Biochemical Characterization of an Invariant Histidine Involved in *Escherichia coli* DNA Topoisomerase I Catalysis*  

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An invariant histidine residue, His-365 in *Escherichia coli* DNA topoisomerase I, is located at the active site of type IA DNA topoisomerases and near the active site tyrosine. Its ability to participate in the multistep catalytic process of DNA relaxation was investigated. His-365 was mutated to alanine, arginine, asparagine, aspartate, glutamate, and glutamine to study its ability to participate in general acid/base catalysis and bind DNA. The mutants were examined for pH-dependent DNA relaxation and cleavage, salt-dependent DNA relaxation, and salt-dependent DNA binding affinity. The mutants relax DNA in a pH-dependent manner and at low salt concentrations. The pH dependence of all mutants is different from the wild type, suggesting that His-365 is responsible for the pH dependence of the enzyme. Additionally, whereas the wild type enzyme shows pH-dependent oligonucleotide cleavage, cleavage by both H365Q and H365A is pH-independent. H365Q cleaves DNA with rates similar to the wild type enzyme, whereas H365A has a slower rate of DNA cleavage than the wild type but can cleave more substrate overall. H365A also has a lower DNA binding affinity than the wild type enzyme. The binding affinity was determined at different salt concentrations, showing that the alanine mutant displaces half a charge less upon binding DNA than an inactive form of topoisomerase I. These observations indicate that His-365 participates in DNA binding and is responsible for optimal catalysis at physiological pH.

Type I DNA topoisomerases transiently cleave the phosphodiester backbone of one DNA strand to allow single- or double-stranded DNA to pass through the break before resealing it. This allows for the interconversion of topological isomers, which is necessary for nearly all cellular transactions of DNA such as replication, transcription, and recombination (1). Type I enzymes are further divided into two subfamilies, type IA and type IB. Type IA DNA topoisomerases form a covalent linkage to the 5'-phosphoryl end of the cleaved DNA, whereas type IB DNA topoisomerases undergo a transesterification reaction to the 3'-phosphoryl end. All topoisomerases cleave the phosphodiester bond via a phosphotyrosine intermediate. For a recent review see Ref. 2.

Creation of the transient break, strand passage, and re-ligation of DNA by type IA topoisomerases is a multistep process. A proposed mechanism (3) has the following steps. 1) The enzyme recognizes and binds a single-stranded DNA region, positioning the DNA in the active site. 2) Cleavage of the single-stranded DNA occurs at the active site, via formation of a covalent bond between the 5'-phosphoryl and the hydroxyl of the active site tyrosine, whereas the 3'-end of the DNA remains non-covalently bound to the enzyme. 3) The enzyme opens to allow passage of the other strand through the gap or gate created by separating the broken ends of the cleaved DNA. 4) Following strand passage, the enzyme closes, trapping the passing DNA inside. 5) Once the gate is closed, the enzyme re-ligates the cleaved strand. 6) The enzyme opens to release both the re-ligated strand and the one that was passed through the gap.

*Escherichia coli* DNA topoisomerase I is a member of the type IA subfamily of topoisomerases (1, 4). The 97-kDa protein is the product of the *topA* gene and is expressed as a single chain of 865 amino acids (5). The three-dimensional x-ray crystal structure of the 67-kDa N-terminal fragment of the enzyme, residues 1–595, has been solved to 2.2-Å resolution (3). It has four domains that form a toroid-shaped molecule. The remaining 30-kDa C terminus confers higher DNA binding affinity (6) and contains a tetracysteine motif that forms a putative zinc finger triplet (7, 8) and a 14-kDa C terminus that enhances DNA binding (9). The active site is located at the interface between domains I and III and is identified by the presence of the catalytic tyrosine, Tyr-319. Near the active site are many highly conserved residues as follows: Glu-9, Asp-111, Asp-113, Glu-115, Tyr-312, Glu-313, Arg-321, and His-365 (Fig. 1). The three-dimensional structure as well as mutagenesis studies suggest that many of these conserved residues are involved in the catalytic mechanism (3, 10, 11). Although Asp-111, Asp-113, and Glu-115 have been implicated in Mg(II) binding (12), in many instances the specific role of particular residues has not been definitively determined.

