Evidence for Mutations That Break Communication between the Endo and Topo Domains in *Nae*I Endonuclease/Topoisomerase†

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ABSTRACT: *Nae*I is a type IIe endonuclease that interacts with two DNA recognition sequences to cleave DNA. One DNA sequence serves as a substrate and the other serves to activate cleavage. *Nae*I is divided into two domains whose structures parallel the two functionalities recognized in *Nae*I, endonuclease and topoisomerase. In this study, we report evidence for mutations that break interdomain functional communication in a *Nae*I−DNA complex. Deletion of the initial 124 amino acids of the N-terminal domain of *Nae*I converted *Nae*I to a monomer, consistent with self-association being mediated by the Endo domain. Deletions within a small region of the C-terminal DNA binding domain of *Nae*I (amino acids 182−192) altered the recognition by *Nae*I of sequences flanking the *Nae*I recognition sequence. Substituting Ala for Arg182 within this region had no apparent effect on DNA binding but greatly reduced the extent of DNA cleavage even though it is not part of the catalytic Endo domain. Substituting Ala for Ile185 reduced the extent of DNA binding about 1000-fold. Substituting Ala for Lys189 altered flanking sequence recognition. Residues 182−192 are away from the Endo domain responsible for cleavage and also face away from the modeled DNA binding faces of the apoprotein crystal structure. We propose that residues 182−192 are part of a web that mediates the flow of information between the *Nae*I Endo and Topo domains.

*Nae*I endonuclease is a homodimer of a 317-amino acid polypeptide, is related to the topoisomerase and recombinase families of proteins by both structure and function, but shows no sequence similarities to either protein family (*1*). *Nae*I is divided into two domains connected by a hinge region (*2*, *3*). The N-terminal "Endo" domain includes amino acids 10−162 and folds such as the core structure of the restriction endonucleases (*3*). The Endo domain contains the endonuclease catalytic region, and mutations within the putative Mg^{2+} binding signature reduce or abolish catalysis without a loss in DNA binding (*4*). The C-terminal "Topo" domain includes amino acids 172−311 and contains a CAP motif present in many DNA binding proteins, including topoisomerase IA (*3*). *Nae*I functions in vitro as either an endonuclease or a topoisomerase/recombinase depending on the identity of the amino acid at *Nae*I position 43 (*5*). Like the topoisomerases and recombinases (*6*−*10*), *Nae*I must bind at least two DNA sequences for activity (*11*−*15*). Single *Nae*I recognition sequences can resist cleavage, and the resistance is overcome by addition of an activator sequence either cis or trans to the substrate sequence (*11*). Activator DNA and resistant substrate DNA are both composed of the same *Nae*I core recognition sequence (GCC/GGC) but have different flanking sequences (*11*, *12*). Activator DNA activates its own cleavage in a sigmoid, concentration-dependent manner (*12*), demonstrating cooperative interaction and that *Nae*I is an allosteric protein. Both kinetic analysis of DNA cleavage by *Nae*I and the *Nae*I crystal structure imply that activator and substrate DNAs bind separate domains of *Nae*I (*3*, *11*, *12*).

To determine how the *Nae*I Topo and Endo domains communicate to enable cleavage, we constructed deletion and point mutations in *Nae*I to find mutations that appear to break communication between the Endo and Topo domains. Deletion analysis is useful for identifying regions of a protein involved with specific functional aspects of that protein. For example, deletion analysis has been used to identify regions of DNA topoisomerases that are either required or dispensable for functional aspects such as DNA relaxation, nuclear localization, and the structural integrity of the protein (*16*−*18*). Deletion of the first 124 residues converted *Nae*I to a monomer, which is consistent with mediation of self-association by the Endo domain (*3*). A region within the Topo domain was identified where mutations affect relative recognition of the flanking sequences that differentiate between substrate and activator DNAs. A point mutation in this region greatly reduced the extent of DNA cleavage without reducing the level of DNA binding even though the mutation lies far from the *Nae*I region that is responsible for phosphodiester hydrolysis.

