DNA Cleavage by *Nae*I: Protein Purification, Rate-Limiting Step, and Accuracy

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ABSTRACT: *Nae*I endonuclease must bind two DNA sites for cleavage to occur. *Nae*I was purified to apparent homogeneity and used to determine the rate-limiting step for DNA cleavage and to measure *Nae*I's specificity for its cognate recognition site. Steady-state cleavage by *Nae*I in the presence of effector DNA (activated) gave values of 0.045 s^{-1} and 10 nM for k_{cat} and K_M for M13 DNA substrate, respectively, but values of 0.4 s^{-1} and 170 nM, respectively, for an M13 DNA fragment substrate. Single-turnover cleavage of M13 DNA demonstrated that DNA strand scission is not rate-limiting for turnover of *Nae*I. Transient kinetic analysis of M13 DNA cleavage by *Nae*I showed an initial burst of substrate cleavage that was proportional to *Nae*I concentration, implying that product release is rate-limiting for turnover of *Nae*I. The *Nae*I effector and substrate binding sites were found to prefer cognate over noncognate sequences by 10^3-fold and at least 40−500-fold, respectively. k_{cat} for noncognate recognition sequence was at least 10^6-fold lower than that for cognate. The specificity of activated *Nae*I, as measured by k_{cat}/K_M, for noncognate recognition sequence was 10^8-fold lower than that for cognate, and over 10^{11}-fold lower when the decreased affinity for noncognate sequence at the effector binding site was taken into account. This specificity is approximately 10^4-fold larger than for any other restriction enzyme measured.

Type II restriction endonucleases exhibit a variety of mechanisms for DNA recognition and cleavage [for a recent review, see Heitman (1993)]. All type II enzymes require Mg^{2+} for DNA cleavage, however, type II enzymes have different requirements for the metal cofactor for DNA binding/recognition. The type II enzymes have been categorized into different subclasses based on this latter requirement. *Eco*RI (McLaughlin et al., 1987; Lesser et al., 1990) and *Rsr*I (Aiken et al., 1991), type IIi, do not require Mg^{2+} for DNA binding specificity, whereas *Eco*RV (Taylor et al., 1991) and *Taq*I (Zebala et al., 1992a,b), type IId, derive much of their specificity at the DNA cleavage level, and require Mg^{2+} for DNA binding specificity. Despite their differences in DNA binding/recognition, the crystal structures of *Eco*RI and *Eco*RV show remarkably similar arrangements of catalytically important amino acid residues at the DNA cleavage site (Selent et al., 1992). These observations suggest that there are potentially several mechanisms for sequence-specific DNA−protein recognition.

*Nae*I endonuclease, from *Nocardia aerocolonigenes*, is a prototype for yet another subclass of restriction enzymes we term type IIe, because members of this subclass require the recognition of a second DNA (effector) site to undergo DNA cleavage. The velocity of *Nae*I-mediated DNA cleavage shows a sigmoidal dependence on the concentration of substrates containing a single *Nae*I recognition sequence, consistent with DNA cleavage being second-order with respect to *Nae*I site concentration (Yang & Topal, 1992). Furthermore, *Nae*I cannot cleave some DNAs with single *Nae*I recognition sequences because of the low affinity of those DNAs for either one of the two *Nae*I DNA binding sites. Addition in *cis* or in *trans* of a second *Nae*I recognition sequence that has a high affinity for the unoccupied DNA binding site activates cleavage of the refractory recognition sequence (Conrad & Topal, 1989; Yang & Topal, 1992). Other members of this expanding subclass include *Nar*I, *Bsp*MI, *Hpa*II, *Sac*II (Oller et al., 1991), *Eco*RII (Krüger et al., 1988), *Atu*BI, *Cfr*9I, *Sau*BMKI, *Eco*57I, and *Ksp*632I (Reuter et al., 1993). These enzymes can be further subdivided on the basis of whether effector DNA activates catalysis (*Nae*I, *Bsp*MI) or substrate binding (*Hpa*II, *Nar*I, *Sac*II) (Oller et al., 1991).