Alanine substitution mutagenesis has been performed on Glu-9, His-33, Asp-111, Glu-115, Gln-209, Glu-313, Thr-318, Arg-321, Thr-322, Asp-323, His-365, and Thr-496 to identify their possible roles (10). Most mutations do not completely abolish relaxation activity. The exception is Glu-9. When substituted with an alanine, it cannot relax supercoiled DNA but retains reduced relaxation ability when replaced with a glutamine (10). Nevertheless, the role that these residues may be playing in the catalytic process has not been resolved. This is partially due to the fact that the role of some residues may not be immediately apparent when they are only tested for relaxation activity or cleavage, and hence it is important to elucidate their involvement in greater detail. For example, several possible roles have been suggested for the invariant histidine residue at position 365 (His-365). It could either act as a general base in acid/base catalysis (13), participate in binding to...
The structure residues at the active site of histidine 365 and other conserved away from the 5 Å to them. The invariant histidine is 5.8 Å to show the location of His-365 in relation to them. The invariant histidine is 5.8 Å away from the 5'-TMP. Direct interaction cannot occur at this distance. This figure was created with MOLSCRIPT (34) and RASTER3D (35).

To determine whether His-365 (a) acts as a general base to abstract the hydroxyl proton from the active site tyrosine, Tyr-319; (b) protonates the 3'-OH of the leaving DNA strand as a general acid; (c) donates a proton to Glu-9 as part of a charge-relay network; or (d) is involved in DNA binding, the residue was mutated to alanine, arginine, glutamine, aspartate, glutamate, and asparagine, and was also tested for its ability to form a hydrogen-bonding network that may allow it to donate a proton to Asp-111 that could then relay the charge to Glu-9 (15) (Fig. 2). Mutagenesis studies have shown that changing the histidine to alanine does not abolish overall enzymatic activity (10), but the mutation results in a reduced level of relaxation activity. This makes residue His-365 an excellent candidate for an in-depth study.

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Materials and Methods
Creation of DNA Topoisomerase I with a Polyhistidine Tag—The topA gene was amplified from plasmid pJW312 (17) via PCR and inserted into the pET15b plasmid from Novagen. A unique NdeI restriction site was introduced with the primer 5'-GGGACCATGAGGATCCTTTGTC-3' and a unique XhoI restriction site was introduced with the primer 5'-CCGCTCGAGTCATTTTTTTCCTTC-3'. The amplified product was inserted in-frame into the multiple cloning site of pET15b between the NdeI site and the XhoI site to create plasmid pHis97. This plasmid encodes the full-length wild-type topoisomerase I protein with the addition of a 6-residue histidine tag and a linker region containing a thrombin cleavage site at the N terminus of the native protein. The accuracy of the cloning was confirmed by DNA sequencing.

Mutagenesis—The Unique Site Elimination kit from Amersham Biosciences was used for the construction of the 67-kDa N-terminal mutants from plasmid pT67, which contains the 67-kDa N-terminal fragment of E. coli DNA topoisomerase I fused to glutathione S-transferase (GST) (18). These mutants were used for structure determination and for the binding assays. Plasmid pHis97 was used as a template for the construction of mutants in the relaxation and cleavage assays. Full-length mutants of the histidine-tagged protein were generated using the QuikChange site-directed mutagenesis kit from Stratagene. Each mutant contained a unique restriction site at the location of interest. Mutant identity was verified via DNA sequencing of the mutation site.

Protein Purification of GST Fusion Proteins—Ten liters of E. coli DH5α cells containing the mutant plasmid were grown at 34 °C in a BioFlo IV fermentor (New Brunswick Scientific Co.). Expression of the protein was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.3 mM. Cells were lysed by a modified
Bribyllyzyme procedure (19) by incubation in 20% sucrose, 50 mM Tris, 25 mM EDTA, 100 mM β-mercaptoethanol, 2.5 μg/ml leupeptin, 2.5 μg/ml pepstatin A, 0.625 mM phenylmethylsulfonyl fluoride, and 0.25 mg/ml lysozyme for 15 min. followed by addition of KCl and Brij-58 to a final concentration of 120 mM and 0.25%, respectively. The protein was dialyzed against an ionic strength of 0.5 and against cell lysate buffer by ultracentrifugation at 113,613 g (40,000 rpm in a Beckman 60 Ti fixed angle rotor). Additional DNA was removed by poly~P precipitation (1% w/v final concentration). The fusion protein was purified using a GST-Sepharose (Amersham Biosciences) affinity column and Sephacryl S-100 (Amersham Biosciences) column, and then the fusion protein was cleaved to remove the GST tag using 0.9 NIH units of thrombin/mg of protein in 150 mM NaCl, 16 mM NaHPO4, 4 mM NaH2PO4 (pH 7.3) for 15 h at room temperature. The cleavage reaction was stopped by addition of phenylmethylsulfonyl fluoride to a final concentration of 1 mM. The cleaved protein was purified away from the non-cleaved protein and free GST by passing the protein through a second GST-Sepharose affinity column and applying the flow-through fraction through a Sephacryl S-100 column to remove the residual thrombin.

