Changing a Leucine to a Lysine Residue Makes *Nae*I Endonuclease Hypersensitive to DNA Intercalative Drugs†

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ABSTRACT: A single amino acid change transforms restriction enzyme *Nae*I to a topoisomerase and recombinase (*Nae*I-L43K) that shows no sequence similarity to these protein families. This transformation appears to result from coupled endonuclease and ligase domains. To further elucidate the relationship between *Nae*I-L43K and the topoisomerase protein family, we studied the effect of the topoisomerase inhibitors on *Nae*I-L43K activity. The intercalative drugs amsacrine, ellipticine, and daunorubicin inhibited *Nae*I-L43K, whereas the nonintercalating drugs camptothecin, VP-16, and oxolinic acid did not. Ethidium bromide also inhibited *Nae*I-L43K, implying that intercalation is responsible for its inhibition. The effects of the intercalative drugs on the DNA cleavage steps of *Nae*I and *Nae*I-L43K were compared. The drugs hardly inhibited DNA cleavage by wild type *Nae*I but completely inhibited DNA cleavage by *Nae*I-L43K. This difference in inhibition demonstrates that the L43K amino acid change sensitized *Nae*I to these drugs. Low concentrations of the intercalative drugs, except for ethidium bromide, enhance production of topoisomerase−DNA covalent intermediates but inhibited production of the *Nae*I-L43K−DNA covalent intermediate. These results imply some unique differences between DNA relaxation by *Nae*I-L43K and DNA topoisomerase. Concomitant with studying inhibition of the cleavage intermediate, *Nae*I-L43K was found to covalently bond with the 5′ end of the cleaved DNA strand.

Restriction enzyme *Nae*I is unusual; it must bind two DNA sequences for activity (Conrad & Topal, 1989; Topal et al., 1991; Yang & Topal, 1992; Baxter & Topal, 1993), and it is linked to the recombinase, topoisomerase, and ligase protein families (Jo & Topal, 1995) by its formation of a covalent intermediate with DNA, by its homology with a DNA ligase-like active site, and by a single amino acid change that transforms it to a topoisomerase and recombinase. The energy stored in the covalent protein−DNA intermediate is used by the topoisomerases for religation, after strand passage, to the same broken DNA end to achieve DNA relaxation. The same energy is used by the recombinases for ligation to a different broken DNA end to achieve recombination. The energy is used by *Nae*I-L43K to achieve these same functions *in vitro* (Jo & Topal, 1995).

The *Nae*I DNA ligase-like active site is most similar to that of human DNA ligase I (Jo & Topal, 1995). The *Nae*I sequence differs from the human ligase active site in one important respect; the lysine (K) that forms the adenylated intermediate essential for catalysis by the DNA ligase active site is not present in *Nae*I. Instead, there is a leucine (L43) at this position. Changing L43 to K43 transforms *Nae*I activity to that of a topoisomerase and recombinase even though *Nae*I has no detectable sequence similarity with the topoisomerase and recombinase protein families (Jo & Topal, 1995). The topoisomerase activity appears to result from endonuclease and ligase domains that are coupled by means of the covalent protein−DNA intermediate.

Because they are needed for DNA replication, transcription, and repair, topoisomerases are highly expressed in rapidly proliferating cancer cells and bacteria and therefore are an important target for anticancer drugs (Drlica & Franco, 1988; Liu, 1989; Reece & Maxwell, 1991; Chen & Liu, 1994; Sinha, 1995; Froelich-Ammon & Osheroff, 1995) and antibiotics. The importance of topoisomerase drugs led to characterization of a wide variety of such agents. These drugs differentiate between mammalian and bacterial topoisomerases and between type I and type II topoisomerases. For example, the DNA intercalative drugs *m*-AMSA, ellipticine, daunorubicin, and the epipodophyllotoxin VP-16 only inhibit eukaryotic topoisomerase II. The mechanism of action of VP-16 differs from that of the DNA intercalative drugs, since VP-16 does not bind DNA. Camptothecin, a mammalian topoisomerase I inhibitor, does not inhibit eukaryotic topoisomerase II. The coumarins (e.g., novobiocin) inhibit both prokaryotic and eukaryotic topoisomerase II through their ability to inhibit ATP hydrolysis (Mizuuchi et al., 1978; Staudenbauer & Orr, 1981).

