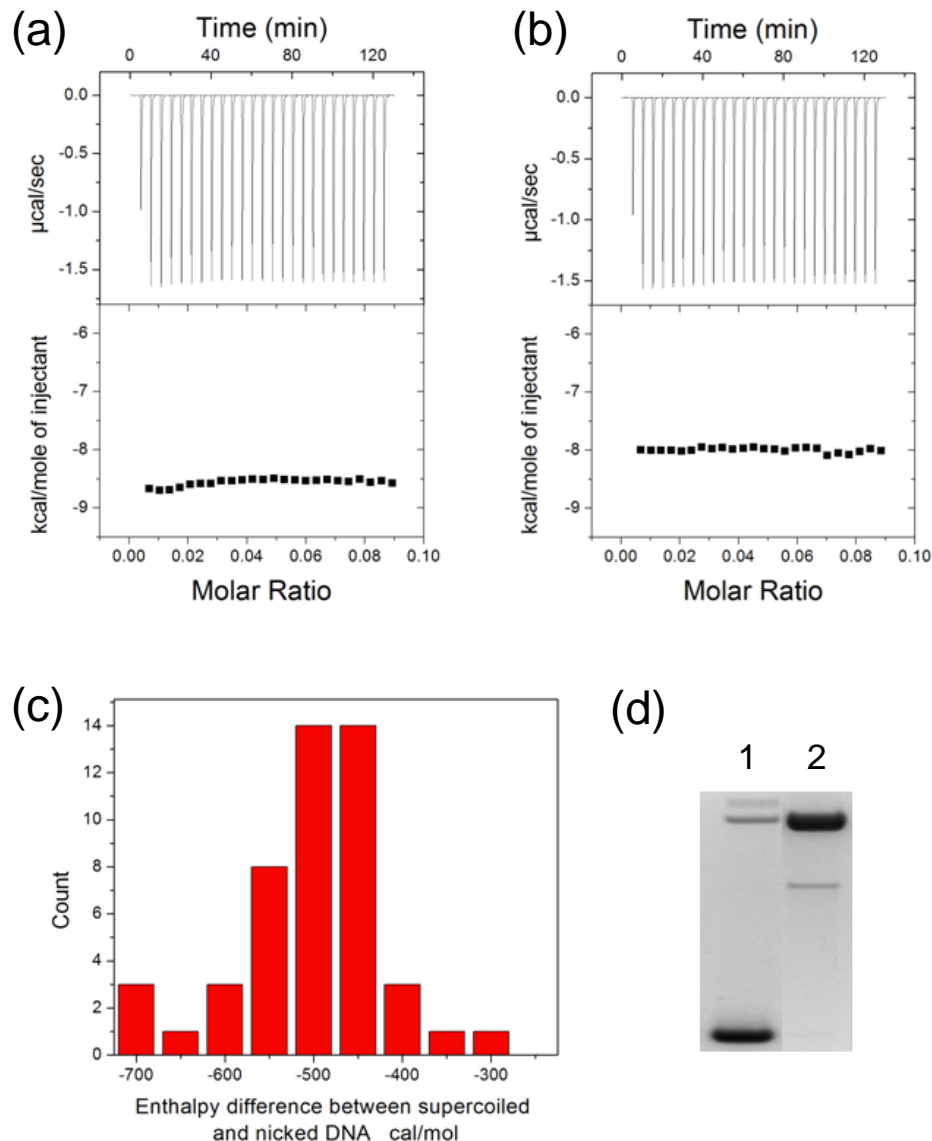


Figure 2.5 Sample raw ITC data for the titration of daunomycin into plasmid pXXZ06 (a) supercoiled and (b) nicked DNA at 25 °C in $1\times$ BPES buffer. Top, each peak corresponds to the heat generated by injection of an aliquot of 5 μL of daunomycin (1mM) into DNA solution (1.7 mL of 1.0 mM (bp)). Bottom, binding isotherm by integration with respect to time; (c) Enthalpy difference between supercoiled and nicked DNA (d) 1% agarose electrophoresis analysis of DNA sample (1) pXXZ06 supercoiled DNA; (2) pXXZ06

nicked DNA.

