Inhibition of Mg$^{2+}$ binding and DNA religation by bacterial topoisomerase I via introduction of an additional positive charge into the active site region

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Received May 5, 2008; Revised June 26, 2008; Accepted July 2, 2008

ABSTRACT
Among bacterial topoisomerase I enzymes, a conserved methionine residue is found at the active site next to the nucleophilic tyrosine. Substitution of this methionine residue with arginine in recombinant Yersinia pestis topoisomerase I (YTOP) was the only substitution at this position found to induce the SOS response in Escherichia coli. Overexpression of the M326R mutant YTOP resulted in ~4 log loss of viability. Biochemical analysis of purified Y. pestis and E. coli mutant topoisomerase I showed that the Met to Arg substitution affected the DNA religation step of the catalytic cycle. The introduction of an additional positive charge into the active site region of the mutant E. coli topoisomerase I activity shifted the pH for optimal activity and decreased the Mg$^{2+}$ binding affinity. This study demonstrated that a substitution outside the TOPRIM motif, which binds Mg$^{2+}$ directly, can nonetheless inhibit Mg$^{2+}$ binding and DNA religation by the enzyme, increasing the accumulation of covalent cleavage complex, with bactericidal consequence. Small molecules that can inhibit Mg$^{2+}$ dependent religation by bacterial topoisomerase I specifically could be developed into useful new antibacterial compounds. This approach would be similar to the inhibition of divalent ion dependent strand transfer by HIV integrase in antiviral therapy.

INTRODUCTION
DNA topoisomerases are ubiquitous enzymes that affect many vital cellular processes by controlling the level of DNA supercoiling and removing the tangling of DNA strands via the concerted cleavage and religation of DNA strands (1–4). Besides playing important roles in removing topological barriers from DNA, these enzymes are also highly relevant targets for anticancer and antibacterial therapy, because compounds that act as topoisomerase poisons can increase the accumulation of the covalent intermediates formed between topoisomerases and cleaved DNA, leading to cell death (5–8). The ability to combat bacterial infection has been seriously challenged by the rapid development of bacterial pathogens that are resistant to all commonly used antibiotics, including fluoroquinolones that target type IIA topoisomerases (8–11).

Type IA topoisomerases represent a promising new target for development of a novel class of antibiotics (12,13). All DNA topoisomerases utilize the hydroxyl group of an active site tyrosine side chain in the nucleophilic attack of the DNA phosphodiester backbone to form the covalent intermediate with the cleaved DNA (4). Type IA and type IIA topoisomerases utilize the TOPRIM DXDXXG motif found in many examples of nucleotidyl transferases (14) to coordinate two Mg$^{2+}$ ions in the active site for catalytic activity. For type IA topoisomerases, Mg$^{2+}$ addition is not required for DNA cleavage, but Mg$^{2+}$ addition is required for the DNA religation step (15,16). Mutation of the conserved Gly residue in the bacterial topoisomerase I TOPRIM motif to Ser has been shown to inhibit DNA religation and was identified by induction of the dinD1::lacZ reporter upon expression of recombinant Y. pestis topoisomerase I with this mutation (17). Following DNA damage in E. coli, RecA protein activated by filament formation with single-stranded DNA interacts with the LexA repressor and stimulates LexA autoproteolysis, resulting in induction of the SOS regulon, including the dinD1 gene, normally repressed by LexA binding to its operator sequence.
The rapid loss of *E. coli* viability following induction of expression of the recombinant Gly to Ser mutant topoisomerase I proves that accumulation of cleavage complex from type IA topoisomerases is bactericidal and validates bacterial topoisomerase I as a target for identification of novel antibacterial compounds (12). A second mutation in bacterial topoisomerase I, with the Met residue that immediately follows the active site tyrosine changed to Val, has been previously found to have no SOS induction or bactericidal effect on its own, but can enhance the cell killing by the TOPRIM Gly to Ser mutation due to the increase in DNA cleavage (18). In this study, all possible substitutions at this position that directly follows the active site tyrosine were examined in vivo using recombinant *Y. pestis* topoisomerase I (YTOP) to determine if any of the mutant YTOP proteins could induce the SOS response because of possible effect of the mutation on DNA religation. The M326R substitution was the only one found to be able to induce the SOS response from the *dinD1* promoter (19). Detailed biochemical analysis of the purified recombinant *Y. pestis* topoisomerase I mutant YTOP-M326R and the corresponding *E. coli* topoisomerase I mutant ETOP-M320R protein showed that significant DNA cleavage by these mutant topoisomerase proteins required the addition of Mg²⁺. More critically, DNA religation and DNA relaxation activities were found to be reduced by this Met to Arg substitution. The shift of optimal pH for relaxation activity to higher values and the reduction of measured Mg²⁺ binding affinity as a result of the mutation indicated that Mg²⁺-dependent DNA religation was compromised by the introduction of an additional positive charge into the active site region. This result has significant implication for the screening and design of novel antibacterial compounds that can cause cell death by inhibiting the religation of the bacterial topoisomerase IA cleavage complex.