The bacterial topoisomerase II drugs include the quinolone (e.g., oxolinic acid and nalidixic acid), which do not inhibit eukaryotic topoisomerases, and coumarin (mentioned above) antibiotics. The primary target of quinolones is thought to be GyrA (Reece & Maxwell, 1991), the breakage−reunion subunit of bacterial DNA topo II.[1]

The differential sensitivities of the prokaryotic and eukaryotic topoisomerases to particular drugs can be reversed or eliminated by single amino acid changes in the topoisomerase (Huff & Kreuzer, 1990; Liu et al., 1994; Elsea et al., 1995; Fujimori et al., 1995; Hsiung et al., 1995). This

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[1] Abbreviations: topo II, type II topoisomerase; EtBr, ethidium bromide.

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implies that the mechanism of drug inhibition is the same no matter what the source of topoisomerase and that differences in sensitivity to particular drugs may be due to differences in drug−protein binding affinity.

The topoisomerase drugs are called topoisomerase poisons because they induce cell toxicity apparently by stabilization of covalent cleavage complexes (Drlica & Franco, 1988; Liu, 1989; Reece & Maxwell, 1991; Chen & Liu, 1994; Sinha, 1995; Froelich-Ammon & Osheroff, 1995). The DNA intercalative topoisomerase poisons, such as *m*-AMSA, daunorubicin, and ellipticine, induce cleavage intermediates at low concentrations *in vitro* and *in vivo* and inhibit DNA cleavage at higher concentrations (Liu, 1989). Ethidium bromide, a strong intercalator that does not induce cell toxicity at low concentrations, does not induce topoisomerase cleavage intermediates but does inhibit topoisomerase activity (Tewey et al., 1984; Liu, 1989).

To further probe the relationship between *Nae*I-L43K and the topoisomerase family, the effects of the different topoisomerase inhibitors on the DNA relaxation activity of *Nae*I-L43K were studied. We report here that the L43K mutation greatly sensitized *Nae*I to the intercalative drugs, that formation of the covalent *Nae*I-L43K−DNA intermediate differs in its response to the intercalative drugs from that of the topoisomerases, and that *Nae*I-L43K covalently links to the 5′ end of the cleaved DNA strand.

EXPERIMENTAL PROCEDURES

Relaxation Assay. The DNA relaxation assay (15 μL) contained 10 mM Tris-HCl (pH 8.0), 20 mM NaCl, 5 mM $MgCl_2$, bovine serum albumin (0.1 mg/mL), and 5.0 mM β-mercaptoethanol. Plasmid pBR322 DNA (11.6 nM) and purified *Nae*I-L43K (as indicated in the figure and table legends) were incubated with topoisomerase inhibitor at 37 °C for 30 min, and the reaction was stopped by addition of SDS to a final concentration of 1%. Reaction mixtures were extracted with 1-butanol to remove drug. Drug was not removed in the experiments shown in Figure 1A to demonstrate relative intercalating abilities. Products were resolved by 1% agarose gel electrophoresis and quantitated by densitometry after staining with ethidium bromide.

End Labeling. *Eco*RI-linearized pBR322 was dephosphorylated with calf intestine alkaline phosphatase and labeled either at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP or at the 3′ end with the large Klenow fragment of *Escherichia coli* DNA polymerase I and [α-^{32}P]-dATP by standard methods.

Quantitative Precipitation. Production of the *Nae*I-L43K−DNA covalent cleavage intermediate was quantitated by precipitation with SDS and KCl as described elsewhere (Liu et al., 1983). Reaction mixtures (50 μL) containing 11.6 nM end-labeled DNA ($\sim$1.2 $\times$ 10^5 cpm/μg) and 0.96 μM *Nae*I-L43K were incubated under relaxation assay conditions and reactions stopped with 100 μL of prewarmed 0.2 M NaOH, 2% SDS, 5 mM EDTA, and salmon sperm DNA (0.5 mg/mL). Protein−DNA complexes were precipitated with 50 μL of 0.25 M KCl, 0.4 M HCl, and 0.4 M Tris-HCl at pH 7.9 on ice for 10 min. Precipitates were centrifuged at 4 °C and then resuspended at 65 °C in 200 μL of 10 mM Tris-HCl (pH 8.0), 100 mM KCl, 1.0 mM EDTA, BSA (50 μg/mL), and salmon sperm DNA (100 μg/mL). The resuspended samples were cooled on ice and recentrifuged and resuspended in 200 μL of water by heating to 65 °C, and their radioactivity was quantitated by liquid scintillation counting.