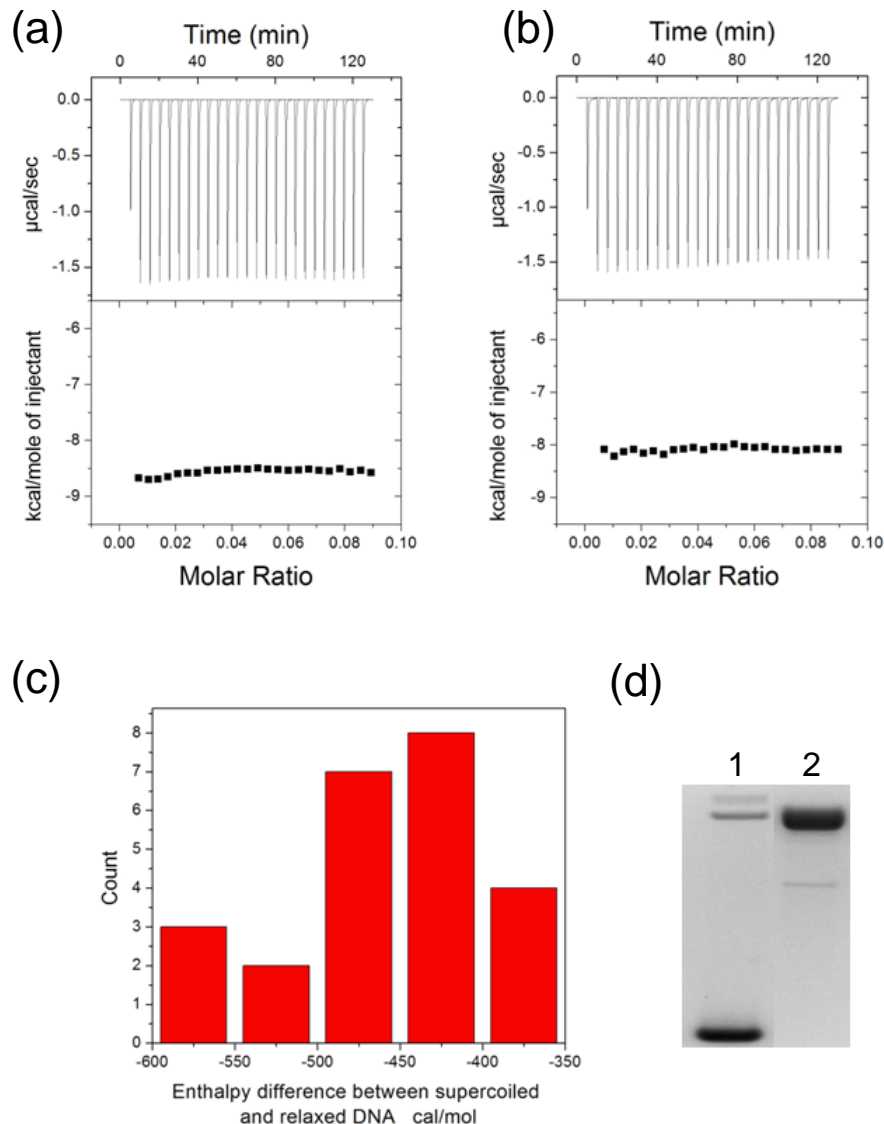


Figure 2.6 Sample raw ITC data for the titration of daunomycin into plasmid pXXZ06 (a) supercoiled and (b) relaxed DNA at 25 °C in 1× BPES buffer. Top, each peak corresponds to the heat generated by injection of an aliquot of 5 μL of daunomycin (1mM) into DNA solution (1.7 mL of 1.0 mM (bp)). Bottom, binding isotherm by integration with respect to time; (c) Enthalpy difference between supercoiled and relaxed DNA (d) 1% agarose electrophoresis analysis of DNA sample (1) pXXZ06 supercoiled

DNA; (2) pXXZ06 relaxed DNA.

