Formation of a Cleavasome: Enhancer DNA-2 Stabilizes an Active Conformation of *Nae*I Dimer

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ABSTRACT: Cleavage of DNA by *Nae*I-type restriction enzymes is stimulated by a DNA element with affinity for the activator site of the enzyme: a cleavage-enhancer DNA element. Measurements of the mobility of *Nae*I activity in comparison with protein standards on gel permeation columns and glycerol gradients demonstrated that *Nae*I, without enhancer, can form a 70 000 MW dimer. The dimer, however, is inactive: it could not cleave the "resistant" *Nae*I site in M13mp18 DNA in the absence of enhancer. In cleavage assays, enhancer stimulated either DNA nicking or DNA cleavage, depending upon *Nae*I concentration, and reduced the *Nae*I concentration required for the transition from nicking to cleavage activity. A gel mobility–shift assay of the interaction of *Nae*I with enhancer showed the formation of two complexes. Results using different sized DNAs and different percentage acrylamide gels for gel mobility–shift analysis implied that the two complexes were caused by *Nae*I monomer and dimer structures rather than one and two DNA binding. Dimer formation increased with the affinity of enhancer for *Nae*I. UV cross-linking "captured" the *Nae*I–enhancer complex; electrophoretic analysis of the cross-linked products showed *Nae*I dimer bound to enhancer. These results imply a model for cleavage enhancement in which enhancer binding stabilizes an active *Nae*I dimer conformation ("cleavasome") that cleaves both DNA strands before dissociating.

*Nae*I is a member of an expanding class of endonucleases that require specific binding to another DNA element for DNA cleavage. These nucleases currently include the type II restriction endonucleases *Bsp*MI, *Hpa*II, *Nae*I, *Nar*I, and *Sac*II (Conrad & Topal, 1989; Oller et al., 1991) and *Eco*RII (Krüger et al., 1988; Gabbara & Bhagwat, 1992) which, according to initial rate studies (Conrad & Topal, 1989; Oller et al., 1991; Gabbara & Bhagwat, 1992), must bind two sites located either on the same DNA molecule (in *cis*) or on two different DNA molecules (in *trans*) to cleave their cognate recognition sites.

The type III endonuclease *Eco*P15 (Meisel et al., 1992) probably binds two sites as well: two of its nonpalindromic recognition sites must be present in an inverse, head-to-head or tail-to-tail, orientation (to give a palindrome interrupted with a variable length spacer) for cleavage to occur. This requirement protects newly replicated DNA, which contains unmethylated sites, from unwanted cleavage. The type I restriction enzymes apparently also bind two sites simultaneously (Rosamond et al., 1979; Studier & Bandyopadhyay, 1988): these enzymes cleave randomly at some distance from their DNA recognition site; DNA loops have been seen in the presence of these enzymes (Rosamond et al., 1979).

Two-site binding enables the enzymes *Bsp*MI, *Hpa*II, *Nae*I, *Nar*I, and *Sac*II to achieve DNA cleavage in two different ways (Oller et al., 1991). The binding of one DNA site by *Hpa*II, *Nar*I, and *Sac*II increases the binding affinity (decreases the K_m) of the enzyme for the second DNA site (without affecting its k_{cat}), resulting in substrate cleavage. On the other hand, binding of a second DNA site by *Nae*I and *Bsp*MI enables cleavage of the first bound site (increases its k_{cat} without affecting its K_m) (Conrad & Topal, 1989; Oller et al., 1991).

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The two *Nae*I DNA-binding sites are nonidentical (Yang & Topal, 1992). They recognize the same core recognition sequence, GCCGGC, but prefer different families of flanking sequences: GGGT*GCCGGC*AGGG is preferred 8-fold more by the activator site but 14-fold less by the substrate site than TTTC*GCCGGC*GTTT. At least part of these preferences resides in extended DNA recognition beyond the core recognition sequence: substitution of pyrimidine or 7-deaza-purine for purine immediately 3′ to *GCCGGC* reduces DNA affinity for the activator site, but not the substrate site, by up to 26-fold, implying that the DNA-binding site requires N-7 base contacts immediately flanking *GCCGGC*.

As might be expected from its two nonidentical DNA-binding sites, *Nae*I protein induces loops in DNAs containing multiple recognition sequences (Topal et al., 1991). Electron microscopy demonstrated multiple DNA loops emanating from *Nae*I molecules bound to DNA. Competition for enzyme binding by DNA fragments containing *Nae*I sites, but not by fragments lacking such sites, greatly diminished looping by *Nae*I. The use of substrate DNAs with single *Nae*I recognition sequences had similar results. Therefore, distant *Nae*I sites, as opposed to random sites, are juxtaposed by the binding of *Nae*I protein (Topal et al., 1991).

The stimulation of DNA cleavage by *Nae*I restriction endonuclease is an example of a DNA process stimulated by an enhancer. Enhancers are defined as DNA elements that stimulate protein–DNA reactions and functions relatively independent of distance and orientation from their site of action. The basis for enhancer function is its ability to interact with distant sites through a DNA looping mechanism that enables multiple sequence elements to bind specific proteins [reviewed by Ptashne (1986) and Schleif (1988)]. Enhancer function is well recognized, yet the details of how stimulation is achieved is not well understood.