**MATERIALS AND METHODS**

**Recombinant topoisomerase I**

Recombinant *Y. pestis* topoisomerase I mutants with all possible substitutions at Met326 were generated by site-directed mutagenesis using the *Pfu* Ultra DNA polymerase (from Stratagene) and pYTOP derived from pBAD/Thio (17) as template. Mutants were obtained first by utilizing an oligonucleotide pair with mixed nucleotides at the codon for Met326, and then with specific oligonucleotides designed for substitutions not identified from the random codon for Met326, and then with specific oligonucleotides designed for substitutions not identified from the random phase LB culture. The recombinant protein in the soluble lysate was allowed to bind to Ni-NTA agarose (Qiagen) by the addition of 0.02% arabinose and 1 mM IPTG to exponential phase LB culture.

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**Expression plasmids**

Expression plasmids were isolated from the induced cultures during protein expression for sequencing of the entire coding region of the recombinant topoisomerase I molecule to ensure that no other mutation was present in the polypeptide.

**SOS induction and cell killing assays**

Plasmid pYTOP derivatives expressing mutant YTOP proteins under the control of the BAD promoter were transformed into *E. coli* JD5 with the chromosomal *dinD1::lacZ* fusion. SOS-inducing mutants were identified as blue colonies by replica plating the transformants onto X-gal plate with 100 µg/ml ampicillin and 0.002% arabinose as previously described (18). To measure the effect of mutant YTOP expression on viability, early exponential phase culture of JD5 transformants in LB medium with ampicillin (100 µg/ml) was induced with 0.2% arabinose for 2 h before serial dilution and plating on LB plates with 2% glucose and ampicillin, followed by overnight incubation at 37°C. The number of colonies obtained from the induced culture was divided by the number of colonies from the control non-induced culture to calculate the survival ratio.

**Topoisomerase activity assays**

DNA relaxation activity of each mutant topoisomerase was compared with the wild-type topoisomerase by the amount of enzyme in serial dilutions needed to observe similar distribution of partially relaxed topoisomers from the supercoiled DNA substrate. The relaxation activity assay was carried out first at pH 8.0 or then at different pH values as described (24). DNA cleavage activity was purified using the ProPur IMAC Midi spin column (Nunc). After addition of the soluble lysate, the column was first washed with 30 column volume of PBS buffer (59 mM sodium phosphate pH 7.4, 300 mM NaCl) with 30 mM imidazole, then with 1 column volume of each of PBS buffer with 50 and 100 mM imidazole. The topoisomerase I proteins were eluted with 1 column volume of PBS buffer with 200 mM imidazole, then 2 column volume PBS buffer with 500 mM imidazole.

