The Acidic Triad Conserved in Type IA DNA Topoisomerases Is Required for Binding of Mg(II) and Subsequent Conformational Change*

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The acidic residues Asp-111, Asp-113, and Glu-115 of *Escherichia coli* DNA topoisomerase I are located near the active site Tyr-319 and are conserved in type IA topoisomerase sequences with counterparts in type IIA DNA topoisomerases. Their exact functional roles in catalysis have not been clearly defined. Mutant enzymes with two or more of these residues converted to alanines were found to have >90% loss of activity in the relaxation assay with 6 mM Mg(II) present. Mg(II) concentrations (15–20 mM) inhibitory for the wild type enzyme are needed by these double mutants for maximal relaxation activity. The triple mutant D111A/D113A/E115A had no detectable relaxation activity. Mg(II) binding to wild type enzyme resulted in an altered conformation detectable by Glu-C proteolytic digestion. This conformational change was not observed for the triple mutant or for the double mutant D111A/D113A. Direct measurement of Mg(II) bound showed the loss of 1–2 Mg(II) ions for each enzyme molecule due to the mutations. These results demonstrate a functional role for these acidic residues in the binding of Mg(II) to induce the conformational change required for the relaxation of supercoiled DNA by the enzyme.

*Escherichia coli* DNA topoisomerase I is the best studied representative of the type IA DNA topoisomerases. This class of enzymes includes the bacterial and archael DNA topoisomerase I and III, reverse gyrase, and yeast and mammalian topoisomerase III, with diverse roles in cellular functions (reviewed in Refs. 1 and 2). Mg(II) is required for the interconversion of DNA topological isomers catalyzed by these enzymes. Comparison of their polypeptide sequences showed that the conserved positions include the acidic residues Asp-111, Asp-113, and Glu-115 (3, 4). When the crystal structure of the 67-kDa N-terminal transesterification domain of the enzyme was published, it was noted (5) that these three acidic residues in the active site are arranged similarly to the three acidic residues known to coordinate two divalent ions in Klenow fragment (6). Mg(II) concentration (15–20 mM) inhibitory for the wild type enzyme are needed by these double mutants for maximal relaxation activity. The triple mutant D111A/D113A/E115A had no detectable relaxation activity. Mg(II) binding to wild type enzyme resulted in an altered conformation detectable by Glu-C proteolytic digestion. This conformational change was not observed for the triple mutant or for the double mutant D111A/D113A. Direct measurement of Mg(II) bound showed the loss of 1–2 Mg(II) ions for each enzyme molecule due to the mutations. These results demonstrate a functional role for these acidic residues in the binding of Mg(II) to induce the conformational change required for the relaxation of supercoiled DNA by the enzyme.

**EXPERIMENTAL PROCEDURES**

Materials—The chemical reagents used were either ultrapure or ACS reagent grade. Solutions were prepared with water first deionized with the Barnstead Nanopure system and then passed over a Bio-Rad chellex 100 resin (100–200 mesh sodium form) to remove any contaminating metal ions. Plasmid DNA was purified by cesium chloride gradient centrifugation.

Mutagenesis—The QuikChange site-directed mutagenesis kit from Stratagene was used for construction of the mutants. The mutants were identified by DNA sequencing.

**Enzyme Expression and Purification**—Wild type topoisomerase I was expressed and purified as described (14). The double and triple mutants studied here were expressed in *E. coli* GP200 (gyrA(Nal) gyrB225 Δ(topA)ณB204) (15) and purified with procedures similar to those used for the wild type enzyme.