**Protein Purification of Polystyline-tagged Protein**—E. coli BL21 cells containing the mutant plasmid of interest were grown at 37°C. Protein expression was initiated by infection of the cells with bacteriophage λ C66 (20). Cells were harvested, frozen in liquid nitrogen, and stored at −70°C. Cells were lysed in the same manner as the GST fusion proteins without including EDTA or the polymin-P precipitation step. Protein was bound to a nickel-nitrilotriacetic acid-agarose column and flushed with 20 mM Tris, 500 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4 (pH 8.0 at 22°C) with an iodinated uracil, 40 mM Tris-HCl (pH 8.0 at 22°C) was separated from membranes and cellular DNA by ultracentrifugation.

**Relaxation Activity Assay**—Relaxation mixtures for salt dependence studies contained 400 ng of supercoiled pBR322, 40 mM Tris-HCl (pH 8.0 at 22°C), 5 mM MgCl2, the amount of KCl indicated, and the indicated amount of protein in a total volume of 20 μL. Relaxation mixtures for the pH profiles contained 400 ng of supercoiled pBR322, 40 mM buffer (MES between pH 5.5 and 6.5, Tris-HCl between pH 7.0 and 8.0 at 22°C) using microfiterfuge tubes from Rainin Instrument Co., Inc. The oligonucleotides were purified by phenol/chloroform/isomyl alcohol (25:24:1) extraction and subsequently precipitated with ethanol. The purified product was stored in 1 mM Tris-HCl (pH 8.0 at 22°C), 0.1 mM EDTA.

**Relaxation of Negatively Supercoiled DNA Requires Magnesium**—As magnesium begins to precipitate out of solution at pH 10, it is impossible to determine accurately relaxation ability at or above pH 10. Although there is no detectable relaxation activity by the wild type protein at pH 10.5 and minimal relaxation ability at pH 10 (data not shown), it is unclear if this lack of activity is pH-dependent or due to loss of magnesium below the threshold needed for relaxation to occur. The pH dependence of E. coli topoisomerase I in relaxing negatively supercoiled DNA suggests that the enzyme undergoes general acid/base catalysis and that either a histidine or a cysteine could be involved. The active sites of E. coli topoisomerase I and topoisomerase III do not have a cysteine (3, 13); the only conserved histidine is located at position 365.

His-365 could participate in general acid/base catalysis as follows: 1) as the acid that donates a proton to the 3’-OH of the leaving DNA strand; 2) as the base that abstracts a proton from the active site tyrosine; or 3) by donating a proton to the putative general acid, Glu-9, via a charge-relay network. To test the ability of the residue to act as the general base, it was mutated to an alanine and an arginine. Polar and acidic substitutions were made to test the ability of the residue to act as a general acid. If His-365 acts as a general acid, then deprotonation of the imidazole ring will increase the activity of the wild type enzyme but a similar change in pH should not affect a mutant enzyme with a polar residue in that location. In addition, should the side-chain act as a general acid, then changing the histidine to an acidic residue will shift the maximal activity to a lower pH.

All the mutants show a shift in maximal relaxation to above pH 8.5 with sharper, more attenuated bell-shaped curves (Fig. 3). Only minimal relaxation activity is exhibited by the mutants below pH 7. The polar and acidic substitutions shifted optimal DNA relaxation to the basic region slightly more than the alanine and arginine mutations with maximal relaxation at pH 9.0–9.5. No increased activity at lower pH was observed for any mutant. Attempts to regain wild type-like pH-dependent activity with H365A by adding exogenous imidazole were unsuccessful (data not shown). Addition of up to 1 mM imidazole results in a decrease in H365A enzymatic activity. In the presence of 100 mM imidazole, the H365A mutant has the same pH profile as in the absence of imidazole (data not shown).

**RESULTS**

*E. coli DNA Topoisomerase I Mutagenesis*—Site-directed mutagenesis was used to change His-365 to alanine, arginine, glutamine, asparagine, glutamate, and aspartate. Expression of these mutants in the full-length version of *E. coli* DNA topoisomerase I was not possible as a GST fusion. DNA sequencing data showed that the plasmid contained the correct sequence. However, induction resulted in a degraded form of the protein (data not shown). Instead, these mutants were made with the polyhistidine-tagged construct. All enzymes were purified to homogeneity (data not shown).