DNA sequences immediately flanking the *Nae*I recognition palindrome affect the affinity of that DNA for the substrate and effector binding sites on the enzyme. The differences in affinity of the two binding sites for the same DNA fragment indicates that the two DNA binding sites are nonidentical (Yang & Topal, 1992). The differential affinities of the two DNA binding sites for DNA were characterized by determining the relative abilities of various 14 base pair DNA fragments to both activate and inhibit M13 DNA cleavage by *Nae*I. From these determinations the intrinsic dissociation constants K_A and K_I for the binding of these DNA fragments to the effector and inhibitor (substrate) binding sites, respectively, were determined (Yang & Topal, 1992).

*Nae*I presents a mechanism for DNA recognition and cleavage different from other type II restriction endonucleases (Yang & Topal, 1992; Baxter & Topal, 1993). In this report, we describe a procedure for the purification of *Nae*I to apparent homogeneity and describe the steady-state and transient kinetic analysis of purified *Nae*I. These analyses indicate the step limiting the rate of DNA cleavage by *Nae*I and enable determinations of the specificity of *Nae*I recognition of cognate and noncognate recognition sequences. The specificity of *Nae*I cleavage as measured by comparison of k_{cat}/K_M and binding affinities between cognate and noncognate substrates was significantly higher than for other enzymes that recognize sequences of similar size but bind only one DNA sequence.

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Table 1: DNA Fragments Used in the Kinetic Analysis of *Nae*I Endonuclease Activity[a]

name	sequence
HA	GGGTGCCGGCAGGG
	CCCACGGCCGTCCC
LA	TTTCGCCGGCGTTT
	AAAGCGGCCGCAAA
M13-14	GTTCGCCGGCTTTC
	CAAGCGGCCGAAAG
HA nc	GGGTCCCGGCAGGG
	CCCAGGGCCGTCCC
LA nc	TTTCCCCGGCGTTT
	AAAGGGGCCGCAAA
M13-14 nc	GTTCCCCGGCTTTC
	CAAGGGGCCGAAAG

[a] Studies of DNA fragments HA, LA, and M13−14 are also presented in Yang and Topal (1992) and Baxter and Topal (1993). The flanking sequences surrounding the *Nae*I recognition site for M13−14 are the same as those surrounding the *Nae*I site in M13mp18. The *Nae*I recognition site is underlined, and bold type indicates difference from the cognate recognition sequence.

MATERIALS AND METHODS

Bacterial Strains. Escherichia coli strain: CAA1 (*F⁻ e14⁻ (mcrA⁻) lacY1 or D(lac)⁶ SupE44 galK2 galT22 mcrA rfbD1 mcrBa hsd($r_k^-m_k^+$) M·MspI⁺*) and NEB786 (CAA1 harboring the plasmid pNEB786) were generously provided by Ellen Guthrie (New England Biolabs, Beverly, MA).

DNAs. Plasmid pNEB786, containing the *Nae*IR gene in the pAGR3 tac expression vector, was isolated from NEB786. M13mp18 RFI DNA was purchased from Boehringer Mannheim (Indianapolis, IN). pBR322 DNA was isolated from HB101 and banded on a cesium chloride density gradient (Sambrook et al., 1989). All oligodeoxyribonucleotides were synthesized using an Applied Biosystems 380 A synthesizer. Synthesized DNAs were further purified by polyacrylamide gel electrophoresis (PAGE) followed by phenol extraction, ethanol precipitation, and desalting with G-25 Sephadex (Sambrook et al., 1989). The sequences of DNA fragments used in this study are listed in Table 1.

Chromatographic Materials. G-25 Sephadex (20−80 μm) used for desalting oligodeoxyribonucleotides, DEAE-Sepharose CL-6B, and S-Sepharose were purchased from Sigma Chemical Co. (St. Louis, MO). Heparin Affi-Gel (heparin agarose) was obtained from Bio-Rad (Richmond, CA).

Other Materials. [γ-^{32}P]ATP was purchased from New England Nuclear (Boston, MA). T4 polynucleotide kinase was purchased from Promega (Madison, WI). Isopropyl β-D-thiogalactopyranoside (IPTG) and phenylmethanesulfonyl fluoride (PMSF) and bovine serum albumin (BSA) were purchased from Sigma.