MATERIALS AND METHODS

Materials. Amylose resin was purchased from New England Biolabs (Beverly, MA). *Escherichia coli* strain CAA1 [F^- *e*14$^-$ (*mcrA*$^-$) *lacY*1 or *D(lac)*^{6} *SupE*44 *galK*2 *galT*22 *mcrA* *rfbD*1 *mcr*Ba *hsd* (*rk*$^-$*mk*$^+$) M·*MspI*$^+$] and plasmid pNEB786, containing the *Nae*IR gene, were obtained from New England Biolabs.

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Protein Purification. Substitution and deletion mutants of *Nae*I constructed in pNEB786 were amplified by PCR (*19*) and inserted into plasmid vector pMALc2 (New England Biolabs). The nucleotide sequences of the amplified DNA were determined by the DNA sequencing facility at University of North Carolina at Chapel Hill to ensure that no unwanted mutations had been introduced by PCR. DNA was inserted downstream from the malE gene, which encodes maltose-binding protein (MBP), in pMALc2 so that expression resulted in production of MBP fused to the N-terminus of each *Nae*I derivative. MBP−*Nae*I (wild-type *Nae*I fusion protein) expression from pMALc2−*Nae*I was previously described (*2*). Plasmid constructs were transformed into *E. coli* strain CAA1, and Amp^r colonies were selected by plating on LB-agar plates with ampicillin (100 μg/mL). Single colonies were selected, and the cells were grown at 25 °C in Luria broth. Expression of the protein was induced by adding IPTG to a final concentration of 0.2 mM at an A_{600} of 0.4−0.6. Cells were harvested by centrifugation 6 h after induction. MBP−*Nae*I protein variants were purified from cell paste to approximately 80% homogeneity using amylose resin chromatography as previously described (*2*). Following purification, samples were centrifuged at 30000*g* for 1 h at 4 °C to remove any particulate matter. Mutants are designated in the text as MBP−*Nae*I(25−317), where the expression in parentheses means that amino acid residues 25−317 of *Nae*I have been fused to MBP. MBP−*Nae*I(R182A) means that *Nae*I protein containing an arginine to alanine substitution at position 182 has been fused to MBP.

DNA Cleavage Assays. DNA cleavage activities of *Nae*I constructs were routinely monitored using pBR322 DNA as a substrate, as previously described (*1*). One unit of activity is defined as the amount of protein required to linearize 0.5 μg of pBR322 DNA at 37 °C in 1 h in a reaction volume of 15 μL. The lowest level of detection was <0.001 unit/ng of protein in a reaction mixture containing 1 μg of protein.

Gel Mobility Shift Assays. Reaction and electrophoresis conditions and analysis of gel mobility shift assays are previously described (*2*). For the determination of apparent K_D values by gel mobility shift assay (*20*), the protein concentration was titrated against 0.2 nM DNA. The apparent K_D is defined as the protein concentration (monomeric form) required to shift 50% of the probe DNA. Assays were performed in duplicate and the results averaged (± values represent the range). The K_D was assigned a value of >2000 nM if 2 μM protein shifted less than 50% of the DNA probe. In gel mobility shift assays not used for the apparent K_D determination, 10 nM DNA was incubated with a given protein concentration. The 14-mer DNA fragments used in gel mobility shift assays were previously described (*12*): GC-rich (GGGTGCCGGCAGGG), AT-rich (TTTCGCCG-GCGTTT), and noncognate (GGGCGCCGGGGGGG). DNA fragments were 5′-end-labeled using T4 polynucleotide kinase and [α-^{32}P]ATP.

Self-Association. Gel permeation chromatography was used to determine the self-association status of MBP−*Nae*I variants. Protein (500 μL of a 1 mg/mL solution) was loaded onto a column (30 cm in height, 1.5 cm internal diameter) filled with either Sephacryl G-200 or Sephacryl G-300 resin. The column buffer was 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 50 mM sodium chloride, 5% glycerol, and 2 mM maltose. The flow rate was 0.4 mL/min. The eluted protein was detected by absorbance at 280 nM. The elution patterns of MBP−*Nae*I and MBP−*Nae*I(169−317) were used as markers for determining the self-association status of other MBP−*Nae*I variants. We previously showed that in solution MBP−*Nae*I exists as dimer and multisubunit aggregates in solution and that MBP−*Nae*I(169−317) is a monomer in solution (*2*).