Stimulation of *Nae*I cleavage offers the opportunity to study a relatively simple form of enhancer process: cleavage enhancer functions with *Nae*I *in vitro*, without additional

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Table I: DNA Fragments Used to Form Complexes with *Nae*I

	no.	name	sequence
1)	LA		TTTTGCCGGCATTT AAAACGGCCGTAAA
2)	HA		GGGTGCCGGCAGGG CCCACGGCCGTCCC
3)	HA-22		TGGTGGGTGCCGGCAGGGTGGG ACCACCCACGGCCGTCCCACCC
4)	HA/B-32[a]		GCTGGTGGBGGGBGCCGGCAGGGBGGGCAGCT CGACCACCACCCACGGCCGBCCCACCCGTCGA
5)	HA-42		GATCGGCTGGTGGTGGGTGCCGGCAGGGTGGGCAGCTCGATC CTAGCCGACCACCACCCACGGCCGTCCCACCCGTCGAGCTAG

[a] B indicates that the fragment contains substitutions of bromodeoxyuracil for thymidine.

proteins, and the enhancer sequence appears to be composed of only the *Nae*I recognition site and the immediate flanking sequences (Yang & Topal, 1992). In this report, we demonstrate that *Nae*I can form an inactive dimer in solution in the absence of enhancer. Enhancer interaction with *Nae*I, on the other hand, stabilizes an active conformation of *Nae*I dimer that is able to cleave its recognition site. The ability of cleavage enhancer to affect activity at another DNA site via induction of allosteric protein changes raises the possibility that such allosteric changes may be a general feature of enhancer function.

MATERIALS AND METHODS

DNAs. M13mp18 RFI DNA and poly(dI/dC) were purchased from Boehringer Mannheim (Indianapolis, IN). All oligodeoxyribonucleotides were synthesized by machine (Applied Biosystems Model 380 A) using reagents from Applied Biosystems (Foster City, CA). Bromodeoxyuracil (BrdU) phosphoramidite was obtained from Biogenics (San Ranch, CA). Synthesized DNAs were purified by polyacryalmide gel electrophoresis (PAGE) followed by phenol extraction, ethanol precipitation, and desalting with G-25 Sephadex. The sequences of the synthetic oligonucleotides are given in Table I.

*Enzymes. Sau*96I and *Hgi*AI were purchased from New England Biolabs (Beverly, MA). *Nae*I was purified by methods to be described elsewhere and was also purchased from NEB. The *Nae*I endonuclease was free of *Nae*I methylase activity as assayed by the *Nae*I preparations inability to interfere with cleavage when incubated with *S*-adenosylmethionine in methylation buffer. One unit of *Nae*I is defined as the amount of enzyme required to cleave completely 1 μg of adenovirus-2 DNA in 1 h at 37 °C in 50 μL of reaction buffer (10 mM Bistris propane HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.0 at 25 °C). T4 polynucleotide kinase was purchased from Promega (Madison, WI), Proteinase K from Boehringer Mannheim, and DNAaseI from Bethesda Research Laboratories (Bethesda, MD).

Other Materials. [γ-^{32}P]ATP was purchased from New England Nuclear (Boston, MA). G-25 Sephadex and DEAE–cellulose were purchased from Sigma (St. Louis, MO). Protein molecular weight standards were supplied by Bethesda Research Laboratories and Sigma. Oyster glycogen was obtained from Boehringer Mannheim.

Molecular Weight Determination by Gel Filtration. The native molecular weight of *Nae*I was determined by gel filtration using a Sephadex G-200 column. Chromatography was carried out with a flow rate of 0.12 mL/min at 4 °C with a buffer containing 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.1 mM β-mercaptoethanol. A standard curve of v_e/v_0 versus log MW (v_e is the peak elution volume for each protein) was determined by chromatographing (independently) blue dextran (for the void volume, v_0), cytochrome *c* (MW 12 500), bovine serum albumin (BSA, MW 68 000), catalase (MW 240 000), and ferritin (MW 450 000). *Nae*I was chromatographed independently and together with marker proteins, and its v_e/v_0 was determined by assaying fractions for DNA cleavage activity as described below.

Molecular Weight Determination by Glycerol Gradient Sedimentation. The native molecular weight of *Nae*I was also examined by glycerol gradient sedimentation. Approximately 100–500 units of *Nae*I stock solution (approximately 1300 units/μg of protein) in 200 μL of storage buffer (20 mM Tris (pH 7.4), 100 mM NaCl, 0.2 mM EDTA, and 2 mM dithiothreitol) was sedimented through a 5-mL 15–35% glycerol gradient in storage buffer at 50 000 rpm for 25 h at 4 °C in a Beckman SW 55 Ti rotor. Five drop fractions were collected from the bottom of the tube by siphoning through a carefully placed capillary tube. The glycerol gradient sedimentation of protein standards (ovalbumin, BSA, and aldolase) was determined independently as markers.

Footprinting Reactions. To characterize the DNA region contacted by *Nae*I binding, a 259-bp DNA fragment radiolabeled at one end was generated by cleavage of M13mp18 RFI DNA first with *Sau*96I, 5′-end-labeling of the *Sau*96I-cut ends by standard methods (Maniatis et al., 1982), and subcleavage of the labeled fragment with *Hgi*AI to ensure radiolabeling of only one end. The product was purified from a polyacrylamide gel by overnight elution in 10 mM Tris-HCl (pH 7.4) and 1.0 mM EDTA, followed by filtration, phenol extraction, and ethanol precipitation.

Binding reactions contained 7.2 mM HEPES (pH 7.9), 7.5 mM $MgCl_2$, 39 mM KCl, 70 μM EDTA, 70 μM EGTA, 0.7 mM dithiothreitol, 6.1% glycerol, and 15 μg of poly(dI/dC) DNA in a volume of 120 μL. Approximately 10 ng (40 000 cpm) of end-labeled 259-mer and varying amounts of *Nae*I (as specified) were added to each reaction. After a 10-min incubation at room temperature, $CaCl_2$ was added to 2 mM, DNAaseI was added (3.2 ng/mL), and the reactions were incubated for 4 min at room temperature. Cleavage was stopped by the addition of 2 vol of stop mix (100 mM NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 0.1% SDS, 100 μg/mL oyster glycogen, and 100 μg/mL proteinase K), followed by incubation at 37 °C for 20 min. The DNA was ethanol precipitated, dried, and resuspended in 3 μL of formamide gel loading buffer (0.1% xylene cyanol FF, 0.1% bromophenol blue, and 10 mM EDTA (pH 8.0) in formamide) and heated to 90 °C for 1 min before loading on an 8% denaturing polyacrylamide gel.