Growth of Cells. All bacterial cell cultures were grown at 37 °C in superbroth (32 g of tryptone, 20 g of yeast extract, 5 g of NaCl per liter of media, adjusted to pH 7.0 with NaOH) supplemented with 100 mg/mL ampicillin. Five-liter cultures were grown a New England Brunswick fermentor set for maximum aeration and inoculated with an overnight culture of NEB786 (100 mL). Two hours after inoculation ($A_{600} \approx 0.5-0.7$), *Nae*I expression was induced by the addition of IPTG to a final concentration of 1 mM. Fermentor agitation was set at 500 rpm, and the culture was grown to stationary phase (≥6 h following induction) and harvested. A typical yield of cells from one 5-L culture was 90−100 g. At least 4 × 5 L cultures grown in the fermentor were used in a single protein preparation. Harvested cells could be stored at −20 °C for several weeks with little apparent loss in protein yield.

Enzyme Activity and Cleavage Assays. All enzyme assays were performed in 20 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and bovine serum albumin (BSA) at 0.1 mg/mL concentration. One unit of *Nae*I enzyme activity was defined as the amount of enzyme required to cleave 1 μg of Adenovirus-2 DNA to completion in a 50-μL reaction mixture in 1 h at 37 °C. Reactions mixtures were incubated at 37 °C, after which, cleavage was terminated either by heat inactivation of the enzyme for 10 min at 65 °C or by addition of EDTA (pH 8.0) to a final concentration of 100 mM. Reactions involving pBR322 or M13 DNA as substrates were separated in 1% agarose gels run in TAE, whereas reactions using 14 base pair DNA fragments as substrates were separated on 20% polyacrylamide gels run in TBE.

Kinetic Analysis. Velocities of cleavage were derived from the fraction of cleavage product observed as a function of time. All velocities were determined in the linear region for cleavage with respect to time. Steady-state rate constants were least squares analysis of double-reciprocal plots of velocity against substrate concentration. The values for the enzymatic turnover number (k_{cat}) of the endonuclease were related to V_{max} through the total enzyme concentration in each experiment.

Other Methods. Reaction products of pBR322 or M13 DNAs were visualized by staining the agarose gels with ethidium bromide (Sambrook et al., 1989) and quantitated through scanning densitometry of the photographic negatives from the gels. Reaction products of 14 base pair DNA substrates were followed through densitometric analysis of the autoradiographs of the separation gels. One of the two complementary oligodeoxyribonucleotides was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Sambrook et al., 1989) prior to annealing. Protein concentrations were determined using known concentrations of BSA according to the method described by Bradford (1976).

RESULTS

*Purification of Nae*I *Endonuclease.* A summary of a purification of *Nae*I endonuclease from 500 g of *Escherichia coli* NEB786 is outlined in Table 2. All steps were performed either on ice or at 4 °C. The isolation buffer used throughout contained 20 mM potassium phosphate pH 6.8, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 5% (v/v) glycerol with no NaCl unless otherwise indicated.

Preparation of Cell-Free Extract. After thawing on ice, 500 g of *E. coli* NEB786 cell paste was suspended in 600 mL of isolation buffer containing 50 mM NaCl and 1 mM PMSF. The cells were disrupted using a Branson 185 Cell Disrupter (output control = 6) for 30 min. The cellular debris was then pelleted at 10000*g* for 45 min and the pellet

Table 2: Purification of *Nae*I Endonuclease from 500 g of *E. coli* NEB786

fraction	step	total protein[a] (mg)	specific activity[a] (units/mg of protein)	recovery[b] (%)
I	cell-free extract	15 000	3000	(100)
II	DEAE Sepharose-Heparin agarose	440	100 000	98
III	DEAE Sepharose	220	200 000	98
IV	heparin agarose	97	440 000	95
V	S-Sepharose	20	1 200 000	53

[a] Total protein and specific activity determined as described under Materials and Methods. [b] Defined as percentage of total *Nae*I activity present in the cell-free extract.

discarded. The resulting cell-free extract (fraction I) was retained.