**The *E. coli* ETOP-M320R topoisomerase I mutant clone was generated by site-directed mutagenesis using pLIC-ETOP as template.** The pLIC-ETOP plasmid was made by ligation-independent cloning (21) of ETOP coding sequence into a vector that allows T7 RNA polymerase dependent expression along with a tobacco etch virus (TEV) protease-cleavable N-terminal hexahistidine tag (22). Expression of the recombinant ETOP was induced in *E. coli* BL21 (from Invitrogen) by the addition of 0.02% arabinose and 1 mM IPTG to exponential phase LB culture. The recombinant protein in the soluble lysate was allowed to bind to Ni-NTA agarose (Qiagen) in PBS buffer pH 8.0 with 10 mM imidazole which was then packed into a column. After extensive washing, the topoisomerase protein was eluted with buffer containing 250 mM imidazole, cleaved with TEV protease, and passed through the Ni-NTA agarose again to remove the histidine tag. Final purification was carried out with a single-stranded DNA cellulose column as described (23).
assayed with a 59-base long 5’end labeled single-stranded oligonucleotide substrate and rejoining of the cleavage product was assessed by the addition of high salt to the DNA cleavage reaction (18,25). The level of DNA cleavage products was analyzed after electrophoresis in a 15% sequencing gel with the PhosphoImager Storm 860. To quantitate the percent DNA cleavage product present, the PhosphoImager signals for the cleavage product and uncleaved substrate in each lane were added to obtain the total substrate signal and control for any difference in loading.

Mg²⁺ binding by wild-type ETOP and ETOP-M320R proteins was assessed by following the decrease in intrinsic tryptophan fluorescence emission upon titration of topoisomerase I protein with MgCl₂ using the CARY Eclipse fluorescence spectrophotometer (17). The fraction of maximal decrease in fluorescence signal observed at each Mg²⁺ concentration was curve fitted for two binding sites in each enzyme molecule with the GraphPad Prism software.

Molecular modeling

The Met to Arg substitution was introduced into the structure of the N-terminal fragment of *E. coli* topoisomerase I (1ECL in Protein Data Bank) with the Mutate function of DeepView-Swiss PDB Viewer (26). The arginine rotamer that had a negative score, indicating that it corresponded to a favorable conformation, was selected from nine rotamer that had a negative score, indicating that it corresponded to a favorable conformation, was selected for energy minimization. The minimized structure was used to generate the electrostatic potential map with a solvent dielectric constant of 80.

RESULTS

Substitution of Met326 in YTOP with Arg resulted in SOS induction and cell killing

All possible substitution mutants of Met326 in YTOP were examined *in vivo* using *E. coli* strain JD5 for the ability to illicit the SOS response when mutant YTOP expression was induced by a low level of arabinose (0.002%). The M326R substitution was the only one found to induce the SOS response from the dinD1 promoter. Measurement of ratio of the number of viable colonies in culture induced by a higher level of arabinose (0.2%) versus the number of colonies in the non-induced cultures was carried out for most of the substitution mutants to confirm that the SOS induction phenotype correlated with the effect of overexpression on viability. Substitutions with Pro (found in bacterial topoisomerase III at this position) and valine (previously found to enhance DNA cleavage without affecting viability) were among those included for comparison. The results (Table 1) confirmed that the M326R substitution in YTOP had the most drastic effect on viability, with ~4 log lower number of viable colony counts 2 h after induction with arabinose when compared to the non-induced culture, and >100-fold lower viability ratio than all the other substitution mutants examined. The cell killing effect was comparable to the previously characterized single G122S mutation in the TOPRIM motif (17).