Cleavage Assay. Cleavage of M13mp18 RFI DNA (100 ng) by *Nae*I was done in a 20-μL volume containing 10 mM $MgCl_2$, 25 mM Nacl, 11 mM Tris-HCl (pH 8.0), 0.01 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 mM dithiothreitol, 0.12 mg/mL bovine serum albumin, 5% glycerol, and enhancer DNA as indicated. Reactions were warmed to 37 °C for 2 min. Various amounts of *Nae*I were added and the reactions incubated at 37 °C for 2 min. The reactions were stopped by a 10-min incubation at 65 °C or by addition of EDTA to 50 mM, both of which were found to completely inactivate the enzyme, and run on a 1% agarose gel. The gel was stained with ethidium bromide and photographed under UV light. Cleavage was measured by densitometry of the photographic

negative. Values were corrected for differences in staining of supercoiled, linear, and nicked-circle DNA molecules.

Gel Mobility–Shift Assay. To characterize the binding interaction of *Nae*I with DNA, radiolabeled DNA fragments were synthesized to contain a central *Nae*I recognition site (GCCGGC). Synthesized oligonucleotides (Table I) were radiolabeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (Maniatis et al., 1982). The complementary oligonucleotides were annealed to give double-stranded DNA fragments and desalted using G-25 Sephadex.

Various amounts of *Nae*I (specified in the figure legends) and labeled DNA fragment were incubated together for 10 min at room temperature in reactions containing 5 mM $CaCl_2$, 5 mM KCl, 10 mM NaCl, and 0.2 mg/mL poly(dI/dC) DNA. Except where noted, the reactions were run on 6% (37.5:1) polyacrylamide/bisacrylamide gels for 2 h at 200 V in a buffer containing 25 mM Tris-HCl, 190 mM glycine, and 1 mM EDTA (pH 8.3).

UV Cross-Linking. To covalently cross-link *Nae*I to the DNA, BrdU-substituted 22-, 32-, and 42-bp DNA fragments with centrally located *Nae*I recognition sites (Table I) were radiolabeled as described above. UV cross-linking reactions were prepared to contain 100 nM end-labeled DNA, 5 mM $CaCl_2$, 5 mM KCl, 10 mM NaCl, poly(dI/dC) DNA (0.2 mg/mL), and 25 units of *Nae*I in a reaction volume of 60 μL. Reactions were incubated for 10 min at room temperature and then exposed to UV light (302 nm) from a distance of 3 cm for varying lengths of time. After UV exposure, the samples were heated to 90 °C in the presence of 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 63 mM Tris (pH 6.8), and 0.001% bromophenol blue. The reactions were then run on a (5% stacking/8% resolving) discontinuous polyacrylamide gel (Laemmli, 1970). Autoradiography revealed protein–DNA complexes. Prestained protein standards were used to generate standard curves for MW versus mobility on SDS–PAGE.

RESULTS

*Nae*I *Contacts the Recognition Palindrome Symmetrically.* To determine the *Nae*I binding site on DNA relative to its recognition site, we footprinted *Nae*I on a 259-bp fragment of M13mp18 DNA containing the poorly cleaved *Nae*I site. The DNA sequence protected from DNAaseI cleavage by *Nae*I binding spanned 24 base pairs (Figure 1). The protected region is placed symmetrically around the 6-mer recognition site. There were no other protected regions apparent upstream or downstream from the recognition site. The symmetry of protection around the palindromic recognition site implies that the subunit structure of *Nae*I has at least dyad symmetry. Such symmetry suggests that the native structure of *Nae*I may be a dimer or tetramer as it is for many enzymes that recognize binding sites with dyad symmetry (Pabo & Sauer, 1984; Harrison & Aggarwal, 1990).

Native Molecular Weight Analysis. The molecular weight (MW) of native *Nae*I endonuclease in solution was determined by gel filtration and by sedimentation through a glycerol gradient. Analysis by gel filtration chromatography was carried out on a Sephadex-G-200 column (Figure 2a). A plot of v_e/v_0 versus log MW was derived from the elution profiles of a number of protein standards and was used to estimate the native MW of *Nae*I. The calculated MW of 70 000 is approximately twice that of an *Nae*I protein monomer (35 200) estimated from the gene sequence (C. Taron, E. van Cott, G. Wilson, L. Moran, B. Slatko, L. Hornstra, J. Benner, R. Kucera, and E. Guthrie, unpublished results).

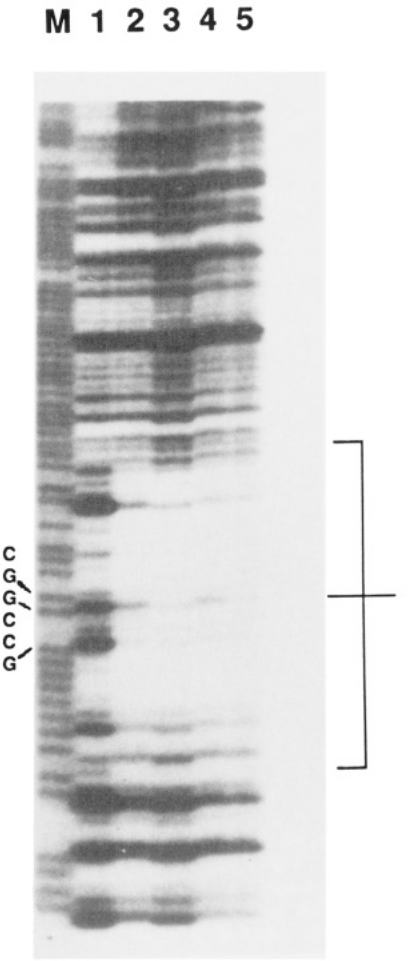

FIGURE 1: DNAse I footprint of *Nae*I complexed to its recognition site in M13 DNA. Complexes between *Nae*I and a 259-bp M13mp18 DNA fragment containing the substrate recognition site were footprinted with DNAse I. Binding reactions were performed as described in Materials and Methods in a 120-μL volume with 10 ng or 40 000 cpm of end-labeled DNA. Increasing amounts of *Nae*I were added to the reactions; lanes 1–5 represent 0, 100, 160, 200, and 240 units of enzyme (specific activity approximately 1300 units/μg of protein), respectively. The marker (M) is a Maxam–Gilbert purine cleavage ladder for sequence refrence (Maxam & Gilbert, 1980).