DEAE Sepharose−Heparin Agarose Chromatography. Fraction I (630 mL) was loaded onto a DEAE Sepharose CL-6B column (4.8 × 30 cm) equilibrated in the isolation buffer containing 50 mM NaCl. The flow-through from this column was loaded directly onto a heparin agarose column (2.5 × 30 cm) equilibrated in the same buffer. The heparin column was then washed with isolation buffer containing 0.2 M NaCl, until the A_{280} of the effluent reached baseline. The heparin column was then washed with isolation buffer containing 0.6 M NaCl, and fractions were collected. The fractions containing *Nae*I activity were pooled (fraction II) and dialyzed against isolation buffer.

DEAE Sepharose Chromatography. Fraction II was loaded onto a DEAE Sepharose CL-6B column (2.5 × 30 cm) equilibrated in isolation buffer. A linear salt gradient (500 mL, 0−0.5 M NaCl in isolation buffer) was used to elute bound proteins on the column. Fractions (8 mL) containing *Nae*I activity, which typically eluted between 0.1 and 0.2 M NaCl, were pooled (fraction III).

Heparin Agarose Chromatography. Fraction III was applied directly onto a heparin agarose column (2.5 × 30 cm) equilibrated in isolation buffer and washed with 300 mL of this buffer. Fractions (8 mL) were collected from a linear gradient (1 L, 0−1 M NaCl) as they eluted from the column. Two major protein peaks as indicated by A_{280} typically eluted from the column. The second peak corresponded with *Nae*I activity and would elute at about 0.3−0.5 M NaCl. These fractions were pooled (fraction IV) and dialyzed against isolation buffer.

S-Sepharose Chromatography. Following dialysis, fraction IV was loaded onto an S-Sepharose column (2.5 × 10 cm) and washed with 100 mL of isolation buffer. Fractions (8 mL) were collected from a linear gradient (1 L, 0−0.5 M NaCl) as they eluted from the column. *Nae*I activity corresponded with the first major protein peak that eluted from this column (about 0.15 M NaCl) as indicated by A_{280}. The fractions containing this peak (fraction V) were pooled and dialyzed against isolation buffer.

SDS−PAGE of fraction V indicated a single protein band with a molecular mass of about 35 kDa of ≥97% purity, consistent with the molecular weight of *Nae*I as indicated by its amino acid sequence (Taron et al., 1994). Typical yields of 15−22 mg of *Nae*I were obtained from cells grown from 20 L of culture. On occasion, fraction V was not completely free of nonspecific nuclease activity. On these occasions, fraction V was loaded onto a heparin agarose column (1.3 × 23 cm) equilibrated in isolation buffer and washed with 100 mL of the same buffer. The protein was then eluted with a linear gradient (500 mL, 0−1 M NaCl). A single peak would elute off at 0.3−0.5 M NaCl, corresponded to *Nae*I activity and MW as indicated by SDS−PAGE, and would be free of nonspecific nucleases. Once free of nonspecific nucleases, the purified enzyme was concentrated using a Centriprep-10 (Amicon, Beverly MA) concentrator and then dialyzed against a storage buffer containing 50 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM DTT, 0.1 mM EDTA, and 50% glycerol. At protein concentrations of ≥100 μg/mL, purified *Nae*I in the absence of BSA lost no detectable activity (<10%) when stored at −20 °C for 6 months. Figure 1 shows a SDS−polyacrylamide gel stained with Comassie Blue with samples from each fraction of the purification outlined above.