RESULTS

Identification of the N-terminal domain of *Nae*I as the endonuclease or Endo domain implies that the C-terminal or Topo domain is the activator domain. Binding of DNA at the Topo domain enables DNA cleavage at the Endo domain. To identify structural elements of *Nae*I involved with communication between the two domains, we expressed a set of *Nae*I protein deletion mutants (Figure 1). *Nae*I deletion mutants expressed as fusion protein were purified and assayed for DNA cleavage activity, DNA binding affinity, and self-association (Figure 1), as described in Materials and Methods. The relative abilities of the *Nae*I protein fragments to bind two separate 14-mer DNA fragments (GC-rich and AT-rich) were determined (apparent K_D values in Figure 1). Both DNA fragments contain the core *Nae*I recognition sequence and differ only in flanking GC-rich and AT-rich nucleotide sequences. The apparent binding constants (K_D) were measured in duplicate and are reported as the average value (± *n* represents range). Cleavage activity was below the level of detection for every deletion mutant that was constructed (<0.001 unit/ng of protein).

MBP−NaeI Fusion Proteins. To determine if fusion with MBP affected the DNA binding ability of wild-type *Nae*I, we determined the binding affinity of MBP−*Nae*I and wild-type *Nae*I [purified as previously described (*2*)] toward GC-rich and AT-rich DNAs. Apparent K_D values for *Nae*I with GC-rich and AT-rich DNAs were 2.7 ± 0.2 and 5.7 ± 0.3 nM, respectively. These values are similar (less than 2-fold different) to the values for MBP−*Nae*I, 1.9 ± 0.3 and 4.0 ± 0.2 nM, respectively (Figure 1). Similarly, fusing MBP to *Nae*I has no apparent effect on *Nae*I DNA cleavage activity (*2*).

NaeI Protein Aggregation. Deletion of the initial 124 amino acids of *Nae*I converted MBP−*Nae*I to the monomer (Figure 1). Other deletion mutants resulted in the formation of soluble aggregates only if amino acids 81−124 in the N-terminal domain were present (Figure 1). To determine if fusion with MBP affects the self-association status of *Nae*I mutants, *Nae*I(169−317) and *Nae*I(1−145) were expressed both fused with and free of MBP. *Nae*I(169−317) and MBP−*Nae*I(169−317) were purified, and both were found to exist as monomers in solution (Figure 1 and ref *2*). MBP−*Nae*I(1−145) was a soluble aggregate in solution. Free *Nae*I-(1−145) formed an insoluble aggregate (results not shown). The different apparent solubility implies that MBP serves to increase the solubility of misfolded forms of *Nae*I. Similar increases in solubility have been reported for other proteins fused to MBP (*21*).

Region 182−192. Deleting 193 amino acids from the N-terminus resulted in a large decrease in the DNA binding affinity compared to deleting 181 amino acids [Figure 1; compare MBP−*Nae*I(193−317) and MBP−*Nae*I(181−317)]. Mutants MBP−*Nae*I(184−317), MBP−*Nae*I(187−