To confirm that native *Nae*I endonuclease is a dimer, the protein was sedimented through a glycerol gradient. Comparison of the mobility of the *Nae*I activity with that of proteins with known S values (Figure 2b) indicated that *Nae*I sedimented as a single band with a Svedberg coefficient of approximately 4.2S. For a globular protein of average partial specific volume and degree of hydration, this S value corresponds to an MW of about 70 000 (Cantor & Schimmel, 1980). This MW is consistent with that determined by gel permeation chromatography and indicates that *Nae*I can form a dimer under conditions of relatively high unit concentration (0.5–5 units/μL). The *Nae*I dimer is inactive in the absence of enhancer DNA, however, since these enzyme concentrations showed negligible cleavage of resistant recognition substrates in reactions lacking enhancer (Conrad & Topal, 1989; Oller et al., 1991).

Effect of NaeI Concentration on DNA Cleavage. The subunit structure of functioning *Nae*I was examined. The effect of increasing *Nae*I concentration on the rate of single- and double-strand cleavage of M13mp18 RFI DNA, with and without enhancer present, was measured. If the formation of a higher order complex is necessary for enzyme activity, then it is possible that the cleavage enhancer works by

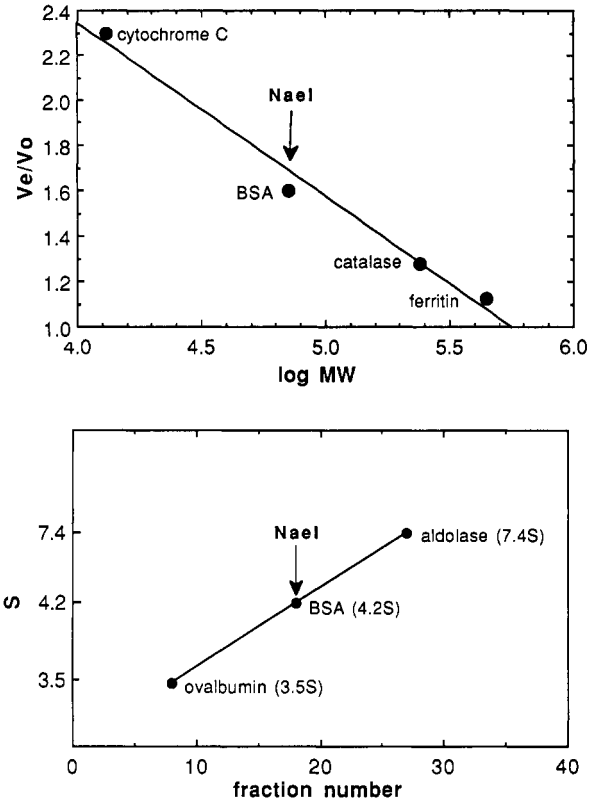

FIGURE 2: Determination of the native molecular weight of *Nae*I in the absence of DNA. (Top, a) *Nae*I was chromatographed through a Sephadex-G-200 column that had been standardized using the proteins indicated. (Bottom, b) *Nae*I was sedimented on a glycerol gradient along with the indicated proteins of known *S* values. The position of *Nae*I activity is indicated.

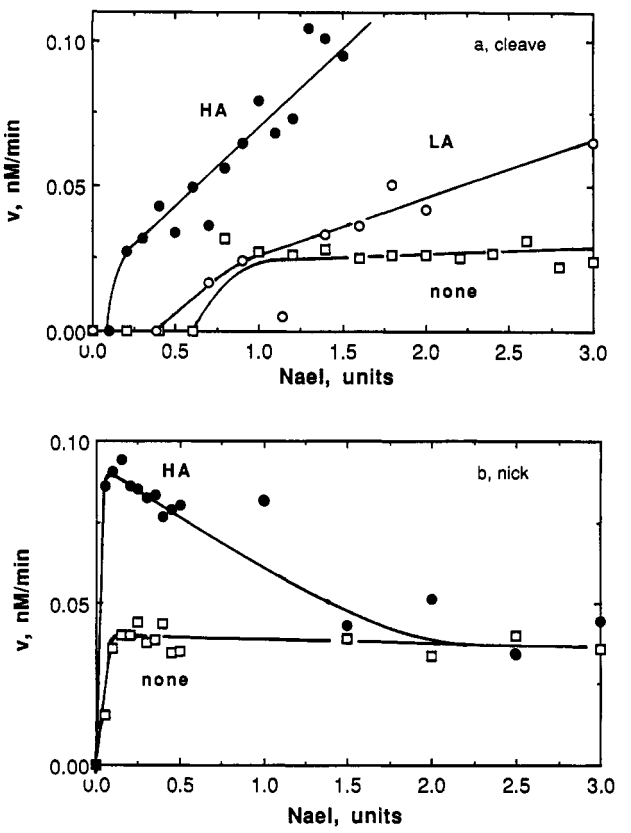

FIGURE 3: Effect of *Nae*I concentration on DNA cleavage reaction velocities. Cleavage reactions with M13mp18 as substrate were performed as described in Materials and Methods with varying concentrations of *Nae*I (1 unit per reaction ≈ 0.5 nmol/L) and analyzed by agarose gel electrophoresis. (Top, a) Double-strand cleavage and (bottom, b) single-strand cleavage (nicking) were measured: (○) low-affinity enhancer (LA) (20.0 nM); (●) high-affinity enhancer (HA) (20.0 nM); (□) no enhancer.

facilitating formation of an active conformation of the *Nae*I dimer. Two 14-bp enhancers [activating enhancer DNAs (A) with higher affinity for the activator site than M13 DNA (Yang & Topal, 1992)] were chosen for this experiment on the basis of their relative affinity for the *Nae*I (E) activator site (K_A = [E][A]/[EA]). Low-affinity [LA: K_A = 210 ± 50 nM and K_i (affinity for the substrate site) = 61 ± 1 nM] and high-affinity [HA: K_A = 17 ± 3 nM and K_i = 310 ± 40 nM] DNA enhancer fragments were used to test the role of the enhancer in the *Nae*I cleavage mechanism (fragments 1 and 2 in Table I). Enhancer DNA, either LA or HA, was added to each reaction to a final concentration of 20 nM. Reaction velocity was determined by analysis of the reaction products on agarose gels (see Materials and Methods); the results were plotted against increasing concentrations of *Nae*I (Figure 3).