**pH Dependence of Supercoiled DNA Relaxation by Wild Type and Mutant Enzymes**—The histidine-tagged and wild type proteins relax supercoiled DNA to the same extent, indicating that N-terminal histidines do not affect activity (data not shown). The wild type enzyme shows maximal relaxation at pH 8 and a broad, bell-shaped pH-dependent curve. Activity ceases to be detectable at pH 5.5 (Fig. 3) and above pH 10 (data not shown). Relaxation of negatively supercoiled DNA requires magnesium (23, 24). As magnesium begins to precipitate out of solution at pH 10, it is impossible to determine accurately relaxation ability at or above pH 10. Although there is no detectable relaxation activity by the wild type protein at pH 10.5 and minimal relaxation ability at pH 10 (data not shown), it is unclear if this lack of activity is pH-dependent or due to loss of magnesium below the threshold needed for relaxation to occur. The pH dependence of *E. coli* topoisomerase I in relaxing negatively supercoiled DNA suggests that the enzyme undergoes general acid/base catalysis and that either a histidine or a cysteine could be involved. The active sites of *E. coli* topoisomerase I and topoisomerase III do not have a cysteine (3, 13); the only conserved histidine is located at position 365.

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Salt Dependence of Supercoiled DNA Relaxation by Wild Type and Mutant Enzymes—Originally, relaxation assays were performed with 120 mM KCl, but His-365 mutants do not fully relax supercoiled DNA at this salt concentration (Fig. 4). The assays show that increasing concentrations of salt inhibit relaxation by the wild type and mutant enzymes. The wild type enzyme can relax negatively supercoiled DNA in the presence of 0–140 mM KCl. The His-365 mutants have maximal enzymatic activity in the absence of salt, have minimal relaxation activity between 60 mM KCl and 120 mM KCl, and lose the ability to relax supercoiled DNA above 120 mM KCl. Wild type E. coli DNA topoisomerase I has full enzymatic activity over a greater range of salt concentrations than the His-365 mutants (Fig. 4).

Successive 5-fold dilutions of wild type and His-365 mutant enzymes were performed to investigate the degree to which the wild type enzyme outperforms mutant enzymes as salt concentration increases. Serial dilutions show that the mutants are ~125-fold less able to relax supercoiled DNA at 60 mM KCl, whereas in the absence of salt, the wild type is only ~25-fold more active. In all cases, the His-365 mutants are less able than the wild type to relax negatively supercoiled DNA as salt concentration increases (Fig. 4).

Mutations Affect Cleaveage of Single-stranded DNA Oligonucleotides—The ability of the H365A and H365Q mutants to cleave single-stranded DNA oligonucleotides was examined with an 8-base oligomer radioactively labeled at the 5’ end. Polydeoxyadenosine with an iodo-uracil as the first base (diUA₃) has been shown previously (16) to have a relatively fast cleavage rate, whereas the iodo-uracil maintains cleavage at the same position in the oligonucleotide. Given that definitive studies of E. coli topoisomerase I (TopoI) cleavage have not been done with this assay to determine the kinetics of the reaction, an attempt was made to assess whether the reaction occurred under single turnover conditions, similar to a Scheme presented by Stivers et al. (25) for vaccinia topoisomerase I.

\[
\text{diUA}_3 + \text{TopoI} \rightleftharpoons \text{diUA}_3 \cdot \text{TopoI} \rightarrow \text{diUA}_3 + A_4 + \text{TopoI}
\]

**SCHEME 1**

In Scheme 1, cleavage is irreversible and occurs under conditions of enzyme excess over DNA. The length of DNA substrate used prevents re-ligation from occurring as the cleaved strand that is non-covalently bound to the enzyme would diffuse away (26). Therefore, in this scheme, the observed rate could in principle either reflect the DNA binding step or the cleavage step in the reaction. Observed rates that are independent of enzyme concentration or reach a maximal rate likely reflect first-order catalytic steps in the mechanism, whereas the binding step should be dependent on enzyme concentration.

To maintain limiting amounts of oligonucleotide, DNA:protein ratios of 1:1, 1:2, 1:4, 1:5, and 1:8 were examined at pH 8. DNA concentration was maintained at 1 μM, whereas enzyme concentration was increased to 1, 2, 3.6, 5.1, and 7.7 μM. Neither the wild type nor H365Q enzymes appear to have concentration dependence regarding the maximal extent of DNA cleavage, suggesting that the assay reports both DNA binding and cleavage inseparably. However, H365A is heavily concentration-dependent up to 500 ng/μl (5.1 μM) with the rate decreasing with increasing enzyme concentration (Fig. 5). This suggests that H365A may undergo a concentration-dependent inactivation that could be due, for example, to enzyme aggregation at high concentration. However, at 500 ng/μl (the enzyme concentration used to determine pH dependence) H365A has reached its maximal reaction rate.