**Relaxation Activity Assay**—Wild type and mutant enzymes were assayed for relaxation activity in 20 μl with 0.5 μg of negatively supercoiled plasmid DNA, 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml gelatin, and the indicated concentration of MgCl₂. Incubation was at 37 °C for 30 min. The reactions were stopped by the addition of 5 μl of 50% glycerol, 50 mM EDTA, and 0.5% (v/v) bromphenol blue. After

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RESULTS

Expression and Purification of the Double and Triple Mutants—Expression of the alanine substitution mutants in E. coli GP200 was confirmed by SDS gel electrophoresis of the soluble lysates followed by Coomassie Blue staining (data not shown). The enzymes were purified to >95% homogeneity (Fig. 1) by the combination of phosphocellulose, hydroxyapatite, and single-stranded DNA agarose column chromatography.

Effect of the Double and Triple Mutations on Relaxation Activity with 6 mM Mg(II) Present—The purified double and triple mutants with the conserved acidic triad in the active site altered by alanine substitutions were diluted serially and assayed for relaxation activity in the presence of 6 mM Mg(II). The results (Fig. 2) showed that their relaxation activities were further diminished under this assay condition when compared with the activities reported for the single mutants (3, 12). No activity was detected for the D111A/D113A double mutant and the D111A/D113A/E115A triple mutant. The D111A/E115A and D113A/E115A double mutants had <10% wild type activity.

The mutant with alanine substitution at Glu-9 has been shown to have >90% loss of relaxation activity (3, 11). Double mutants with alanine substitutions at Glu-9 and one of the acidic triad had no detectable relaxation activity at 6 mM MgCl2 (Fig. 2).

Double Mutants Involving the Acidic Triad Require Higher Mg(II) Concentration for Maximal Relaxation Activity—The effect of increasing Mg(II) concentration on the relaxation activity was compared between the wild type enzyme and the double and triple alanine substitution mutants. Maximal relaxation activity for the double mutants involving the acidic triad was observed at 15 mM MgCl2 (Fig. 3). With wild type enzyme, such high concentration of MgCl2 resulted in inhibition of relaxation activity. Previous studies showed that as little as 1.5–2.5 mM was sufficient for maximal activity for the wild type topoisomerase I (13, 18). Relaxation activity could not be restored for the D111A/D113A/E115A triple mutant even at 20 mM MgCl2, and only partial relaxation of the input DNA can be achieved with the D111A/D113A mutant. The increased Mg(II) concentration had much less of an effect on the relaxation activity of the double mutants involving alanine substitution at Glu-9 and one of the acidic triad residues (Fig. 3). There is a slight enhancement of the relaxation activity of the E9A/D111A and E9A/D113A mutants at 15 mM MgCl2. The E9A/E115A mutant remained inactive.

To further compare the activity of the double mutants involving the acidic triad at the high Mg(II) concentration of 15 mM versus the wild type activity at 6 mM Mg(II), time course of the relaxation reaction was monitored. The results (Fig. 4) showed that 30 min were required for the DNA incubated with the double mutants to approach maximal relaxation, whereas the wild type enzyme reaction was nearly complete at 10 min.

Reduced Mg(II) Binding Stoichiometries for the Acidic Triad

FIG. 1. SDS gel of purified E. coli DNA topoisomerase I wild type (wt) enzyme and mutants with the indicated residues changed to alanine. MW stds, molecular weight standards.

FIG. 2. Relaxation activity of the alanine substitution mutants in the presence of 6 mM MgCl2. Wild type (wt) and mutant enzymes (with the indicated residues changed to alanine) of the indicated amount were added to a standard relaxation reaction with 6 mM MgCl2 present. C, no enzyme added.
constant of around 0.3 mM for the binding of 2 Mg(II). Titration (Fig. 5) as described previously (16) yielded a dissociation

A tryptophan fluorescence (13). Analysis of the fluorescence data

mational change in topoisomerase I detectable by decreased

have previously shown that Mg(II) binding result in a confor-

tration activity of the wild type (wt) and mutant enzymes (with

ated with the relaxation activities observed. The D111A/D113A/

E115A mutant, which did not show any activity under all

conditions tested, had a Mg(II) binding stoichiometry barely

above the background. The D111A/E115A and D113A/E115A

mutants retained binding of around 1 Mg(II) per enzyme mol-

ecule, whereas the double mutant D111A/D113A, with the

more severely reduced activity, had significantly lower Mg(II)

binding stoichiometry.