The velocity curves demonstrated that a critical concentration of enzyme had to be present before double-strand cleavage activity was possible. That is, a significant lag in cleavage activity existed until a particular concentration of enzyme was obtained. The lag was characterized by a burst of nicking activity at the lowest *Nae*I concentrations used (Figure 3b), indicative of formation of an initial (ES) complex between substrate and *Nae*I monomer. After the lag, a burst of cleavage activity was seen, implying cooperative formation of a higher order *Nae*I structure (E_2S) capable of double-strand cleavage. The nicking activity was specific for the *Nae*I site since it was not observed in control reactions containing DNA (pUC18) lacking an *Nae*I site (B. K. Baxter and M. D. Topal, unpublished results).

Three effects of enhancer were evident: enhancer decreased the lag in *Nae*I concentration required for double-strand cleavage, it stimulated cleavage once the critical concentration of *Nae*I was obtained (Figure 3a), and it stimulated the nicking reaction at enzyme concentrations too low for double-strand cleavage (Figure 3b). The effects of enhancer increased with its affinity for the activator site of *Nae*I.

Visualization of NaeI–DNA Complexes Using Gel Mobility–Shift Assays. The transition from nicking activity to cleavage activity with increasing *Nae*I concentration (Figure 3) implied two different structures of *Nae*I: a monomer able to cleave one strand of the recognition site (nick) and a dimer able to cleave the recognition site by a concerted mechanism. Both of these structures require enhancer for activity. To visualize the higher order complex in the absence of cleavage, we used gel mobility–shift assays in the presence of calcium. The substitution of calcium for magnesium eliminates cleavage by several restriction enzymes (Fox, 1988). We found that calcium buffers reduced cleavage to a negligible amount in *Nae*I reactions, but specific binding was maintained.

End-labeled HA enhancer at a final concentration of 1.0 nM was incubated with increasing amounts of *Nae*I (Figure 4a). One protein–DNA complex, complex I, was seen when as little as 5 units of *Nae*I was added. At higher concentrations of enzyme, a slower migrating complex, complex II, appeared. Substitution of 1.0 nM LA enhancer in place of 1.0 nM HA enhancer (Figure 4b) in these experiments gave predominantly complex I over the range of *Nae*I concentrations tested. Densitometric analysis of the bands indicated that, at 150 units of *Nae*I, 18% of the shifted LA DNA is in complex II compared to 42% of the shifted HA DNA under identical

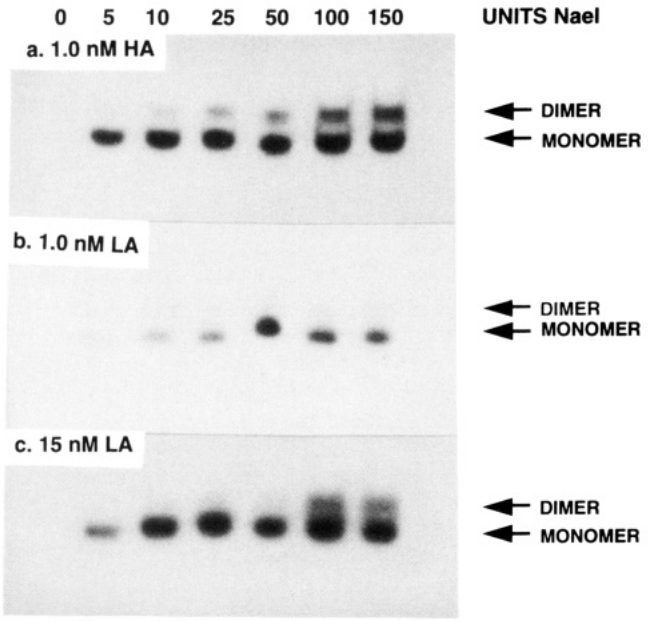

FIGURE 4: Analysis of *Nae*I–enhancer complexes by gel mobility–shift assay. Gel mobility–shift reactions were done as described in Materials and Methods. *Nae*I was added as indicated. In all lanes, unbound probe ran off the gel. *Nae*I–enhancer complexes, located by autoradiography, are indicated; relative amounts of shifted DNA in each complex were determined by densitometry (see text): [HA DNA] = 1 nM; [LA DNA] = 1 nM; [LA DNA] = 15 nM. *Nae*I and labeled DNA fragment were incubated together for 10 min at 25 °C in reactions containing 5 mM $CaCl_2$, 5 mM KCl, 10 mM NaCl, and poly(dI/dC) (0.2 mg/mL). Reaction products were separated on 6% (37.5:1) polyacrylamide/bisacrylamide gels for 2 h at 200 V in a buffer containing 25 mM Tris-HCl, 190 mM glycine, and 1 mM EDTA (pH 8.3). Identical amounts of reaction were loaded in each gel, all three gels (a, b, and c) were autoradiographed for 3 h, and the specific activity of the ^{32}P-labeled DNA was 7.8×10^{17} cpm/mol of HA fragment and 6.2×10^{17} cmp/mol of LA fragment. The different mobilities of the two complexes are constant from panel to panel in the figure.

conditions. Increasing the LA enhancer concentration to 15 nM shifted the equilibrium toward complex II: 33% of the shifted LA DNA was associated with complex II in the 150-unit reaction.