Nevertheless, the rate and extent of the reaction can be observed (Fig. 6). At any pH value, the overall extent of cleavage is greater by the alanine mutant than by the wild type or the glutamine mutant. The wild type and glutamine mutant do not cleave at pH 6, whereas the alanine mutant has minimal activity. After 2 h, it appears that both the wild type and
glutamine mutant have reached their maximal extent of cleavage at pH 7 and 8. In contrast, H365A does not. Given the standard deviation between three experiments, it would appear that the wild type enzyme has a low extent of cleavage at pH 7–9 but reaches its maximal rate more rapidly than either of the mutants (Fig. 6). A better way to compare the cleavage activities is to examine the rate of the reaction. Assuming that the reaction is first-order and fitting the data to a single exponential (Equation 2),

\[
\%\text{Cl} = \%\text{Cl}_0 \cdot (1 - e^{-kt})
\]

where \(\%\text{Cl}\) is the fraction cleaved, \(\%\text{Cl}_0\) is the maximal fraction cleaved, \(k\) is the observed rate, and \(t\) is time, enzymatic rates can be compared at different pH values. It can be seen that the wild type is more active at pH 7 and 8, and it follows the same dependence as for relaxation. The cleavage activity of H365A and H365Q lacks pH dependence (Fig. 7).

**Effect of Mutations on Binding Affinity**—A mechanism of action has been proposed in which the closed enzyme must open to allow binding of single-stranded DNA to a groove leading to the active site. After binding, the enzyme can cleave the single-stranded DNA. As binding of single-stranded DNA must occur before cleavage, measuring the binding affinity of wild type and mutant enzymes allows for a clearer picture of the mechanism of action and the role of various active site residues in binding and cleavage.

To determine whether the greater sensitivity to salt and loss of relaxation ability in the His-365 mutants was a result of a loss or gain of DNA binding ability, fluorescence polarization measurements were used to look at DNA binding affinity. This technique has been used previously to look at binding constants of 22- and 44-base oligonucleotides bound to full-length wild type *E. coli* DNA topoisomerase I under steady-state conditions (27). The 30-kDa C terminus of *E. coli* DNA topoisomerase I is involved in DNA binding (8), but by looking at only the 67-kDa N-terminal fragment of the protein the possible contribution toward binding affinity by the putative zinc finger regions or DNA binding by the 14-kDa C terminus is eliminated. This allows for assessment of the DNA binding affinity only by the main body of the protein (the DNA binding region associated with cleavage and re-ligation) and is a useful assay in quantitatively determining binding affinity at only the active site.

One concern when measuring binding affinity is the rate of oligonucleotide cleavage; it depends on the length and sequence of the substrate (28, 29), so while an incubation period of 3 min allows for binding, minimal amounts of product would be expected to be formed even when the reaction is saturated with enzyme. It is also possible that whereas cleavage may occur, the
product does not dissociate from the enzyme. Product formation was not observed with the wild type enzyme under these conditions in the first 5 min or in low salt concentrations (data not shown). In addition, an inactive Y319F mutant has the same binding affinity (223 ± 42 nM) with an 8-base oligonucleotide as the wild type protein under these conditions (Table I), suggesting that oligonucleotide binding can be recorded accurately under these experimental conditions. Oligonucleotide binding by type I topoisomerases requires a minimum of 7 or 8 bases (28, 29).

Given the size of the oligonucleotide used in the experiment, it is unlikely that more than one enzyme binds to a single substrate. Therefore, 1:1 stoichiometry can be assumed. By using an 8-base oligonucleotide containing two cleavage sites for *E. coli* DNA topoisomerase I, at low salt concentration (5 mM KCl), the wild type enzyme has a binding constant of 201 ± 32 nM; H365A has a binding constant of 244 ± 23 nM, and H365R has a binding constant of 96 ± 11 nM (Fig. 8).

As salt concentration increases, the binding affinity of topoisomerase I decreases (Table I). This is to be expected as the

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**Fig. 5.** Dependence of single-stranded DNA oligonucleotide cleavage on enzyme concentration by wild type, H365A, and H365Q mutant *E. coli* DNA topoisomerase I. A, wild type; B, H365A; and C, H365Q. 100 (■), 200 (●), 350 (▲), 500 (△), and 750 ng/μl enzyme (†) concentrations corresponding to DNA:protein ratios of approximately 1:1, 1:2, 1:4, 1:5, and 1:8, respectively, were tested at pH 8 and 5 mM NaCl. Results are from the average of three separate experiments. The fraction of oligonucleotide cleaved is normalized to percent of total counts. Neither the wild type enzyme nor H365Q exhibit concentration-dependent activity under these conditions. As enzyme concentration increases, H365A is less able to cleave single-stranded DNA. Note that the scale of the y axis for the wild type and H365Q enzymes is much smaller than for H365A. The overall extent of cleavage for H365A is much greater.