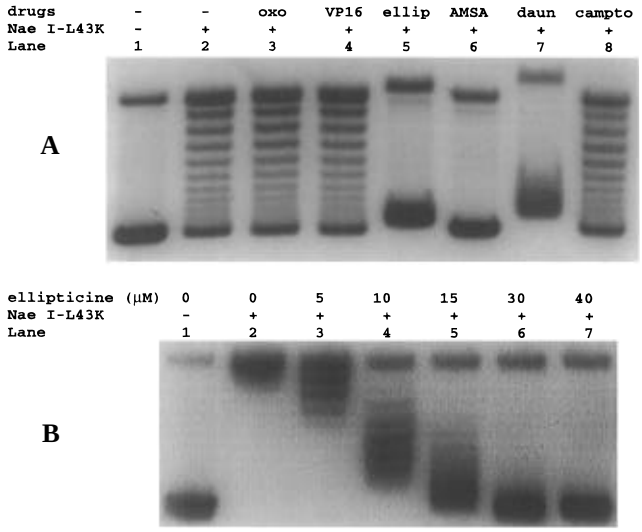

FIGURE 1: Effect of topoisomerase drugs on *Nae*I-L43K. (A) Electrophoresis in the presence of drug. All lanes contain *Nae*I-L43K (0.14 μM), DNA substrate (11.6 nM pBR322), and a 100 μM concentration of the topoisomerase inhibitor indicated except for lane 1, which is a no enzyme control: lane 2, no inhibitor; lane 3, oxolinic acid; lane 4, etoposide (VP16); lane 5, ellipticine; lane 6, *m*-AMSA; lane 7, daunorubicin; and lane 8, camptothecin. The variation in mobility of supercoiled substrate measures the relative intercalation abilities of the various drugs. Reaction conditions for DNA relaxation are described in Experimental Procedures. (B) Electrophoresis after removal of drug with butanol. The effect of increasing concentrations of ellipticine was measured after removal of the drug to eliminate artifacts caused by DNA mobility changes due to drug intercalation. Reaction conditions were identical to those in A, except the *Nae*I-L43K concentration was 0.96 μM: lane 1, DNA control; lane 2, DNA relaxation by *Nae*I-L43K in the absence of drug; and lanes 3−7, inhibition of DNA relaxation by increasing concentrations of ellipticine, as indicated. Reaction conditions are described in Experimental Procedures.

RESULTS AND DISCUSSION

To determine the effect of the topoisomerase drugs on *Nae*I-L43K, a variety of these drugs were tested for their abilities to inhibit *Nae*I-L43K relaxation of pBR322 (Figure 1A). Figure 1A demonstrates complete inhibition of *Nae*I-L43K by a 100 μM concentration of the DNA intercalative drugs ellipticine (lane 5), *m*-AMSA (lane 6), and daunorubicin (lane 7). The drugs were not removed from the reaction products so that we could compare the electrophoretic mobilities of the supercoiled DNAs with and without drug (compare lanes 1 and 5−7). The relative mobilities of the supercoiled DNAs indicate that daunorubicin, ellipticine, and *m*-AMSA prefer to intercalate in that order.

To compare the relative abilities of the different drugs to inhibit *Nae*I-L43K, the relative concentrations of each drug needed to inhibit 50 and 100% of *Nae*I-L43K relaxation of pBR322 (IC^r_{50} and IC^r_{100}, respectively) were determined (Figure 1B and Table 1). IC^r_{50} is defined as the apparent concentration of drug needed to retard the center of the distribution of topoisomer bands, under saturating enzyme conditions (0.96 μM *Nae*I-L43K), approximately half the distance between fully supercoiled and fully relaxed mobilities (between 5 and 10 μM ellipticine in Figure 1B). IC^r_{100} is defined as the concentration of drug needed for complete

Table 1: Concentrations of Topoisomerase Drugs That Inhibit DNA Relaxation by *Nae*I-L43K by 50 and 100%

prokaryotic inhibitor	IC^r_{50} (μM)	eukaryotic inhibitor	IC^r_{50} (μM)	IC^r_{100}[a] (μM)
oxolinic acid	>500[b]	VP-16	>500[b]	>500
nalidixic acid	>500	ellipticine	10 ± 2	35 ± 5
novobiocin	>500	daunorubicin	4 ± 1	6 ± 1
		m-AMSA	25 ± 2	45 ± 5
		camptothecin	>500	>500
		EtBr[c]	nd[d]	5 ± 1