To ensure that the *Nae*I–DNA interactions indicated by the gel mobility–shift assays were specific for the *Nae*I recognition site, the gel mobility–shift assay using 1.0 nM labeled HA DNA was repeated in turn in the presence of 100 nM unlabeled HA DNA, LA DNA, and 30-bp DNA fragment lacking the *Nae*I recognition site. The HA and LA DNAs, which both contain an *Nae*I site, competed away the two complexes, whereas the DNA lacking an *Nae*I recognition site had no effect (not shown).

*Nae*I can bind at least two recognition sequences simultaneously (Topal et. al., 1991). Therefore, the two protein–DNA complexes observed in Figure 4 could have been caused by either multiple binding of DNA to *Nae*I or multiple *Nae*I subunits binding to DNA. However, the ability to visualize multiple protein subunits interacting with DNA by gel electrophoresis is very sensitive to gel pore size and therefore to the percentage gel (Lane et al., 1992). Since changing from 6% (Figure 4) to 15% polyacrylamide gels (Figure 5) abolished the mobility shift of one of the *Nae*I–DNA complexes, that complex was probably caused by higher order protein structure rather than equilibria between binding one and two DNAs.

In addition, when two different size (22- and 42-bp) DNA fragments were complexed with *Nae*I in the same reaction and analyzed by the gel mobility–shift assay, two bands were

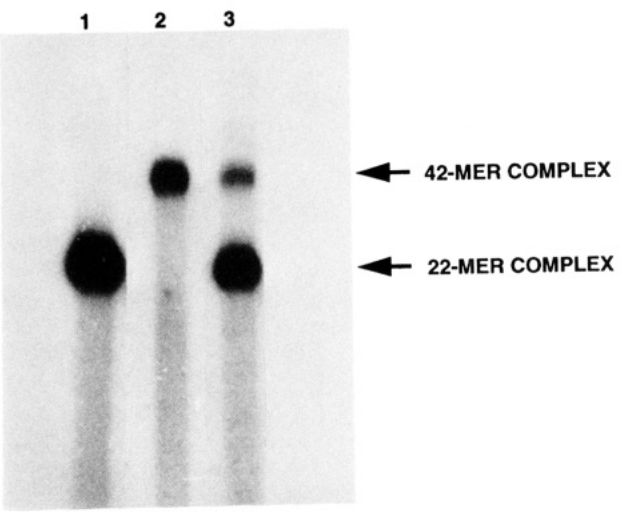

FIGURE 5: *Nae*I complexes with two different length enhancer DNAs. Binding reactions were done as described (Materials and Methods) in a 30-μL volume with 50 units of *Nae*I. The labeled oligoduplexes used in this experiment were 22- and 42-bp DNAs (fragments 3 and 5 in Table I). The total DNA concentration in each reaction was 40 nM. Reaction products were run on a 15% polyacrylamide gel. Lane 1: 22-mer DNA complex with *Nae*I. Lane 2: 42-mer DNA complex with *Nae*I. Lane 3: Mixed DNA population (20 nM each of 22-mer and 42-mer) complexes with *Nae*I.

observed corresponding to the 22- and 42-bp complexes with *Nae*I. No intermediate mobility band for a complex containing both DNA fragments was observed (Figure 5, lane 3). Since the heterologous complex could have comigrated with one of the homogeneous complexes, both bands in lane 3 were excised from the gel, extracted with TE buffer containing 2% SDS, and electrophoresed on 20% polyacrylamide. The bands were homogeneous with respect to the DNA fragment: no heterologous complex was detected. These results show that under these reaction conditions only one DNA was bound per *Nae*I complex and therefore provide further evidence that complexes I and II in Figure 4 involve *Nae*I monomer and dimer, respectively.

UV Cross-Linking of NaeI Bound to DNA. The above results imply an *Nae*I nucleoprotein complex involving *Nae*I dimer and enhancer element. To capture this structure and to characterize the MWs of the complexes formed by *Nae*I and DNA, *Nae*I was cross-linked to DNA using UV light. HA enhancer was extended to 32 base pairs. The sequences flanking the *Nae*I recognition sequence were synthesized to contain four BrdU substitutions for thymidine (fragment **4** in Table I). This DNA was radiolabeled and incubated with *Nae*I under gel mobility–shift conditions. The reactions were exposed to UV light for varying lengths of time and run on an SDS discontinuous gel with protein molecular weight standards to examine the size of the protein subunits attached to the DNA (Figure 6). With an HA site and saturating amounts of enzyme, the UV dose required to cross-link both subunits of *Nae*I dimer to the DNA should be the square of the dose required to cross-link just one of the subunits. The first complex to appear over time was seen after only 5 min of UV exposure. With increasing UV light dosage, a slower migrating species appeared at between 60 and 120 min of exposure time. The small variation from the expected square relationship is attributed to variation in the amounts loaded on the gel. The slower migrating species had an apparent molecular weight (determined using protein standards) approximately twice that of the species appearing first, implying that it involved *Nae*I dimer bound to DNA.

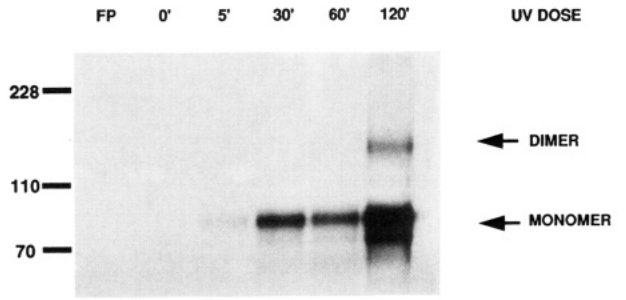

FIGURE 6: UV cross-linking of *Nae*I–enhancer complex. Cross-linking assays were done as described in Materials and Methods using a 32-bp BrdU-substituted DNA fragment (fragment 4 in Table I). Reactions contained poly(dI/dC) DNA (0.2 mg/mL) to eliminate nonspecific binding. Reactions were incubated for 10 min at room temperature and then exposed to UV light (302 nm) from a distance of 3 cm for the time indicated. After UV exposure the samples were heated to 90 °C in the presence of 2% SDS and run on a (5% stacking/8% resolving) discontinuous polyacrylamide gel (Laemmli, 1970). Autoradiography revealed protein–DNA complexes. The reaction shown in the first lane contains only free probe (run off the gel). The MWs for the monomer and dimer bands were determined using protein standards (whose mobilities are indicated).