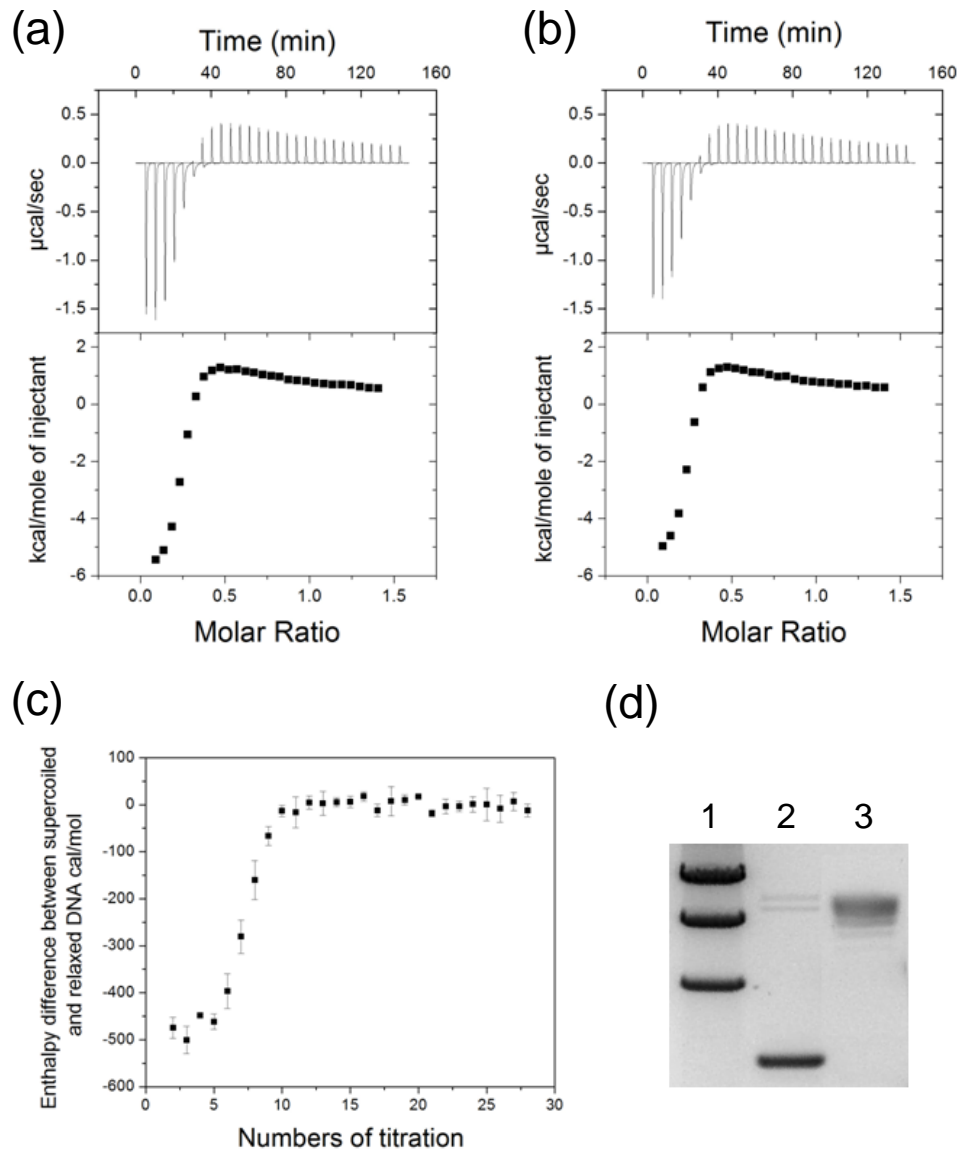


Figure 2.7 Sample raw ITC data for the titration of daunomycin into plasmid pXXZ06 (a) supercoiled and (b) relaxed DNA at 25 °C in 1× BPES buffer. Top, each peak corresponds to the heat generated by injection of an aliquot of 10 μL of daunomycin (1mM) into DNA solution (1.7 mL of 0.15mM (bp)). Bottom, binding isotherm by integration with respect to time; (c) Enthalpy difference between supercoiled and relaxed DNA (d) 1% agarose electrophoresis analysis of DNA sample (1) Marker, λ DNA Hind

III digested; (2) pXXZ06 supercoiled DNA; (3) pXXZ06 relaxed DNA.