[a] IC^r_{50} and IC^r_{100} were determined as described in the text using the DNA relaxation assay described in Experimental Procedures using 0.96 μM *Nae*I-L43K. Values are mean ± variance from at least two determinations. [b] Showed no inhibition up to 500 μM. [c] Ethidium bromide (EtBr) is a DNA intercalator that inhibits both prokaryotic and eukaryotic topoisomerases but does not stimulate covalent complexes. [d] Not determined.

Table 2: Percentage of End-Labeled Cleaved DNA Coprecipitated in Complex with *Nae*I-L43K[a]

reaction conditions	3′-end-labeled DNA	5′-end-labeled DNA
DNA only	1.0 ± 0.1	0.9 ± 0.1
DNA and *Nae*I-L43K		
without Mg^{2+}	1.0 ± 0.1	0.9 ± 0.1
with Mg^{2+}	5.7 ± 0.2	0.9 ± 0.1

[a] Values are mean ± variance from at least two determinations. Linkage of *Nae*I-L43K to either 3′- or 5′-end-labeled DNA was determined by quantitative precipitation as described in Experimental Procedures.

inhibition (between 30 and 40 μM ellipticine in Figure 1B). We used identical reaction conditions, identical concentrations of pBR322, and identical concentrations of *Nae*I-L43K in each reaction. Therefore, a lower IC^r_{50} and IC^r_{100} for one particular drug compared to another indicates qualitatively that the particular drug is a better inhibitor of *Nae*I-L43K topoisomerase activity. Reaction conditions were chosen to completely relax pBR322 in the absence of drug (Figure 1B, lane 2). The drugs were removed from the reaction mixtures by butanol extraction prior to electrophoresis for quantitation of reaction products. Removal of drug gave supercoiled DNA with mobilities identical to that without drug present during the reaction (Figure 1B, compare lanes 1 and 7). Amsacrine (*m*-AMSA), daunorubicin, and ellipticine were the only drugs tested found to significantly inhibit DNA relaxation by *Nae*I-L43K at relatively low drug concentrations (Table 1). Daunorubicin was most effective, followed in order by ellipticine and *m*-AMSA. This is also the order of their DNA-intercalating abilities as shown above. Intercalation appeared to be a common factor in *Nae*I-L43K inhibition. Therefore, we tried ethidium bromide, a good intercalator that inhibits topoisomerases by intercalation into DNA and doesn't stimulate formation of the covalent topoisomerase−DNA intermediate (Tewey et al., 1984). Ethidium bromide at low concentrations inhibited *Nae*I-L43K DNA relaxation (Table 1) and formation of the covalent *Nae*I-L43K−DNA intermediate (not shown).

To determine the step in *Nae*I-L43K topoisomerase activity inhibited by the DNA intercalative drugs, production of the covalent intermediate was measured by quantitative precipitation of the labeled intermediate with SDS and KCl as described in Experimental Procedures. NaOH and SDS were used to interrupt the reaction of *Nae*I-L43K first with a DNA fragment labeled only at the 5′ end of one strand and second with the same DNA fragment labeled only at the 3′ end of

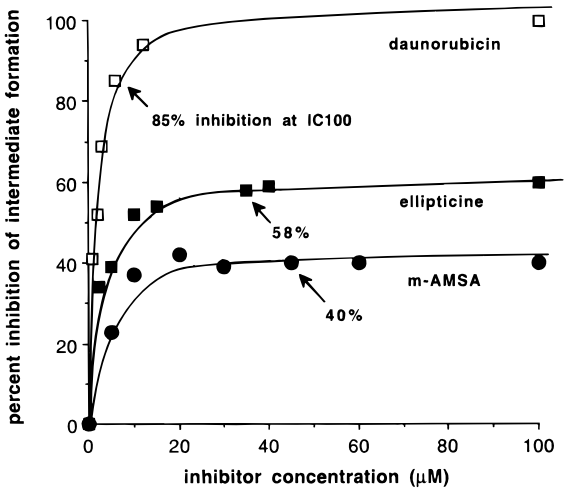

FIGURE 2: Inhibition of formation of the covalent *Nae*I-L43K−DNA intermediate by the eukaryotic topoisomerase II inhibitors: □, daunorubicin; ■, ellipticine; and ●, *m*-AMSA. The percent inhibition at the respective IC^r_{100} for each drug is indicated. The covalent intermediate was quantitated using quantitative precipitation with 3′-end-labeled DNA in the presence of the indicated concentration of drug, as described in Experimental Procedures.