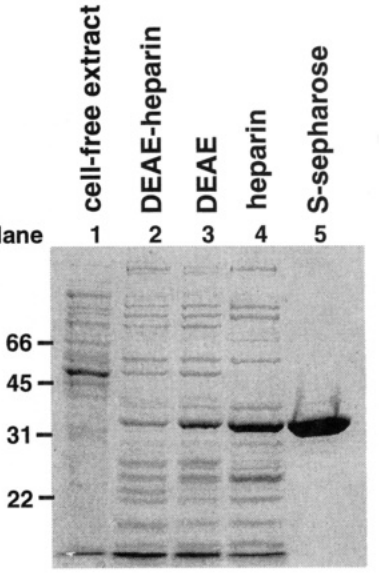

FIGURE 1: SDS-polyacrylamide gel (15%) stained with Commassie Blue. Shown are fractions I−V from the purification scheme for *Nae*I as outlined under Results. Thirty micrograms of total protein was loaded in each lane. Mobilities of prestained, molecular weight standards (Bio-Rad) are indicated: BSA (66), ovalbumin (45), carbonic anhydrase (31), trypsin inhibitor (22).

Catalytic Properties of NaeI Endonuclease. *Nae*I endonuclease obeys Michaelis−Menten kinetics for the cleavage of resistant DNA substrates in the presence of effector DNA (Conrad & Topal, 1989). In this work, the values for the steady-state turnover number k_{cat} and K_M for purified *Nae*I in the presence of effector DNA were determined using both covalently closed M13mp18 RFI circles and a 14 base pair DNA fragment M13−14 (see Table 1) as substrates. Figure 2 shows the extent of cleavage of ^{32}P-labeled M13−14 at 30 min with respect to increasing concentrations of M13−14. Figure 3 shows the double-reciprocal plot of velocities of cleavage against substrate concentration: Activated *Nae*I obeys Michaelis−Menten kinetics for both substrates. Table 3 lists values for k_{cat} and K_M determined for these substrates of *Nae*I in the presence of 100 nM effector HA at 37 °C. The 14 base pair DNA fragment M13−14 possesses a value for k_{cat} that is 9-fold greater than that of M13 DNA, whereas

Table 3: Values of the Steady-State Parameters k_{cat} and K_M for *Nae*I Endonuclease with Different Substrates

substrate	k_{cat} (s^{-1})	K_M (nM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{st} (s^{-1})
M13mp18[a]	$(4.5 \pm 0.5) \times 10^{-2}$	10 ± 3	$(5 \pm 2) \times 10^6$	≥ 0.2
M13−14[a]	$(4 \pm 0.4) \times 10^{-1}$	$(1.7 \pm 0.2) \times 10^2$	$(2.4 \pm 0.5) \times 10^6$	nd
M13−14 nc[b]	$(3 \pm 1) \times 10^{-7}$ [c]	$(1-3) \times 10^4$	$(7-40) \times 10^{-3}$	nd

[a] Reaction conditions used are as described under Materials and Methods, with 0.21 nM *Nae*I and 100 nM DNA fragment HA. [b] With 100 nM *Nae*I. [c] Determined from velocities of cleavage reactions performed at 100 and 200 μM substrate. All values listed are determined from at least three independent experiments.

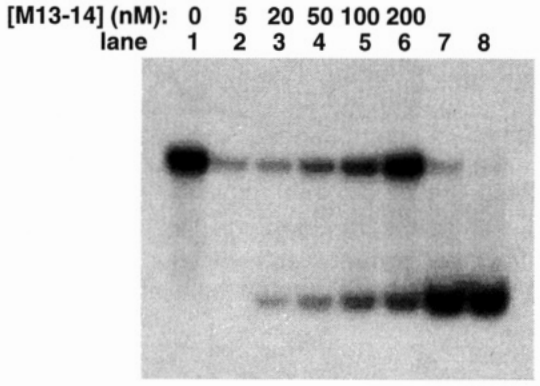

FIGURE 2: Resolution of cleavage products of M13−14 by electrophoresis on 20% polyacrylamide. Reaction conditions are described under Materials and Methods. ^{32}P-labeled M13−14 was incubated with 0.21 nM *Nae*I and 100 nM HA. (Lane 1) M13−14 only. (Lanes 2−6) *Nae*I cleavage of M13−14 in the presence of 5, 20, 50, 100, and 200 nM M13−14, respectively. (Lanes 7 and 8) M13−14 cut to completion by *Nae*I in the presence of 100 nM HA and 100 nM HA thiolated between the scissile phosphate bond, respectively.