Mg(II) Binding Followed by Change in Fluorescence—We

have previously shown that Mg(II) binding result in a confor-
mational change to topoisomerase I detectable by decreased

tryptophan fluorescence (13). Analysis of the fluorescence data

(Fig. 5A) as described previously (16) yielded a dissociation

constant of around 0.3 mM for the binding of 2 Mg(II). Titration

of similar concentrations of MgCl$_2$ resulted in much lower

change in tryptophan fluorescence emission for the double mu-

tants (Fig. 5). Maximal change in fluorescence was achieved

with the wild type enzyme at around 2 mM Mg(II), with no

further significant drop in fluorescence at up to 20 mM Mg(II).

However, a second decrease in fluorescence could be observed

for the double mutants D111A/E115A and D113A/E115A be-
tween 10–15 mM Mg(II), suggesting the binding of a second

Mg(II) at the the higher Mg(II) concentrations. Titration of

Mg(II) did not result in significant change in fluorescence emis-

sion for the triple mutant even at 20 mM MgCl$_2$ (Fig. 5B), in

agreement with abolishment of Mg(II) binding and relaxation

activity for this mutant.

Mg(II) Binding to Topoisomerase I Results in Change in

Glu-C Digestion Pattern—Besides monitoring the change in
tryptophan fluorescence, we have also developed a proteolytic

assay using Glu-C digestion to detect the conformational

change in topoisomerase I upon binding of Mg(II). As shown in

Fig. 6, the presence of as little as 0.5 mM Mg(II) topoisomerase I

resulted in accumulation of a 14-kDa proteolytic fragment not

seen in the time course of digestion in the absence of Mg(II).

The yield of this 14-kDa product was further enhanced when

Mg(II) concentration was increased to 2 mM.

The 14-kDa product was not seen when the D111A/D113A and

D111A/D113A/E115A mutants were digested with Glu-C in

the presence of 2 mM Mg(II) (Fig. 7). For the D111A/E115A and

D113A/E115A mutants, the level of the 14-kDa product

formed was about 3-fold lower than that from the wild type

enzyme.

Identification of the 14-kDa Glu-C Digestion Product—The

exact weight of the 14-kDa Glu-C digestion product was deter-
mained by mass spectrometry to be 14,498. This is almost iden-
tical to the mass predicted for the Glu-C digestion product from

residue 736 to 862 (14,509.5). The masses of the in-gel Lys-C

digestion products of this 14 kDa fragment (Table II) confirmed

this identification.

### DISCUSSION

Type IA and type II DNA topoisomerases share similarity in

their requirement for Mg(II) in the enzyme catalytic mechan-

ism. When the sequences of these two classes of enzymes are

compared, acidic residues corresponding to Glu-9, Asp-111,

Asp-113, and Glu-115 of E. coli DNA topoisomerase I are found

to be conserved (3, 4). A role for Glu-9 in the breaking and

rejoining of DNA strand has been proposed (3, 12). However,

conversion of one of the acidic triad Asp-111, Asp-113, or Glu-

115 to alanine only resulted in partial loss of enzyme activity,

agreement with abolishment of Mg(II) binding and relaxation

activity for this mutant.