At 120 min exposure time, lower MW complexes appear as well. These are caused by degradation of *Nae*I, since they are specific for the *Nae*I site as shown by competition with unlabeled DNA fragments with and without the *Nae*I site (not shown). Only the DNA fragments containing the *Nae*I site competed away all of the bands.

To estimate the size of the protein subunits in the complexes observed, these experiments were repeated using BrdU-substituted HA DNA fragments of different lengths (Figure 7). The resulting mobilities on SDS–PAGE were linearly dependent on the size of the DNA fragment. The approximate molecular weights of the *Nae*I complexes were determined from a standard curve (Figure 7a). The molecular weight of native *Nae*I protein alone was estimated by extrapolation of the mobility of the complexes to that containing a DNA fragment 0 bp in length (Figure 7b). In this way, the molecular weight of *Nae*I monomer was estimated to be ~45 000, which compares reasonably well to its known MW of ~35 200 estimated from the *Nae*I DNA sequence. This same method estimated the molecular weight of *Nae*I in the higher order complex to be approximately 91 000, confirming that the slower migrating species contained *Nae*I dimer.

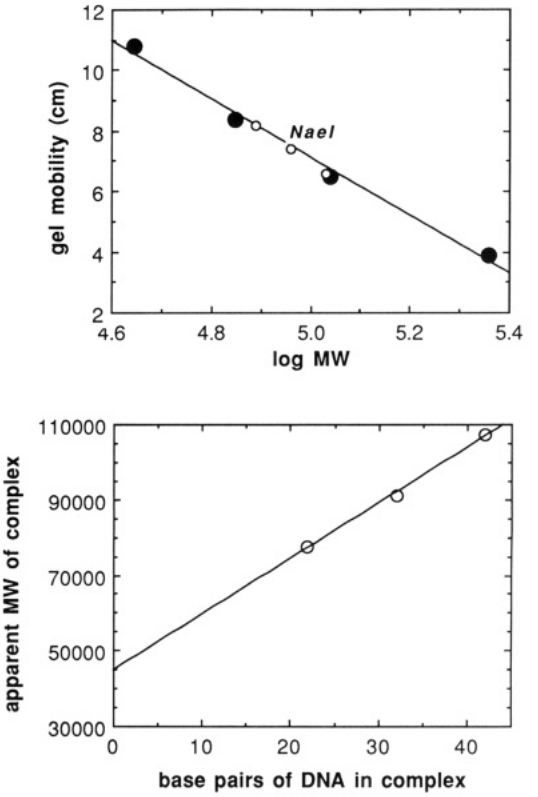

FIGURE 7: Determination of MW of *Nae*I in the UV-cross-linked *Nae*I–DNA Complex. UV cross-linking conditions are described in Figure 6; complexes were cross-linked for 120 min. DNA fragments were 22, 32, and 42 bp long: all contained the same core sequence and BrdU substitutions. (Top, a) The MWs of *Nae*I–DNA (○) complexes were determined using a standard curve (●), by SDS–PAGE, as described in Figure 6. (Bottom, b) A plot of the respective MWs of the complexes versus the length of the DNA in each complex gives the apparent molecular weight of the protein in the complex by extrapolation to 0 length DNA.

DISCUSSION

On the one hand, gel permeation chromatography and glycerol gradient sedimentation demonstrated that, in the absence of enhancer, *Nae*I can form a dimeric structure. This *Nae*I dimer is inactive, however, since in the absence of cleavage enhancer high concentrations of *Nae*I demonstrate very low levels of cleavage (Figure 3a; Conrad & Topal, 1989; Oller et al., 1991).

On the other hand, the reaction rate, gel mobility–shift, and UV cross-linking studies of the interaction of *Nae*I with DNA described above revealed a set of interactions between active *Nae*I conformers and DNA that depended on the presence of cleavage enhancer. Enhancer stimulated nicking activity at low concentrations of *Nae*I at which no double-strand cleavage activity was evident: this result implied an active enhancer–*Nae*I monomer complex able to cleave only one strand of the recognition site. At higher *Nae*I concentrations, enhancer stimulated double-strand cleavage activity with concomitant loss of nicking activity: this result implied formation of a dimeric structure, at the expense of monomer, able to cleave both strands of the recognition site.

The interaction of enhancer with *Nae*I in the absence of substrate cleavage was visualized by a gel mobility–shift assay. An equilibrium between the monomer–enhancer and dimer–enhancer complexes was demonstrated by the ability to shift the population of the two complexes by changing the *Nae*I concentration. UV cross-linking of the gel–shift assay products and determination of the molecular weight of the protein in the cross-linked products by SDS–PAGE provided additional evidence for the dimeric form of *Nae*I bound to enhancer.

*Nae*I shows only low levels of activity in the absence of cleavage enhancer, even though it binds substrate. The presence of enhancer stimulates cleavage by increasing k_{cat} (Conrad & Topal, 1989; Yang & Topal, 1991). These are the hallmarks of a classic allosteric mechanism in which binding at one site enables cleavage at the other by affecting the relative positioning of substrate and catalytic site (Monod et al., 1965). According to the two-site allosteric mechanism proposed for *Nae*I cleavage of DNA (Yang & Topal, 1992), the low background activity observed in the absence of enhancer is consistent with the low level of occupation of the enhancer-binding site by substrate. We showed here that the *Nae*I–enhancer complex is an equilibrium between monomer and dimer structures. The allosteric model dictates that both the monomer and dimer species must exist as equilibria of active and inactive forms. The binding of enhancer stabilizes the active forms of the enzyme.