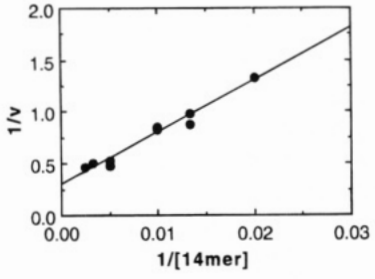

FIGURE 3: Lineweaver−Burke plot for the kinetics of *Nae*I-mediated cleavage of M13−14. Reaction conditions are described under Materials and Methods, in the presence of 0.21 nM *Nae*I and 100 nM HA.

the apparent K_M for M13−14 is 17-fold greater than that seen for M13 DNA. k_{cat}/K_M for M13 DNA is 2-fold higher than that observed for M13−14.

*Single Turnover and Transient Kinetic Analysis of DNA Cleavage by Nae*I. The first-order rate constant of the single turnover for DNA cleavage (k_{st}) was determined at saturating *Nae*I concentrations to compare its value with the steady-state turnover number k_{cat}. Similar values for k_{cat} and k_{st} would imply chemistry of double-strand scission is rate-limiting for enzyme turnover. With 20 nM *Nae*I, 2 nM M13 DNA, and 100 nM effector HA, M13 DNA was completely cleaved in $\leq$15 s (results not shown), which indicates that the half-time for the double-stranded cleavage of enzyme-bound M13 DNA is $\leq$4 s. This result indicates that k_{st} for M13 DNA cleavage by *Nae*I is ≥ 0.2 s^{-1} (Table 3) and is at least 5-fold greater than k_{cat} (Table 3) with M13 DNA as a substrate.

M13 DNA cleavage by *Nae*I was examined to see if a transient phase of rapid product formation (a burst of cleavage) preceded the steady state. Such a burst of product formation is characteristic of product release being rate-limiting for enzyme turnover in the steady state. Figure 4 shows the plot of the concentration of cleavage product against time for experiments performed at two different concentrations of *Nae*I. The kinetics for M13 DNA cleavage in Figure 4 are consistent with a burst of product formation in the first 30 s of the reaction, during which the linear M13 DNA product appears more rapidly than in the subsequent steady state. The magnitude of this cleavage burst was measured by back-extrapolation of the steady-state phase to zero time as shown in Figure 4. The amount of substrate cleaved during the burst was proportional to *Nae*I concentration and was approximately equal to half of the concentration of total enzyme present in reaction at 10.3 nM M13 DNA. No significant amount of open circle (nicked) M13 DNA accumulated during these experiments (results not shown).

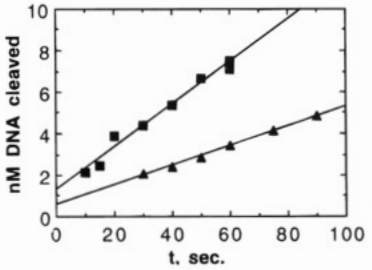

FIGURE 4: Transient kinetics of *Nae*I-mediated M13mp18 cleavage. Reaction conditions are described under Materials and Methods. The reaction mixture contained 2.1 nM (■) and 1 nM (▲) *Nae*I and 10.3 nM M13−14. The y intercepts are 1.3 nM and 0.6 nM for these two cleavage reactions, respectively. The reaction was initiated by the addition of HA to a final concentration of 100 nM. Aliquots (5 μL) were withdrawn at time intervals, and the reaction was quenched by mixing with 1 μL of 500 mM EDTA. Cleavage products were then separated on agarose gels and quantitated as described under Materials and Methods.

Recognition and Cleavage of Noncognate DNA Sequences. A noncognate recognition site, CCCGGC, defined as differing from cognate by a single base pair, was examined for its ability to act as a substrate and for its ability to act as an effector and inhibitor M13 DNA cleavage by *Nae*I. The ability to activate and inhibit M13 DNA cleavage was quantitated by determining the values of the dissociation constants that measure the affinity of the DNA fragment for the effector binding site (K_A) and the substrate binding site (K_I). Table 4 lists values of K_A and K_I determined as described previously (Yang & Topal, 1992) for M13 DNA cleavage by *Nae*I. Activation was observed for HA nc, but could not be detected for LA nc. HA nc has a value for K_A of 75 μM, and the lower limit of K_A for LA nc is 150 μM. Values of K_I for LA nc and M13−14 nc were determined from the competitive inhibition of *Nae*I-mediated M13 DNA cleavage activated by HA. The values of K_I for the three DNA fragments containing noncognate recognition sites have similar values of 11−15 μM.