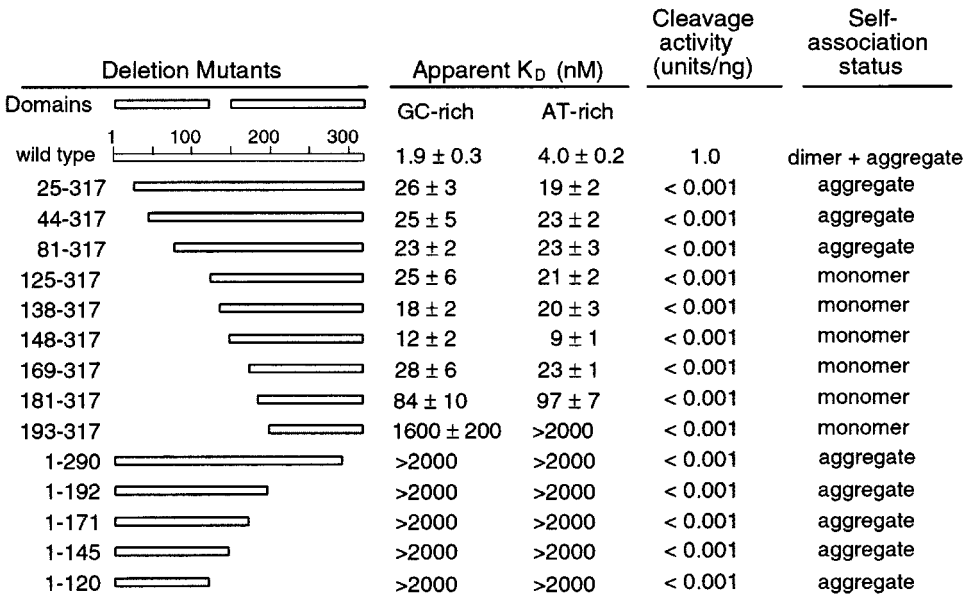

FIGURE 1: Effects of deletion mutations on DNA cleavage activity, apparent DNA binding coefficients (K_D) toward GC-rich and AT-rich DNAs, and oligomeric association of *Nae*I protein. The relative locations of *Nae*I domain structures previously identified by limited proteolysis (*2*) are shown at the top of the first column, as a point of reference. The *Nae*I amino acid residues contained in each fusion protein relative to the full-length *Nae*I polypeptide are indicated on the left. K_D is reported as the average of two determinations with ± *n* values representing the range.

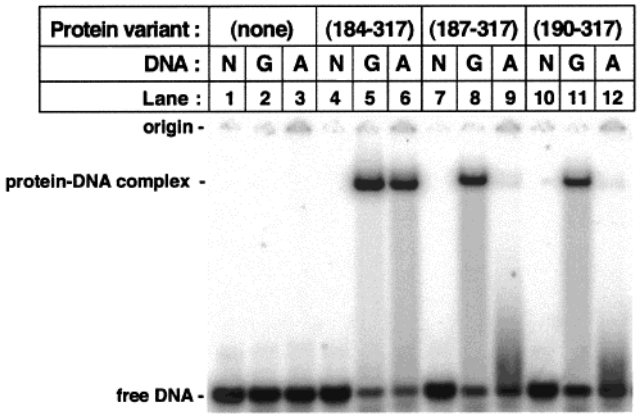

FIGURE 2: Gel mobility shift assay comparing DNA binding properties of MBP−*Nae*I(184−317), MBP−*Nae*I(187−317), and MBP−*Nae*I(190−317). The DNA concentration was 10 nM, and the protein concentration of each MBP−*Nae*I mutant was 300 nM. The assays were performed as described in Materials and Methods. The gel is 6% polyacrylamide, and the bands were visualized using a Storm PhosphorImager. The DNA probes were either the noncognate DNA fragment, differing from the cognate fragment by one base pair (N); the GC-rich DNA fragment (G); or the AT-rich DNA fragment (A).

317), and MBP−*Nae*I(190−317) were constructed to dissect the region between residues 181 and 193 because it lies away from the proposed DNA binding faces (*3*) of *Nae*I. The mutant proteins were purified and their relative DNA binding properties compared by gel mobility shift assay (Figure 2). Deletion mutant MPB−*Nae*I(184−317) showed a small preference for binding GC-rich DNA (1.2-fold). That preference increased with each further deletion in this region (Figure 2 and Table 1).

Noncognate DNA, which differs from the cognate *Nae*I recognition sequence by a single nucleotide (GCCGGG versus GCCGGC), was used to probe for nonspecific DNA binding under our conditions. Nonspecific DNA binding was not detected from MBP−*Nae*I(184−317), MBP−*Nae*I(187−317), and MBP−*Nae*I(190−317) (Figure 2, lanes 4, 7, and 10). Similarly, nonspecific DNA binding was not detected from full-length MBP−*Nae*I or from any other *Nae*I mutant protein used in this study (data not shown).