**Fig. 6.** Cleavage of single-stranded DNA oligonucleotides at varying pH values by wild type, H365A, and H365Q mutant *E. coli* DNA topoisomerase I. A, wild type; B, H365A; and C, H365Q. A comparison of the ability of each enzyme to cleave an 8-base oligonucleotide as pH increases is shown. Results are from the average of three separate experiments. Fraction of oligonucleotide cleaved is normalized to percent of total counts. H365A cleaves a greater percentage of the total DNA than the wild type or H365Q enzymes.
Fig. 7. Comparison of the single-stranded DNA oligonucleotide cleavage rate versus pH values by wild type (■), H365A (▲), and H365Q (○) mutant E. coli DNA topoisomerase I. The rate of oligonucleotide cleavage by H365A and H365Q is not dependent upon pH in contrast to the wild type enzyme. The rate (fraction of oligonucleotide cleaved per min) is plotted as a function of pH.

Table I: Binding affinities of 67-kDa N-terminal mutants acquired by fluorescence polarization

| KCl (mM) | Y319F | H365A | H365R |
|----------|--------|-------|-------|
| 5        | 223 ± 42 | 244 ± 24 | 96 ± 11 |
| 20       | 237 ± 61 | 419 ± 84 | 203 ± 24 |
| 40       | 1480 ± 432 | 1087 ± 248 | 592 ± 211 |
| 60       | 3121 ± 2741 | >7000 | 858 ± 187 |
| 80       | 3368 ± 1868 | ND* | ND |

* ND, not determined.

Fig. 8. Equilibrium binding isotherms for an 8-base oligonucleotide to wild type (■), H365A (▲), and 365R (○) mutant E. coli DNA topoisomerase I monitored by fluorescence polarization. In the presence of 5 nm KCl, 2 nm DNA was titrated with increasing concentrations of enzyme and incubated for 3 min to reach equilibrium. Wild type $K_d = 201 ± 32$ nm, H365A $K_d = 244 ± 24$ nm, and H365R $K_d = 96 ± 11$ nm.

Counterion condensation will compete with the protein for binding to the phosphodiester backbone of the DNA (30). Counterion condensation theory allows for the determination of the number of charges in the ionic atmosphere around the single-stranded DNA that will be displaced upon binding of the enzyme (31). The salt dependence is related to the binding affinity by Equation 3,

$$ \Delta \log K = \log c = N\psi $$

where $K$ is the binding constant; $N$ is the number of anions displaced by the enzyme that is considered an $N$-valent cation; $c$ is the concentration of the monovalent salt; and $\psi$ is the fraction of a counterion thermodynamically associated per phosphate in the absence of the enzyme. The logarithm of the binding constant has a linear relationship to the logarithm of the salt concentration. Thus, the slope equals $N\psi$. For infinitely long single-stranded DNA in aqueous solution containing only monovalent cations, $\psi = 0.71$ (32), and from calculating the slopes obtained by plotting the log of the binding affinity versus the log of the salt concentration (Fig. 9), it can be determined that T67-Y319F displaces ~1.54 charges; T67-H365A displaces ~0.89 charges, and T67-H365R displaces ~1.25 charges. In this instance, it has been assumed that binding of the divalent cation displaces 2 univalent ions (33), and the 8-base oligonucleotide used to determine binding affinity is not infinitely long. Therefore, the number of counterions released can only be approximated.

**DISCUSSION**

Relaxation of negatively supercoiled DNA by type IA DNA topoisomerases is a multistep catalytic process. Elucidation of the roles of different residues during various steps is necessary for a full understanding of the reaction. Although substitution of the invariant histidine at position 365 does not abolish activity, changing the residue shifts the pH dependence of negative supercoiled DNA relaxation to basic pH, affects the rate and pH dependence of single-stranded DNA cleavage, and changes the DNA binding affinity of E. coli topoisomerase I.

The three-dimensional crystal structure of the 67-kDa N-terminal fragment of E. coli topoisomerase I complexed with nucleotides suggests that the histidine may be involved in DNA...
binding (14). Changing the distance between the side-chain of this residue and the DNA phosphate backbone or the charge of the residue may affect DNA binding and may result in partial or complete loss of function. Although it would be expected that the substitution of an alanine for the histidine would diminish DNA binding ability, substitution of a positively charged residue, such as arginine or lysine, may enhance it, and acidic residues may reduce it. Alternatively, if the only role of His-365 is to maintain the orientation of the regional side-chains via a hydrogen-bonding network, then polar mutations should not greatly affect the ability of the enzyme to bind DNA.