Table 4: Values of K_A and K_I for *Nae*I-Mediated M13mp18 Cleavage of Various DNA Fragments with Cognate and Noncognate Recognition Sites

DNA fragment	K_A (nM)	$\Delta\Delta G$ (K_A)[b] (kcal/mol)	K_I[c] (nM)	$\Delta\Delta G$ (K_I)[b] (kcal/mol)
HA[a]	17	5.2	310	2.2
HA nc	7.5×10^3		1.1×10^4	
LA[a]	137	>4.3	22	4.0
LA nc	$>1.5 \times 10^5$		1.5×10^4	
M13−14[a]	83		97	3.0
M13−14 nc	not determined		1.2×10^4	

[a] Values from Yang and Topal (1992). [b] Calculated from the ratio of noncognate to cognate at 37 °C. [c] Determined from the ability of each DNA fragment to competitively inhibit activated *Nae*I cleavage of M13 DNA.

We also characterized the cleavage of noncognate recognition sequences by *Nae*I. In the presence of 100 nM enzyme, 100 μM ^{32}P-labeled noncognate DNA substrate (M13−14 nc) and effector with a thiolated scissile bond to prevent significant cleavage of the effector (Topal & Conrad, 1992), significant noncognate substrate cleavage was only observed after 5 h: after 20 h, less than 5% of the noncognate DNA substrate had been cleaved. The concentration of effector was kept below its K_D (310 ± 40 nM; Yang & Topal, 1992) for the substrate binding site, to avoid significant inhibition of DNA cleavage because of competition between effector and substrate DNAs for binding to the substrate binding site. The velocities for DNA cleavage at 100 and 200 μM noncognate DNA substrate were identical. No DNA cleavage could be observed at noncognate substrate concentrations below 20 μM in our assays. These results indicated that the cleavage observed at ≥100 μM noncognate DNA was at saturating substrate concentrations (V_{max} conditions). Therefore, the value for k_{cat} could be determined directly from the velocity of cleavage at ≥100 μM noncognate substrate. These results also defined a range for the value of K_M for the noncognate sustrate based on our lower limit of detection and the lack of cleavage at 20 μM [DNA] and V_{max} at ≥100 μM (Table 3). Values of k_{cat} and K_M for the cleavage of the noncognate substrate are listed in Table 3: k_{cat} is 10^6-fold smaller than for cognate, whereas K_M for noncognate is between 50- and 200-fold larger than cognate (lower affinity).

DISCUSSION

The purification procedure for *Nae*I endonuclease outlined here provides apparently homogeneous enzyme in good yield. Although simpler protocols for the purification of restriction endonucleases yield preparations sufficiently pure for many purposes [e.g., Greene et al., (1978)], further purification is necessary for structural determinations. The quantitative yields of protein from this procedure are suitable for physical analysis such as crystallization and X-ray structure determination.

Under denaturing conditions, the endonuclease behaves as a monomeric species with a molecular mass of 35 kDa consistent with the 35 200 MW calculated from the amino acid sequence translated from the gene coding for the endonuclease (Holtz & Topal, 1994; Taron et al., 1994). On the basis of the densitometric analysis of the cell-free extract shown in Figure 1, *Nae*I accounts for about 2% of the total cytosolic protein present in cells that have been induced with IPTG. This suggests a 50-fold purification by our procedure. A 400-fold increase in specific activity, however, is indicated in Table 2. This suggests that either *Nae*I is over represented in cell-free extract by densitometry because of comigrating proteins or that the conditions of the cell-free extract inhibit *Nae*I activity, lowering the apparent specific activity reported for fraction I of Table 2.