Table 1: DNA Cleavage Activity and Apparent K_D Values of Deletion Mutants MBP−*Nae*I(184−317), MBP−*Nae*I(187−317), and MBP−*Nae*I(190-317)

deletion mutant	GC-rich[a] K_D (nM)	AT-rich K_D (nM)	bias[b]
*Nae*I(184−317)	330 ± 40	360 ± 30	1.2 ± 0.2
*Nae*I(187−317)	480 ± 40	1100 ± 200	2.4 ± 0.7
*Nae*I(190−317)	500 ± 20	2600 ± 400	5.3 ± 1.1

[a] GC-rich and AT-rich refer to 14mer DNA fragments containing the *Nae*I recognition sequence with GC- and AT-rich flanking sequences, respectively, as defined in Materials and Methods. [b] Bias of MBP−*Nae*I deletion mutant for binding GC-rich DNA over AT-rich DNA equals (AT-rich K_D)/(GC-rich K_D), where GC-rich and AT-rich K_Ds are apparent K_Ds determined as described in Materials and Methods.

Alanine Scanning. The involvement of individual amino acid residues from positions 182−190 within the context of the full-length *Nae*I polypeptide was investigated using alanine-scanning mutagenesis (except position A188). The mutant MBP fusion proteins were assayed for cleavage activity and DNA binding affinity (Table 2). A temperature-sensitive mutation in this region (A188T) was previously seen to result in a reduced level of DNA binding (*4*). Three amino acid substitutions within this region (R182A, I185A, and K189A) had significant effects on either catalysis or binding and catalysis (Table 2). The relative location of these residues within the apo-*Nae*I crystal structure (*3*) is shown in Figure 3.

DISCUSSION

*Nae*I endonuclease/topoisomerase is a two-domain enzyme that requires DNA binding at one domain to activate DNA cleavage at the other domain. The activator can also serve

Table 2: Effect of Alanine Substitution Mutations on DNA Cleavage Activity and Apparent K_D Values of MBP−*Nae*I

mutant	DNA cleavage activity (units/ng)	GC-rich[a] K_D (nM)	AT-rich K_D (nM)	bias[b]
wild type	1.0	1.9 ± 0.3	4.0 ± 0.2	2.1 ± 0.5
R182A	<0.01	2.5 ± 0.5	3.9 ± 0.3	1.6 ± 0.5
D183A	0.5	1.9 ± 0.3	5.6 ± 0.6	3.0 ± 0.9
Q184A	1.0	1.5 ± 0.3	3.7 ± 0.4	2.6 ± 0.8
I185A	<0.01	1500 ± 200	3100 ± 400	2.1 ± 0.6
F186A	0.05	18 ± 4	32 ± 8	2.0 ± 0.9
S187A	0.5	2.0 ± 0.4	5.8 ± 0.4	3.1 ± 0.8
K189A	0.05	28 ± 5	260 ± 50	9.4 ± 4.1
S190A	0.5	6.3 ± 0.7	8.0 ± 1.0	1.3 ± 0.3

[a] See the footnote of Table 1. [b] Bias defined in footnote b of Table 1.

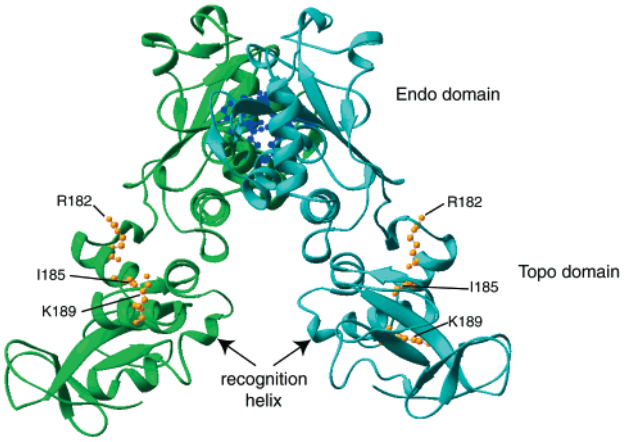

FIGURE 3: Ribbon diagram representing the three-dimensional structure of the *Nae*I dimer. One subunit is shown in light blue and the other in green. The dimer associates in a "head-to-head" manner. The Endo domain of each subunit is shown toward the top of the figure. The Endo domains interact to mediate dimerization and form the catalytic site. Amino acid residues E70, D86, D95, and K97 are defined by structural superposition to be involved with metal binding and catalysis (*3*) and are shown in dark blue as ball-and-stick models. The putative recognition helix of a helix−turn−helix DNA binding motif within the Topo domain of each subunit is indicated. These two recogntion helices face each other with dyad symmetry to form a cleft for DNA binding, which is the putative activator DNA binding site (*3*). Residues R182, I185, and K189 are shown in yellow as ball-and-stick models. Each of these residues is at least 20 Å from the catalytic site, yet R182A reduces the level of DNA cleavage without affecting DNA binding. K189 comes closest to the putative activator binding site and is about 12 Å from the nearest residue in the recognition helix of the helix−turn−helix DNA binding motif.