One way to examine the ability of a protein to bind DNA is to examine the dependence of the activity on salt concentration, as positively charged ions can mask the negatively charged phosphate backbone. Above 140 mM KCl, supercoil relaxation ability is fully inhibited in the wild type enzyme, whereas the mutants are inactive above 60 mM KCl. This is consistent with previously published results on H365A where relaxation assays were performed under low salt conditions (10). The His-365 mutants are ~125-fold less active than negative supercoils in the presence of 60 mM KCl but only ~25-fold less active to relax negative supercoils in the presence of little or no salt, suggesting that the histidine plays a role in DNA binding.

FP shows that the alanine mutant displaces half a charge less than the phenylalanine mutant (Y319F) from the ionic atmosphere surrounding the DNA. Both the Y319F and H365R both displace over a full charge. This suggests that changing the net charge at the location of the conserved histidine affects the valency of the interaction between protein and DNA. This supports the hypothesis that His-365 is involved in DNA binding at the active site. Also consistent with this hypothesis are binding affinities obtained from FP experiments that show that the histidine to alanine mutation results in a lower binding affinity, whereas substitution with arginine results in a higher binding affinity for single-stranded oligonucleotides. It is likely that His-365 does not bind DNA directly but rather helps other residues perform this task. The presently available data do not definitively determine whether the interaction between the histidine and DNA is direct or indirect in E. coli DNA topoisomerase I. However, the recently published structure of E. coli DNA topoisomerase III complexed with single-stranded DNA shows that the analogous histidine creates a pocket of positive charge for the phosphate that is proximal to the scissile bond and is involved in a hydrogen-bonding network (15). Although the histidine is still too far away to contact the phosphate directly, the histidine could influence the local environment, making binding more likely.

At similar salt concentrations, supercoiled DNA relaxation is affected in the same manner by the alanine and arginine substitutions, yet the alanine mutant has a binding affinity approximately 2-fold lower than the wild type, and the arginine mutant has a binding affinity 2-fold higher than the wild type. Binding too tightly probably results in a reduced level of activity due to the inability of the enzyme to release DNA. Binding too loosely may result in a reduced level of activity because the DNA is less able to attach initially. The results suggest that the histidine is very important in maintaining the local charge environment or correctly positioning the side-chains of other residues and enhancing DNA binding.

The structure of E. coli topoisomerase III complexed with single-stranded DNA (15), and the results of the biochemical assays involving the His-365 mutant enzymes suggest that the invariant histidine could be part of a charge-relay network that can protonate a putative general acid, Glu-9. In the structure of the topoisomerase III-DNA complex, His-381 (the His-365 equivalent) is part of a hydrogen-bonding network involving Glu-7, Lys-8, and Asp-103 (Glu-9, Lys-13, and Asp-111 in E. coli topoisomerase I) (Fig. 2) (15). The structure supports a role for Glu-9 as a general acid that donates a proton to the '3'-oxygen of the scissile bond during the cleavage and abstracts a proton from the '3'-hydroxyl during religation. This mechanism requires the protonation of Glu-9 at physiological pH. One possibility is that Glu-9 could be protonated by His-365 via a charge-relay mechanism. First, His-365 could donate a proton to Asp-111; second, the aspartate relays the charge to Glu-9; and finally, the protonated glutamate can proceed to act on the single-stranded DNA bound at the active site (Fig. 2). During re-ligation, the proton would move in the reverse direction, traveling via Asp-111 back to His-365.

Wild type protein relaxes supercoiled DNA and cleaves oligonucleotides optimally at pH 8 and loses activity below pH 5.5. This ability to relax supercoiled DNA is lost gradually, producing a broad bell-shaped curve. The pH dependence suggests that type IA topoisomerases may use general acid/base catalysis to cleave DNA, and a histidine or a cysteine could act as a general acid or base. The lack of a cysteine at the active site points to histidine as the likely candidate. Furthermore, the shift in optimal relaxation activity from neutral to basic pH upon removal of the invariant histidine indicates that His-365 is necessary for activity at physiological pH, although this may be a result of the electrostatic effect of His-365 on nearby residues, for example, lowering the pH of Lys-13, raising the pH of Glu-111, or lowering the pH of Tyr-319.