Table 1. Effect of induction of recombinant *Y. pestis* topoisomerase I protein (YTOP) with various selected substitutions at Met326 on viability

<table>
<thead>
<tr>
<th>Substitution at Met326</th>
<th>Induced/non-induced relative viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0.17 ± 0.11</td>
</tr>
<tr>
<td>Arg</td>
<td>0.00015 ± 0.000014</td>
</tr>
<tr>
<td>Asn</td>
<td>0.073 ± 0.022</td>
</tr>
<tr>
<td>Asp</td>
<td>0.018 ± 0.003</td>
</tr>
<tr>
<td>Gln</td>
<td>0.14 ± 0.09</td>
</tr>
<tr>
<td>Glu</td>
<td>0.026 ± 0.005</td>
</tr>
<tr>
<td>His</td>
<td>0.18 ± 0.16</td>
</tr>
<tr>
<td>Lys</td>
<td>0.054 ± 0.006</td>
</tr>
<tr>
<td>Pro</td>
<td>0.075 ± 0.018</td>
</tr>
<tr>
<td>Ser</td>
<td>0.081 ± 0.027</td>
</tr>
<tr>
<td>Thr</td>
<td>0.13 ± 0.036</td>
</tr>
<tr>
<td>Trp</td>
<td>0.017 ± 0.009</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.099 ± 0.065</td>
</tr>
<tr>
<td>Val</td>
<td>0.11 ± 0.01</td>
</tr>
</tbody>
</table>

Exponential phase cultures of *E. coli* JD5 transformants were treated with 0.2% arabinose for 2 h at 37°C to induce the expression of recombinant YTOP from the BAD promoter. Viable colony counts were determined by serial dilution and overnight incubation on LB + ampicillin + 2% glucose plates. The number of colonies from the induced culture was divided by the number of colonies from the control non-induced culture to obtain the relative viability. The results represent the average and standard deviation from at least 3 measurements.

The Arg substitution led to the loss of >90% of relaxation activity

Relaxation assays using negatively supercoiled DNA as substrate (Figure 1) showed that the Arg substitution resulted in the greatest loss of relaxation activity (~16-fold) among the YTOP Met326 substitution mutant proteins purified and characterized. This suggested that the cell killing effect of the YTOP-M326R mutant protein might be due to disruption of the catalytic cycle of the enzyme. Three other mutant YTOP proteins also had substitutions bringing in a side chain expected to be fully charged at physiological pH. The Lys substitution had the next largest reduction in relaxation activity (~8-fold). The Asp and Glu substitutions had a relatively minor effect on the relaxation activity (~2–4-fold lower). While the Lys, Asp, Glu substitutions resulted in some reduction in relaxation activity, their effects on viability was >100-fold lower when compared with the Arg substitution. Wild-type level relaxation activity was exhibited by the Ser, Thr and Pro substitution mutant enzymes.

YTOP-M326R and ETOP-M320R had proficient DNA cleavage activity in the presence of Mg²⁺ but showed deficiency in DNA religation