as the substrate. Cleavage of the activator shows a sigmoidal dependence on activator concentration, demonstrating cooperativity between domains, and reveals the allosteric nature of *Nae*I DNA cleavage (*12*). Deletion and point mutations were constructed to investigate communication between the Endo and Topo domains of *Nae*I to achieve cleavage.

NaeI Protein Aggregation. Deletion mutants containing amino acids 81−124 gave soluble aggregates of *Nae*I protein. The apo-*Nae*I crystal structure shows that residues 81−124 are involved in formation of the hydrophobic core of the Endo domain and contain the putative *Nae*I catalytic Mg^{2+} binding site (*3*). Perturbations in structure that expose this region probably result in aggregation due to exposure of internal hydrophobic amino acids. The aggregates apparently are soluble due to the presence of MBP (*21*). Aggregation due to partial N-terminal deletions of the Endo domain appears to have little influence on the Topo domain. The aggregated mutants with intact Topo domains showed strong sequence-specific DNA binding (Figure 2 and Table 1).

Deletion of the first 124 amino acids of *Nae*I to give deletion mutant *Nae*I(125−317) converted *Nae*I to a monomer in solution. The result is consistent with the Endo domain mediating self-association. The apo-*Nae*I crystal structure shows monomer interactions mediated by Endo domain α-helix 4 which contains amino acids 59−77 (*3*). The Endo domain of *Nae*I is unstable on its own. It forms aggregates that show no detectable DNA cleavage or DNA binding affinity (Figure 1). Aggregation and removal of the activating Topo domain probably interfere with Endo domain function: The apo-*Nae*I crystal structure shows that the Endo domain contains the substrate binding and catalytic sites (*3*). Deletions within the *Nae*I Endo domain gave 12−15-fold decreases in the binding affinity for GC-rich DNA versus approximately 6-fold decreases in binding affinity for AT-rich DNA relative to that of wild-type *Nae*I. Deletion of the entire Endo domain and most of the hinge region decreased the level of binding less than partial deletions of the Endo domain (Figure 1). This difference implies that deletions within the Endo domain result in protein distortion and unfolding so that the domain remnants interfere with the accessibility of DNA to bind to the *Nae*I Topo domain. The partial deletions of the Endo domain contained DNA recognition loop R2 (*3*). Deletion of that loop to complete deletion of the Endo domain restored a small amount of DNA binding. H5, B8, and the hinge loop attach R2 to the Topo domain. It is possible that R2 by means of this relatively unstructured and long tether can swing down and partially compete with and interfere with Topo domain DNA binding. Thus, removal of R2 shows a small improvement in DNA binding for the Topo domain.

Region 182−192. Deletion mutant *Nae*I(1−292), which lacks 27 amino acids from the C-terminus of the Topo domain, abolished detectable DNA binding (Figure 1). Deletions from the other end of the Topo domain [see deletion mutants *Nae*I(148−317), -(169−317), and -(181−317) in Figure 1] showed a gradual increasing loss of DNA binding. It was not until removal of amino acids 182−192 that the extent of DNA binding was greatly reduced. Additional deletions in this area and quantitation of binding to different DNA fragments demonstrate that this region (182−192) has an effect on the relative binding of GC-rich versus AT-rich DNAs.

Alanine Scanning. Alanine was substituted for each of the amino acids from positions 182−190 (except A188). Alanine substitution for amino acids R182, I185, and K189 altered *Nae*I interactions with DNA. R182A reduced the level of DNA cleavage more than 100-fold with no significant effect on DNA binding. I185A reduced the level of binding to both GC-rich and AT-rich DNAs nearly 1000-fold, with concomitant loss of cleavage activity. K189A reduced the level of binding of AT-rich DNA 65-fold and GC-rich DNA 14.7-fold, resulting in a 4.5-fold bias over the wild type toward binding GC-rich DNA.