Table 2.1 Enthalpy change for DNA intercalator (Syringe, 1.0 mM) -- plasmid DNA pXXZ06 (Cell, 1.0 mM) ITC titrations

	Ethidium Bromide			Daunomycin		
	ΔH (cal/mol)	$\Delta\Delta H$ (cal/mol)	ΔH_s (cal/mol)	ΔH (cal/mol)	$\Delta\Delta H$ (cal/mol)	ΔH_s (cal/mol)
Supercoiled DNA	7945 \pm 42	—	—	8538 \pm 85	—	—
Relaxed DNA	7049 \pm 211	896 \pm 253	12433 \pm 3510	8086 \pm 49	452 \pm 134	14793 \pm 4386
Nicked DNA	7120 \pm 52	825 \pm 94	11423 \pm 1302	8030 \pm 92	508 \pm 177	16625 \pm 5793

Table 2.2 Enthalpy change for DNA intercalator (Syringe, 1.0 mM) -- plasmid DNA pXXZ06 (Cell, 0.15 mM) ITC titrations

	Ethidium Bromide			Daunomycin		
	ΔH (cal/mol)	$\Delta\Delta H$ (cal/mol)	ΔH_s (cal/mol)	$\Delta\Delta H$ (cal/mol)	$\Delta\Delta H$ (cal/mol)	ΔH_s (cal/mol)
Supercoiled DNA	7439 \pm 51	—	—	5432 \pm 44	—	—
Relaxed DNA	6601 \pm 45	838 \pm 96	11603 \pm 1329	4954 \pm 40	478 \pm 84	15643 \pm 2749
Nicked DNA	6670 \pm 66	769 \pm 117	10648 \pm 1620	—	—	—

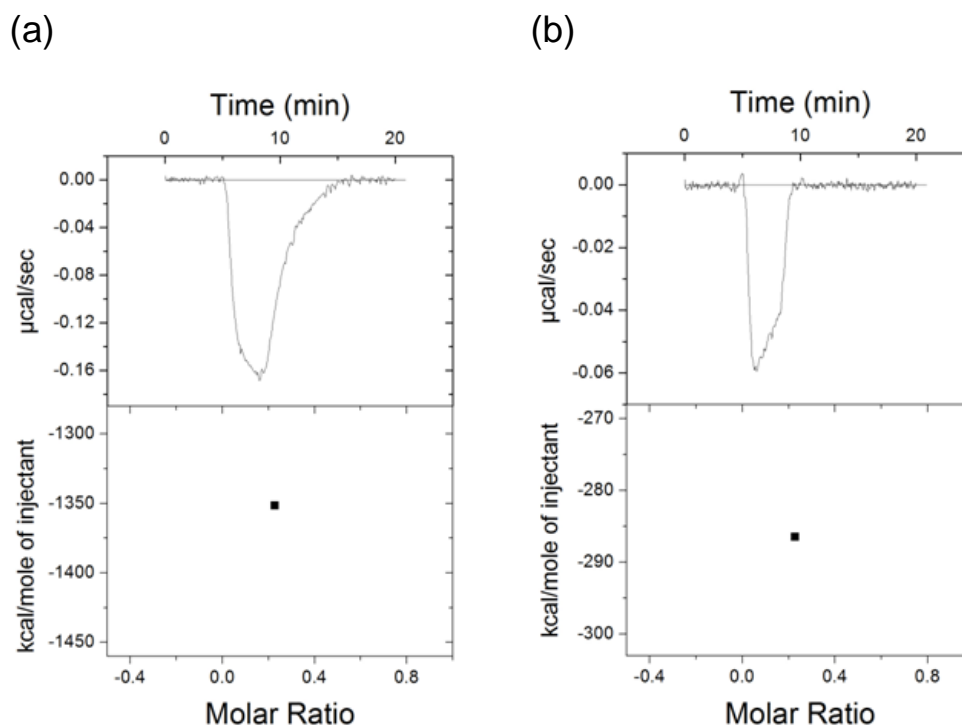


Figure 2.8 Sample raw ITC data for the titration of plasmid pXXZ06 supercoiled DNA into *E. coli* topoisomerase I at 37 °C in (a) Buffer I; (b) Buffer II. The peak corresponds to the heat generated by injection of 100 μ L of 1.5 mM (bp) DNA solution into *E. coli* topoisomerase I (1.7 mL of 0.1 μ M).

pXXZ06 was determined to be -0.06 which is equivalent to 26 superhelical turns. Using this value, the DNA supercoiling enthalpy of pXXZ06 is 39.1 kcal/mol, which is substantially greater than these values determined from the intercalator-unwinding assays. This discrepancy may result from the slow and complex properties of the relaxation reaction of *E. coli* topoisomerase I and may also come from the DNA-binding enthalpy of *E. coli* topoisomerase I binding to the negatively supercoiled DNA templates. Further studies are needed to investigate what causes the difference.