that same strand. The NaOH was to denature the cleavage product. The SDS−protein complex was precipitated with KCl. As shown in Table 2, end-labeled DNA was detected in the precipitated covalent intermediate only when 3′-end-labeled DNA was used. This result demonstrates that *Nae*I-L43K covalently bonds with the newly created 5′ end of the cleaved DNA strand. The 5′ end of the DNA is used for covalent bond formation by eukaryotic type II topoisomerase, prokaryotic topoisomerase (Wang, 1985), and the resolvase/invertase family of recombinases (Craig, 1988). The 3′ end of the DNA is the choice of eukaryotic type I topoisomerase (Wang, 1985) and the integrase family of recombinases (Craig, 1988).

Using the quantitative precipitation assay, ellipticine and *m*-AMSA, over the full range of concentrations studied, were found to only partially inhibit formation of the covalent *Nae*I-L43K−DNA intermediate (Figure 2). This inhibition demonstrates that these drugs partially inhibit the cleavage step in the *Nae*I-L43K DNA relaxation pathway. Therefore, the intercalative drugs at their IC^r_{100} must also inhibit steps following the cleavage step in the *Nae*I-L43K DNA relaxation pathway to achieve complete inhibition of DNA relaxation. Daunorubicin, a strong DNA intercalator (Figure 1A), was the only drug tested that could completely inhibit the cleavage step. The drugs at low concentrations generally enhance production of the covalent topoisomerase−DNA intermediate (Drlica & Franco, 1988; Liu, 1989; Reece & Maxwell, 1991; Chen & Liu, 1994; Sinha, 1995; Froelich-Ammon & Osheroff, 1995) by interfering with the strand passage and religation steps of the relaxation pathway. This interference traps a reversible "cleavage complex" that can be isolated as a covalent intermediate by treatment with SDS. Ethidium bromide does not enhance production of the covalent topoisomerase−DNA intermediate; this is believed to be due to its DNA intercalative mechanism of topoisomerase inhibition (Tewey et al., 1984). Thus, our results point to a difference between *Nae*I-L43K and topoisomerase inhibition. The intercalative drugs partially inhibit DNA cleavage and also inhibit steps after DNA cleavage in the *Nae*I-L43K reaction pathway apparently without trapping the

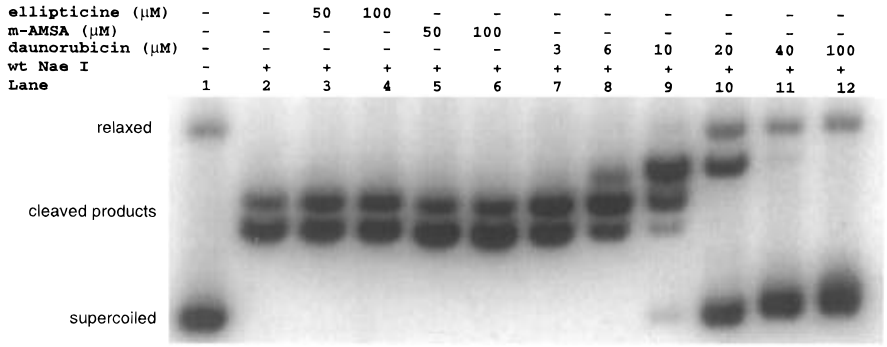

FIGURE 3: Effect of *m*-AMSA, ellipticine, and daunorubicin on DNA cleavage by *Nae*I: lane 1, pBR322 DNA only; lane 2, with *Nae*I; lanes 3 and 4, 50 and 100 μM ellipticine; lanes 5 and 6, 50 and 100 μM *m*-AMSA; and lanes 7−12, 3, 6, 10, 20, 40, and 100 μM daunorubicin. Cleavage reactions were identical to relaxation assays except for 3.7 nM *Nae*I.