Amino acids 182−192 are positioned in the apo-*Nae*I crystal structure away from the two DNA binding faces of *Nae*I (Figure 3). I185 forms hydrophobic interactions with other side chains at the interior of the Topo domain and may play a significant structural role in forming the domain.

K189A may either indirectly affect interactions between *Nae*I and flanking DNA sequences, by affecting the general structure of the Topo domain, or directly be involved with flanking sequence interactions. K189 is about 12 Å away from the nearest amino acid residue within the putative DNA recognition helix of the helix−turn−helix DNA-binding motif, placing it within reasonable proximity to directly interact with the flanking DNA sequence. However, the entire structure and distances may significantly change during DNA binding. In the apo*Nae*I structure (*3*), R182 in the Topo domain is more than 20 Å away from the putative catalytic site in the Endo domain of *Nae*I. Yet, R182A reduces the level of cleavage more than 100-fold with no significant effect on DNA binding. It is possible that R182 is directly involved with DNA cleavage, but it would require a drastic conformational change in protein structure upon DNA binding. More likely, substitution of R182A blocks the pathway through which the binding of activator DNA induces cleavage activity by a conformational change in the protein. R182 is found within a region of the protein shown here to be important to interactions between *Nae*I and flanking DNA sequences (Figure 2 and Table 1). It is these interactions that determine whether a DNA may mediate activation of DNA cleavage (*12*), presumably by transmitting a conformational change to the catalytic site. We interpret these results to mean that this region (182−192) of the *Nae*I protein is involved in the transfer of information between the two *Nae*I domains.

Restriction Endonucleases. In the crystal structure of *Fok*I, the active site of *Fok*I is held away from the DNA (*22*). This conformation may explain how *Fok*I regulates its cleavage activity until needed by requiring a conformational change in the protein upon DNA binding to bring the active site to the DNA (*22*). The majority of type II restriction endonucleases whose structures are known, such as *Bam*HI, *Eco*RI, *Eco*RV, and *Pvu*II, are homodimers such as *Nae*I and contain a single DNA binding site. Specific binding of DNA at this site leads to changes in both protein and DNA conformations (induced fit) that leads to activation of the catalytic center located at the binding site (*23*). The Endo domain of *Nae*I has a high degree of structural similarity to the DNA binding site and catalytic motif of the type II endonucleases (*3*). For *Nae*I, specific binding at the catalytic site is insufficient for cleavage; instead, binding of a second DNA (at the activator binding site) enables the enzyme to cleave substrate, presumably by conformation changes transmitted to the catalytic site from the Topo domain.

Multidomain Proteins. In allosteric enzymes, effector binding to one subunit may mediate conformational changes in a closely associated catalytic subunit to affect catalysis. Transfer of information may also take place by interdomain communication within the same polypeptide of a multidomain protein. Known examples include methionyl-tRNA synthetase (*24*), histidyl-tRNA synthetase (*25*), 6-phospho-fructo-2-kinase/fructose-2,6-biphosphatase (*26*), and groEL (*27*) among others. In some cases, such as methionyl-tRNA synthetase, specific amino acid residues involved with transfer of information have been identified. Methionyl-tRNA synthetase has two major domains: the N-terminal or active site domain, which interacts with the acceptor arm of $tRNA^{Met}$ and performs aminoacylation, and the C-terminal domain, which interacts with the anticodon-containing arm of the tRNA (*24*). Interactions between the C-terminal domain and the anticodon-containing arm of the tRNA transmit conformational changes to the active site domain, allowing for efficient aminoacylation. Specific amino acid residues have been identified that do not directly interact with the anticodon stem−loop portion or the acceptor arm, yet block efficient aminoacylation. These amino acids are apparently involved with a network that facilitates communication between the C-terminal domain and the active site domain (*24*). Similarly, in this study we have identified a region of *Nae*I that may be part of a network or web of amino acids that mediate transfer of information between the Topo (activator) domain and the Endo (catalytic) domain.

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