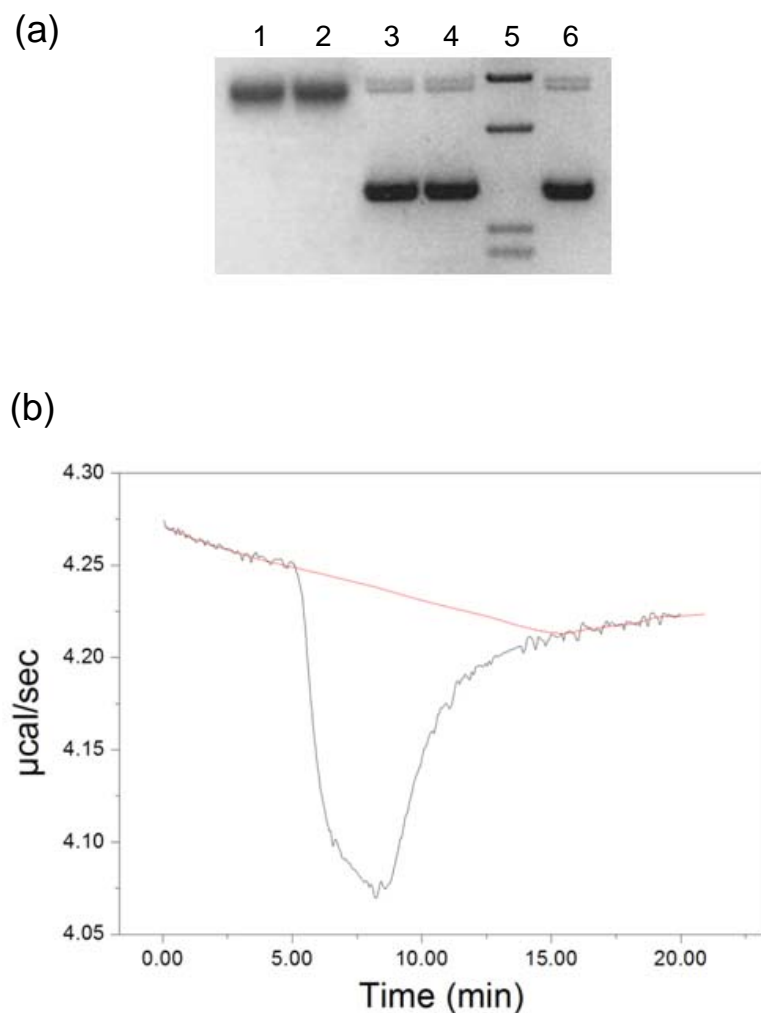


Figure 2.9 ITC result for the titration of plasmid pXXZ06 supercoiled DNA into *E. coli* topoisomerase I at 37 °C. (a) 1% agarose electrophoresis analysis of DNA samples after ITC titration in (1-2) Buffer I; (3-4) Buffer II; (5) Marker, λ DNA Hind III digested; (6) pXXZ06 supercoiled DNA before ITC titration; (b) Sample raw ITC data for the titration of plasmid pXXZ06 supercoiled DNA into *E. coli* topoisomerase I at 37 °C in Buffer I.

2.5. Conclusion:

By utilizing the isothermal titration calorimetry (ITC), the interaction of both (1) DNA intercalator (ethidium bromide and daunomycin) & plasmid DNA (negatively supercoiled/ relaxed/ nicked) pXXZ06 and (2) *E. coli* topoisomerase I & negatively supercoiled plasmid DNA were investigated. Because of the different unwinding angle can be introduced by ethidium bromide and daunomycin at 26 and 11 degrees, respectively, the enthalpy change of the formation DNA supercoiling can be deduced. The enthalpy change of the formation DNA supercoiling was estimated in a range of 10648 ± 1620 to 16625 ± 5793 cal/mol, which is consistent with the results obtained from a previous study. The ITC results from interaction between *E. coli* topoisomerase I and negatively supercoiled plasmid DNA, although influenced by other possible complicated factors, are also comparable to the result above. Further studies are needed to investigate more details about the difference between them.

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