To investigate whether the invariant histidine could act as proton acceptor or donor, His-365 was also mutated to alanine, arginine, asparagine, glutamine, aspartate, and glutamate. The arginine mutation not only adds a positive charge but also increases bulk, and it may disturb any hydrogen bonds made by the histidine. The polar nature of asparagine and glutamine maintains the hydrogen bonding abilities of the N51 and N22 atoms present in the histidine but prevents action of the residue as either a general acid or base. If His-365 acts as a proton acceptor or donor, then the alanine and polar substitutions might result in an enzyme that is insensitive to pH changes. In addition, the substitution of an acidic or basic residue at this site may result in shifting the optimal relaxation condition toward the pKₐ of the new side-chain. However, polar, basic, and acidic substitutions at this site result in a shift of the optimal pH to ~9, inconsistent with a direct role in acid/base catalysis. One possible explanation for this shift is that it may reflect the titration of a different group at the active site, such as Tyr-319 or Lys-13.

If His-365 acts as part of a charge-relay network to protonate Glu-9, then changing the residue to alanine or a polar residue would prevent protonation, and the resulting mutants would be expected to have reduced activity and lack pH dependence in this scenario. The rise in optimal pH for the relaxation reaction may reflect not only the loss of modulating activity of the histidine but also the possible action of a different group acting as the general acid in place of Glu-9. Another possibility is that the shift in optimal pH reflects the protonation of Tyr-319. If His-365 solely participates in hydrogen bonding and does not participate in a charge-relay mechanism, then the pH dependence should be unaffected by the presence of a polar residue at this site. In addition, the polar mutations should not exhibit increased sensitivity to increasing salt concentrations. This was not observed; the polar mutants exhibit a shift in relaxation optimal pH and an increased sensitivity to salt. Although the acidic substitutions might allow for a charge relay mecha-
nism, this would occur at a much lower pH. A shift to a lower optimal pH was not seen in the conditions tested. In fact, the pH profile of the acidic substitutions is similar to that of the other mutants.

A comparison of the observed rates of oligonucleotide cleavage indicates that the glutamine and alanine mutants lack pH dependence. The greater extent of oligonucleotide cleavage at pH 9 but at a slower observed rate suggests that the activity may be the result of protonation of a basic residue. The alanine mutant cleaves a greater fraction of the substrate present, yet it is less able to bind DNA than the wild type, suggesting that changing the invariant histidine to an alanine changes the end point of the reaction and the final equilibrium state between cleaved and uncleaved DNA. For example, H365A may lose the cleaved DNA after the enzyme opens. This would allow the alanine mutant to cleave more DNA in the assay as the non-cova-}

lently bound strand may leave the active site more readily. Conversely, the ability of the glutamine mutant to cleave more DNA in the assay as the non-cova-

lently bound strand may leave the active site more readily. Conversely, the ability of the glutamine mutant to participate in regional hydrogen bonding may help bind the phosphate backbone and prevent DNA loss. Although the alanine mutant is able to cleave more substrate, the rates of cleavage by the wild type and glutamine mutant enzymes are still greater, suggesting that the ability to bind DNA accelerates the approach to equilibrium and inhibits rapid substrate turnover by preventing loss of the substrate, a possibly lethal state in vivo. The lower DNA binding affinity of the alanine mutant also supports this possibility. It has been suggested that when oligonucleotides are cleaved, the final nucleophile is water, whereas in the case of relaxation, the final nucleophile is the 3'-OH group of the DNA backbone which is 5' of the scissile bond (26). This may also account for the possibility of H365A losing DNA more readily in the cleavage reaction.

The ability to substitute a number of different residues at position 365 without complete loss of activity suggests that an alternate mechanism of cleavage and re-ligation to the one proposed here exists. His-365 is important because changing this residue results in a less efficient enzyme, but the system appears to contain redundancy. For example, it has been reported that changing Glu-9 to a glutamine results in an enzyme with normal levels of DNA cleavage and rejoining ability but reduced relaxation activity (10). An explanation for these observations is that, as when His-365 has been replaced, Lys-13 may act as the general acid. The hydrogen-bonding network, important for orienting the substrate, is maintained, but a glutamine cannot donate or receive a proton. Another residue may substitute for Glu-9, the general acid, as long as the structure and charge environment of the region is maintained. This would also be consistent with the mutant cleavage activity at higher pH.

A different pH profile, decreased ability to relax supercoiled DNA, increased sensitivity to salt, and the change in binding affinity by the mutants suggests that the invariant histidine at position 365 has a dual role in E. coli topoisomerase I enzymatic activity. His-365 not only affects DNA binding but also is responsible for the optimal pH for relaxation activity. His-365 does not play the role of a general catalytic acid or base, but its loss may affect the ability of the putative general acid, Glu-9, from acting on single-stranded DNA. Clearly, a redundant mechanism of action exists, but His-365 is absolutely conserved because it allows the enzyme to be optimally active under physiological conditions and maintains a balance between substrate and product by modulating binding affinity and cleavage ability.

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