To determine the step of the catalytic cycle disrupted by the Met to Arg substitution, DNA cleavage and religation activities of YTOP-M326R were analyzed using an oligonucleotide substrate labeled with ³²P at the 5’-end. DNA cleavage by wild-type YTOP did not require addition of Mg²⁺. In contrast, cleavage by YTOP-M326R could not be detected unless Mg²⁺ was added to the reaction (Figure 2A). The cleavage activity of YTOP-M326K mutant protein was also dependent on added MgCl₂. This dependence of added Mg²⁺ for DNA cleavage to be
observable was also found previously to be a property of YTOP-G122S. Based on densitometry analysis of results from three experiments, the amount of cleavage product observed for YTOP-M326K in the presence of 2 mM MgCl₂ was ~4-fold lower than that of YTOP-M326R. For wild-type YTOP addition of 2 mM MgCl₂ decreased the level of DNA cleavage product observed because some of the cleavage product was religated. However, the level of cleavage product observed for wild-type YTOP in the presence of 2 mM MgCl₂ was still around 2-fold higher than that of YTOP-M326K. Rejoining of DNA cleavage product by wild-type YTOP was both more rapid, and more complete when compared to YTOP-M326R (Figure 2B). The rejoining of cleavage product by YTOP-M326K was also reduced in rate, but was more complete than YTOP-M326R. DNA cleavage and religation by the previously characterized YTOP-G122S mutant was also included in Figure 2 for comparison. While extent of DNA religation inhibition by the M326R mutation was not as complete as the G122S mutation (Figure 2B), there was ~4-fold more DNA cleavage product formed in the presence of 2 mM Mg(II) by YTOP-M326R mutant when compared to YTOP-G122S (Figure 2A). Therefore the level of cleavage complexes accumulated in vivo by these two mutants may be comparable. These observations reflect that DNA cleavage is not the rate determining step of relaxation, so YTOP-M326K showed greater relaxation activity than YTOP-M326R even though DNA cleavage activity was lower for YTOP-M326K. If DNA rejoining was inhibited, the extent of DNA cleavage product accumulated in the presence of Mg⁺⁺ would be expected to correlate with the observed bactericidal effect due to the prolonged presence of the covalent cleavage complex in vivo. The amount of cleavage product accumulated by the YTOP-M326K mutant topoisomerase I when induced by arabinose in vivo was probably insufficient to induce the SOS response and cause much significant bacterial cell death. Even though DNA rejoining by the YTOP-M326R mutant topoisomerase I was not completely inhibited as in the case of YTOP-G122S, the higher level of cleavage complex formed initially still resulted in significant extent
of overall cleavage complex accumulation and can trigger the cell-killing process.

The ETOP-M320R mutant protein with the same Met to Arg substitution as YTOP-M326R was also expressed and purified. It exhibited 12–16-fold lower relaxation activity than wild-type ETOP (Figure 3A). Experiments with 5'-end labeled oligonucleotide substrate (Figure 3B) showed that DNA cleavage by ETOP-M320R was very low in the absence of added Mg\(^{2+}\), but the level of cleavage products formed in the presence of 2 mM MgCl\(_2\) was much higher than that of wild-type ETOP and the previously characterized ETOP-M320V mutant protein which had normal DNA religation activity (18). After addition of high salt to the DNA cleavage reaction, DNA religation by wild-type ETOP enzyme was extremely rapid and observed to be complete at 10 s (Figure 3C). The M320R mutation not only affects the level of DNA cleavage product that accumulate, but it also takes longer time ( >2 min) to reach the maximal level. This longer life time of the DNA cleavage complex in vivo would then result in greater level of DNA cleavage complex accumulated on the chromosome in vivo, making it more likely to initiate the bactericidal pathway.

Shift in optimal pH for relaxation activity of ETOP-M320R

The recombinant ETOP-M320R protein had sufficient residual activity to allow the pH profile of the relaxation reaction to be determined. The results (Figure 4) showed that wild-type ETOP showed a broad pH optimum between pH 6.5 and 8.5 as previously reported, with a symmetrical bell shape curve (24,27). Substitution of Met320 with Arg resulted in a shift in maximal relaxation activity to pH 8.5–9.0, and a non-symmetrical bell shape curve that had a sharp drop-off in relaxation activity between pH 9.0 and pH 9.5. This can be interpreted that while ionization states of the other catalytic residues limited the enzyme relaxation activity below pH 6.0 or above pH 9.0, the positive charge of the newly introduced Arg side chain in ETOP-M320R mutant protein prevented the relaxation activity except when the electrostatic potential of the active site region became less positive at the high pH values. Analysis of the relaxation reaction of products from ETOP-M320R in agarose gel containing ethidium bromide (Supplementary Figure 1) showed that the reaction products near the top of the topoisomer ladders formed at pH 8.5–9.0 corresponded to closed relaxed DNA, and not nicked DNA molecules. The Arg substitution probably resulted in increased processivity. Analysis of the pH dependence of DNA cleavage in the presence of Mg\(^{2+}\) also showed a shift to the basic pH values (Supplementary Figure 2).