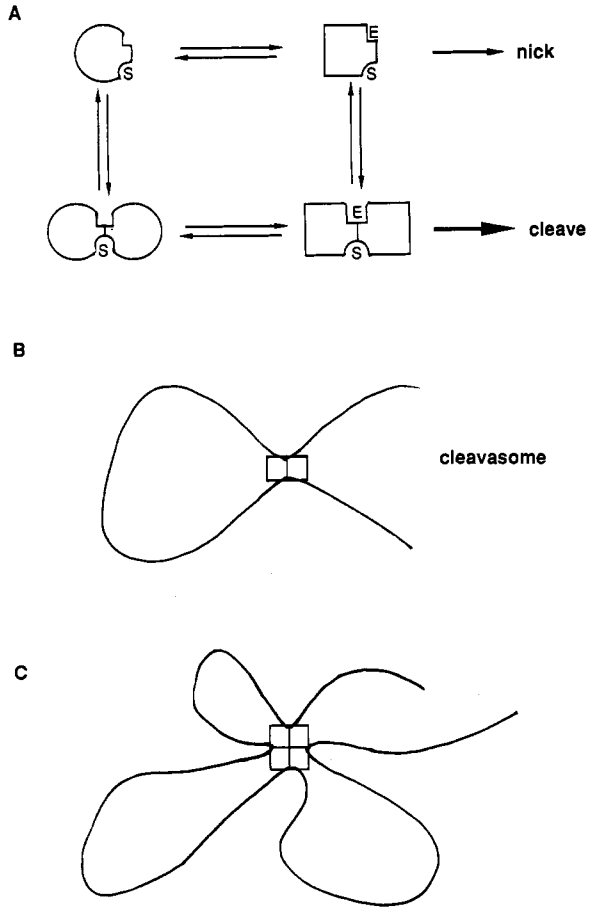

FIGURE 8: Model of the interaction between *Nae*I and enhancer and substrate DNAs. (A) Equilibria between active and inactive *Nae*I quaternary structures in the presence of enhancer DNA. Substrate is arbitrarily shown bound first; initial rate measurements imply that the order of binding is random and is driven by the affinity of enhancer and cleavage elements for their respective sites (Yang & Topal, 1992). The circle and square represent unactivated and activated forms of *Nae*I, respectively. Semicircular and square enzyme binding sites represent substrate (S) and enhancer (E) binding sites, respectively. (B and C) *Cis* interactions between distant *Nae*I sites and *Nae*I. See text for a description of the evidence to support the scheme outlined in A–C.

A model that is consistent with all of these results is presented in Figure 8. In this model, *Nae*I monomers bind sequentially to either substrate or enhancer in a manner similar to LexA repressor binding of operator (Kim & Little, 1992). The bound *Nae*I monomer and dimer exhibit equilibria between active and inactive conformers. We applied the concerted transition model of Monod et al. (1965) to our proposed equilibrium between *Nae*I allosteric conformers. Application of this model conserved the symmetry relationships implied by a dimer that recognizes DNA-binding sites with dyad symmetry. Heterodimer species containing one active and one inactive subunit, however, are possible (Koshland et al., 1966).

Initial cleavage rates indicated that activated *Nae*I has two nonidentical DNA-binding sites (Yang & Topal, 1992). Our model indicates two binding sites per both the *Nae*I monomer and dimer. This speculation is based on the ability of the *Nae*I monomer to bind enhancer and substrate, but only nick its recognition site. The fully active substrate-binding site, which is able to cleave both strands of the recognition sequence, is formed only with formation of the *Nae*I dimer and occupation of the enhancer-binding site.

Our model, for *cis* interactions (Figure 8B,C) on a DNA molecule containing both enhancer and cleavage elements, predicts that two dimers may be able to interact to give a tetrameric structure (Figure 8C). This prediction is based on electron microscopic visualization of the interaction of *Nae*I with pBR322 DNA (Topal et al., 1991), which contains four *Nae*I recognition sites. This interaction induced four-leaf as well as two-leaf clover structures. Studies are in progress to investigate the possibility of an *Nae*I tetramer.

The equilibrium between active and inactive conformers predicts that, in principle, negative regulators as well as enhancers are possible for DNA cleavage. Since a DNA element binding to the active form of *Nae*I stabilizes that form over the inactive form, it is just as possible that a DNA element can be found for an active or activated enzyme that stabilizes the inactive form. Such behavior would manifest itself in cleavage rate studies as noncompetitive inhibition. The model predicts the possible interaction of protein with different DNA elements to shift activity up or down.

Our results argue that, to cleave DNA, *Nae*I requires the formation of a multi-subunit complex whose active conformation is stabilized by the binding of an enhancer element (Figure 8B). We term this nucleoprotein complex a cleavasome by analogy with the intasome (Richet et al., 1986), transpososome (Surette et al., 1987), invertasome (Heichman & Johnson, 1990), and resolvosome/synaptosome (Sanderson et al., 1990) nucleoprotein complexes in which a nucleic acid component is an integral part of the machinery. Recently, possible homology was found between one of the members of the *Nae*I-type endonucleases, *Eco*RII, and the int family of recombinases (Topal & Conrad, 1993).

Other proteins that process DNA require binding of multiple DNA sites. The mechanisms that govern site-specific recombination (Gellert & Nash, 1987; Moitoso de Vargas et al., 1989, Hughes et al., 1990), initiation of DNA replication (Dodson et al., 1985), and control of transcription of the eukaryotic genome (Knight et al., 1991; Li et al., 1991; Mastrangelo et al., 1991; Su et al., 1991) require multiple DNA–protein interactions to achieve precise site-specificity. It is not surprising, therefore, that a cleavasome would be required to achieve precise and accurate cleavage of one short sequence out of many in the cell. Thus, our results appear to extend the notion of a specialized nucleoprotein structure for specificity in localized DNA transactions (Echols, 1986, 1990) to site-specific double-strand cleavage of DNA.

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