cleavage complex as found for the topoisomerases. The most likely explanation for this difference is twofold: drug destabilization of the *Nae*I-L43K−DNA complex and the transient nature of the covalent *Nae*I-L43K−DNA intermediate. Intercalating drugs destabilize the interaction of restriction enzymes with DNA (Goppelt et al., 1981; Hardwick et al., 1984). *Nae*I-L43K is only one amino acid change away from a restriction enzyme. Therefore, drug intercalation can inhibit formation of the covalent intermediate. In addition, restriction enzymes like *Nae*I have evolved to use water to displace the covalent protein−DNA intermediate, whereas the topoisomerases have evolved to use the other cleaved end of the DNA for displacement. Thus, the covalent intermediate of *Nae*I-L43K may be more transient than that of the topoisomerases. The transient nature of the intermediate predicts accumulation of nicked DNA during the relaxation reaction as the covalent intermediate is hydrolyzed. Nicked DNA was observed to accumulate during *Nae*I-L43K DNA relaxation reactions without inhibitor present (Jo & Topal, 1995). The nicked product accumulated even in the presence of concentrations of drug that completely inhibited DNA relaxation (Figure 2B, compare lanes 1 and 7). This accumulation of nicked product in the presence of drug at IC^r_{100} implies that drug inhibited religation better than it inhibited formation of the covalent intermediate.

It is also possible that the difference in inhibition of topoisomerase and *Nae*I-L43K is related to the sequence specificity of *Nae*I-L43K. It has been proposed that topoisomerase-bound sequences must be cleaved first to enable drug intercalation at the cleavage site (Capranico et al., 1990; Freudenreich & Kreuzer, 1993), which then inhibits DNA strand passage and religation by topoisomerase. Therefore, it is possible that the difference in the drug effect on the *Nae*I-L43K versus topoisomerase covalent intermediate lies with the sequence specificity of *Nae*I for a GC-rich sequence that may already be a good target for intercalation (Quigley et al., 1980; Timsit et al., 1989; Kamitori & Takusagawa, 1992).

Whereas the effects of the DNA intercalative drugs on *Nae*I-L43K were dramatic, the same drugs showed much less ability to inhibit wild type *Nae*I (Figure 3). *m*-AMSA and ellipticine, at concentrations up to 100 μM, showed almost no inhibition of DNA cleavage by *Nae*I. Daunorubicin at concentrations higher than 40 μM showed complete inhibition of *Nae*I cleavage reactions. Since the only difference between *Nae*I and *Nae*I-L43K is the single amino acid change, substituting lysine for leucine-43 sensitized DNA cleavage to inhibition by these drugs just as it transformed *Nae*I activity from restriction endonuclease to topoisomerase and recombinase.

We have little evidence about the basis for the hypersensitivity to the DNA intercalative drugs of *Nae*I-L43K compared to that of wild type *Nae*I. In principle, however, it could be due either first to an increase in affinity of the drugs for the binding pocket composed of protein, including at least in part the ligase-like active site, and DNA or second to a reduced ability of *Nae*I-L43K to recognize the drug−DNA complex. The first possibility, less likely because of the lack of drug-stimulated formation of the covalent *Nae*I-L43K−DNA intermediate, would be consistent with models of the topoisomerase drug binding pocket (Zechiedrich & Osheroff, 1990; Roca et al., 1993; Freudenreich & Kreuzer, 1994), with the ability of single amino acid changes to reverse the sensitivity of topoisomerases to certain drugs (Huff & Kreuzer, 1990; Liu et al., 1994; Elsea et al., 1995; Fujimori et al., 1995; Hsiung et al., 1995), and with evidence that the intercalative drugs can interact with topoisomerase in the absence of DNA (Nabiev et al., 1994; Froelich-Ammon et al., 1995). We favor the second possibility. Considering the radical change in activity brought on by the L43K amino acid change (Jo & Topal, 1995), it is not unreasonable that *Nae*I and *Nae*I-L43K have significantly different abilities to recognize the drug−DNA complex. Assuming the likely scenario that *Nae*I evolved from a topoisomerase/ligase, the K43L change probably sacrificed ligase activity to gain an efficient sequence-specific endonuclease function needed by the cell for protection against foreign DNAs. The opposing L43K change gains ligase activity but may alter and destabilize the enzyme−DNA complex. Drug intercalation may further destabilize the enzyme−DNA complex (Goppelt et al., 1981) so that catalysis is no longer detectable.

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