Decreased Mg\(^{2+}\)-binding affinity for ETOP-320R due to introduction of additional positive charge into the topoisomerase active site

The positive charge introduced into the active site region of YTOP and ETOP from the Met to Arg substitution could affect binding of Mg\(^{2+}\), and the decrease in Mg\(^{2+}\)-binding affinity would account for the inhibition of DNA religation. To test this hypothesis, binding affinity of ETOP-M320R was measured by monitoring the decrease in emission of the intrinsic tryptophan fluorescence upon binding of Mg\(^{2+}\) and subsequent conformational change (28) (Figure 5). The results from curve fitting of three sets of data for two Mg\(^{2+}\)-binding sites in each enzyme molecule showed that the dissociation constants calculated for ETOP-M320R (K\(_{d1}\) = 6.4 \(\mu\)M, K\(_{d2}\) = 348.1 \(\mu\)M) were several fold higher than those reported previously for wild-type ETOP (K\(_{d1}\) = 1.3 \(\mu\)M, K\(_{d2}\) = 75.2 \(\mu\)M) (18). In contrast, the Met to Val substitution had no significant effect on the Mg\(^{2+}\)-binding affinity of ETOP (18).

Modeling of the effect of the Met to Arg substitution on the ETOP active site

The Met to Arg substitution was modeled into the previously determined structure of E. coli topoisomerase I N-terminal fragment (1ECL). The electrostatic potential map of the minimized mutant structure (Figure 6) showed that presence of an arginine residue at position 320 caused a significant increase of positive electrostatic potential and a decrease of the negative electrostatic potential around the side chains of the acidic triad of Asp111, Asp113 and Glu115 responsible for binding of the two Mg\(^{2+}\) ions.
These changes in electrostatic potential in the active site region due to the Met to Arg substitution would be expected to affect the Mg\(^{2+}\) binding affinity of the mutant topoisomerase and inhibit DNA religation.

**Substitutions of Met326 that increased DNA cleavage by YTOP**

We have previously observed that the Met to Val substitution in YTOP and ETOP enhanced DNA cleavage without inhibiting DNA religation, and the overall relaxation activity was maintained at wild-type level (18). The DNA cleavage activity of the other purified mutant YTOP proteins included in Figure 2 were analyzed to determine if any of these substitutions could also enhance the DNA cleavage by YTOP. YTOP-M326T and YTOP-M326P were found to form more DNA cleavage products than wild-type YTOP (Figure 7). The effect of the Thr substitution was very similar to the Val substitution, with around 2-fold increase in DNA cleavage product in the absence of added MgCl\(_2\). The Pro substitution produced a ~3-fold increase in DNA cleavage in the absence of added MgCl\(_2\). Similar to the Val substitution, the Thr and Pro substitutions did not increase the amount of cleavage product significantly when 2 or 5 mM MgCl\(_2\) was added to the cleavage reaction, and the mutations had no effect on the relaxation activity. Therefore overexpression of these mutant YTOP proteins did not induce the SOS response or result in bacterial cell death. Methionine is the conserved residue at this position for bacterial topoisomerase I while proline is the conserved residue for bacterial and eukaryotic topoisomerase III (29).