**TABLE I**

| Enzyme Magnesium content atom/molecule enzyme |
|---------------------------------------------|
| Wild type 2.32 ± 0.03                        |
| D111A/D113A 0.22 ± 0.02                     |
| D111A/E115A 1.11 ± 0.01                      |
| D113A/E115A 1.08 ± 0.01                      |
| D111A/D113A/E115A 0.01 ± 0.01                |

Incubation and dialysis conditions were as described under “Experimental Procedures.” The average of two determined values were shown here.
Coordination sites may well be easily tolerated by the enzyme, whereas the effect of the loss of two Mg(II) coordination sites would be much more severe for the binding affinity of Mg(II) at the position required for relaxation activity. The results shown here suggest that the double mutations D111A/E115A or D113A/E115A primarily affect the binding of the second Mg(II) required for relaxation activity (13). In the wild type enzyme, the acidic triad may bind a third Mg(II) at 15–20 mM Mg(II) that could lead to inhibition of relaxation activity.

![Fluorescence titration of wild type and mutant topoisomerase I with increasing concentrations of MgCl₂.](image)

**Fig. 5.** Fluorescence titration of wild type and mutant topoisomerase I with increasing concentrations of MgCl₂. The enzymes were present at 1 μM. Emission at 335 nm was plotted (excitation at 295 nm). A, Mg(II) concentrations up to 1 mM. B, Mg(II) concentrations up to 20 mM.

![Effect of Mg(II) on the Glu-C digestion pattern of E. coli DNA topoisomerase I.](image)

**Fig. 6.** Effect of Mg(II) on the Glu-C digestion pattern of *E. coli* DNA topoisomerase I. Glu-C digestion was carried out with 0, 0.5, or 2 mM MgCl₂ present for the indicated lengths of time. The digestion products were analyzed by 15% SDS-polyacrylamide gel electrophoresis. The asterisk indicates the position of the 14 kDa digestion product.

![Comparison of Glu-C digestion patterns of wild type (Wt) and mutant topoisomerase I in the absence and presence of Mg(II).](image)

**Fig. 7.** Comparison of Glu-C digestion patterns of wild type (Wt) and mutant topoisomerase I in the absence and presence of Mg(II). HM, high molecular weight standards; LM, low molecular weight standards.
Double mutants involving Glu-9 and one of the acidic triads have no detectable relaxation activity at 6 mM Mg(II). Increased Mg(II) concentration had only a slight effect on the relaxation activity. This supports the interpretation that even though lower Mg(II) binding affinity may contribute to the decreased activity seen for the double mutants involving Glu-9, the major reason for activity deficiency is likely to be due to the effect of the Glu-9 mutation related to DNA cleavage/religation (3, 12).

The Glu-C digestion patterns of all of the mutants examined here are quite similar in the absence of Mg(II). Therefore, the alanine substitutions did not lead to a severe change of enzyme folding that may account for the loss of activity. Addition of Mg(II) did not lead to the appearance of a 14-kDa fragment in the Glu-C digestion of the D111A/D113A and D111A/D113A/ E115A mutants This supports the role of the acidic triads in binding to Mg(II) and resulting in a conformational change in the enzyme.

The effect of double alanine substitutions at Asp-111 and Asp-113 was more severe in both the relaxation assay and the Glu-C digestion assay than the double alanine substitutions at one of these residues and Glu-115. In contrast, the effect of the single alanine substitution on relaxation activity was more severe at Glu-115 than at Asp-111 and Asp-113 (3). This indicates that Asp-111 and Asp-113 may have overlapping roles in Mg(II) binding, but elimination of both functional groups would greatly diminish Mg(II) binding. Mutation at Glu-115 may also influence another aspect of enzyme function, aside from binding of Mg(II) and the resulting conformational change.

The 14-kDa Glu-C digestion product that is preferentially exposed to further Glu-C digestion. Biochemical analysis of the role of the acidic triad in the relaxation reaction is consistent with this interpretation.

The results presented here established a functional role for the acidic triad conserved in type IA and type IIA topoisomerases. The DXXD motif residues Asp-111 and Asp-113 are clearly involved in binding of Mg(II) and the resulting enzyme conformational change. Besides additional structural information on the enzyme, identification of other amino acid residues needed for this Mg(II)-induced conformational change would be helpful for elucidation of the dynamic action of the topoisomerase activity.

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