**DISCUSSIONS**

Previous work (18) showed that substituting valine for the conserved methionine adjacent to the active site tyrosine
of bacterial topoisomerase I could enhance DNA cleavage, indicating that this position can influence the balance of cleavage-religation by type IA topoisomerases. In this study, all possible amino acid substitutions of Met326 of YTOP were examined in vivo to determine if any amino acid substitution could induce the SOS response in order to identify substitutions that could inhibit religation of DNA. The Arg substitution was the only one found to induce the SOS response from the dinD1 promoter in vivo. This mutant topoisomerase had >100-fold greater effect on cell viability than the other 12 substitution mutant enzymes that were further characterized for the consequence of their overexpression on bacterial cell viability. Biochemical characterization of the purified YTOP-M326R and ETOP-M320R mutant topoisomerase proteins showed that DNA relaxation activity was greatly reduced because of the effect of Arg substitution on the DNA relaxation step. The introduction of an additional positive charge into the active site region of ETOP due to the mutation resulted in a shift of the optimal pH for relaxation activity to higher pH values, indicating an inhibitory effect from the positive charge of the arginine side chain. Measurement of the topoisomerase intrinsic tryptophan fluorescence showed that the arginine substitution reduced the Mg$^{2+}$-binding affinity, accounting for the inhibition of DNA religation. This is consistent with the molecular modeling results showing the change in electrostatic potential in the active site ETOP-M320R, resulting in a shift of optimal pH for the relaxation reaction to higher values, indicating an inhibitory effect from the positive charge of the arginine side chain.

Figure 4. The Met to Arg substitution in ETOP shifted the optimal pH of the relaxation reaction to higher values. Effect of pH on the relaxation activity of wild-type ETOP (15 ng, ~0.15 pmol) and ETOP-M320R (300 ng, ~3 pmol) was determined by first equilibrating the enzyme in 5 μl with buffer of MES (pH 5.0–7.0), Tris (pH 8.0–8.5), or CHES (pH 9.0–9.5) at 100 mM before the addition of 8 μl of DNA (0.5 μg, ~0.17 pmol) plus MgCl₂, followed by incubation at 37 °C for 30 min.

Figure 5. Reduction in Mg$^{2+}$-binding affinity as a result of the M320R substitution in ETOP. Decrease in intrinsic tryptophan fluorescence upon binding of Mg$^{2+}$ measured for wild-type ETOP and ETOP-M320R was curve fitted for binding of two Mg$^{2+}$/enzyme molecule with the GraphPad Prism program.
for catalysis and the divalent ions–enzyme interaction results in a protein conformational change (31,33,34). Strand transfer by HIV-1 integrase can be inhibited by small molecules that chelate the divalent metal ions in the enzyme active sites (35–37). It might be possible to target DNA religation by bacterial type IA topoisomerase employing analogous approaches of interfering with the divalent metal ion interactions in the enzyme active site.

Even though divalent metal ions have such broad physiological roles, the FDA approval of raltegravir (MK-0518) that chelates the active site metal ions of HIV-1 integrase for clinical treatment of AIDS (38) provides evidence that highly specific targeting of enzyme–metal ion interaction can be achieved for therapeutic applications.

**Figure 6.** Modeling of the electrostatic potential around the active site of ETOP and ETOP-M320R. Stereo view of electrostatic potential maps were generated with DeepView-Swiss PDB Viewer after energy minimization using a solvent dielectric constant of 80. The maps were contoured at $-1.8 kT/e$ (red) < $0.0 kT/e$ (white) $< 1.8 kT/e$ (blue) where $k$ = Boltzmann constant, $T$ = absolute temperature, $e$ = charge of a proton. Residues Asp111, Asp113, Glu115, Tyr319, Met320/Arg320, Arg321 are shown.

**Figure 7.** Comparison of enhancement of DNA cleavage by Val, Thr and Pro substitutions at Met326 of YTOP. DNA cleavage products were analyzed after the incubation of 400 ng of enzyme (4 pmol) with 0.5 pmol of 5'-32P-labeled oligonucleotide substrate in the presence of the indicated concentration of MgCl2 at 37°C for 30 min.
SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We are grateful to Prof. James M. Berger at University of California Berkeley and Mary Coons in his group for providing us with the TEV protease expression plasmid, LIC cloning vector and valuable advice. Funding is provided by National Institutes of Health (GM54226, AI06933 to Y.T.) Funding to pay the Open Access publication charges for this article was provided by National Institutes of Health.

Conflict of interest statement. None declared.

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