Determination of the Rate-Limiting Step for NaeI-Mediated DNA Cleavage. The value of k_{cat} for *Nae*I cleavage of M13 DNA (Table 3) at 37 °C is at least 5-fold smaller than k_{st}. These results imply that the chemistry of double-stranded DNA scission cannot be predominantly rate-limiting for the steady-state turnover of *Nae*I and that a nonchemical step either prior to or subsequent to DNA strand scission is the slow step.

To determine the rate-limiting step, we examined transient-phase product formation prior to the steady-state. With M13 DNA as substrate, there is an initial "burst" of product formation prior to steady-state as shown in Figure 4. The magnitude of the burst was determined by back extrapolating the steady-state phase of the reaction to zero time; the magnitude of the burst was found to depend on the concentration of *Nae*I present in the reaction. This *Nae*I-dependent burst of product formation observed in the transient phase is consistent with product release being partially or completely rate-limiting for the steady-state turnover of *Nae*I when cleaving M13 DNA.

The conversion of the enzyme−substrate complex ES to products can be outlined as shown in

$$ES \xrightarrow{k_1} EP \xrightarrow{k_2} E + P \qquad (1)$$

k_1 represents the microscopic rate constant for the DNA cleavage event, whereas k_2 represents the microscopic rate constant for product release. The magnitude of the burst of product formation π can be described by eq 2 [eq 4.79 in Fersht (1985)].

$$\pi = [E]_o(k_1(k_1 + k_2))^2 \qquad (2)$$

Since $[E]_o = [E] + [ES]$, burst magnitude depends on [ES] and the relative magnitudes of k_1 and k_2. A burst will be observed if the concentration of S is sufficiently high (e.g., $[S] \geq K_M$) and if $k_1 \geq k_2$, such as when product release is rate-limiting for k_{cat}. On the other hand, if $k_1 < k_2$, such as in the case when chemistry or a nonchemical step prior to product release is rate-limiting, π in eq 2 will approach 0, and no discernible burst of product formation will be observed. The magnitude of the bursts of product formation shown in Figure 3 for M13 DNA cleavage is half of the total enzyme concentration at 1 nM and 2.1 nM *Nae*I. The experiments shown in Figure 4 were performed at a concentration of M13 DNA near K_M (10.3 nM), so $[E] \approx [ES]$, and, according to eq 2, the burst of product formation should be half of the total enzyme concentration in the reaction if a step following chemistry in rate-limiting, such as is observed in these experiments. These observations are all consistent with the conclusion that product release is rate-limiting for *Nae*I-mediated M13 DNA cleavage.

A similar burst of product formation in the transient phase was observed for the cleavage of pAT153 by *Eco*RV and has been attributed to rate-limiting product release (Halford & Goodall, 1988). It has also been suggested that product

release is rate-limiting for the steady-state turnover of *Eco*RI during DNA cleavage (Modrich & Zabel, 1976; Terry et al., 1987).

The Effect of Substrate Length on k_{cat} and K_M. Under steady-state conditions ([E] ≪ [S]) and at concentrations of effector DNA that saturate *Nae*I, we have determined the values of K_M and k_{cat} for cleavage of both a large supercoiled DNA substrate and for a small linear DNA substrate (Table 3). k_{cat} for M13−14 is almost 10-fold greater than k_{cat} for M13 DNA, even though the flanking sequences surrounding the *Nae*I recognition site of M13−14 are identical to those that surround the recognition site in M13mp18. This observation suggests that product release for M13−14 cleavage by *Nae*I is significantly faster than product release for *Nae*I cleavage of M13 DNA. This difference cannot be attributed to DNA supercoiling: cleavage of linear M13 DNA by activated *Nae*I exhibits identical reaction kinetics to the supercoiled substrate (results not shown). The observed value of k_{cat} for *Nae*I cleavage of M13−14, 0.4 s^{-1}, is consistent with chemistry being predominantly rate-limiting, since k_{st} is ≥ 0.2 s^{-1}. It can be concluded from these results that product release from *Nae*I is significantly rate-limiting for *Nae*I cleavage of M13. In addition, the rate of product release from *Nae*I is distinctly substrate length dependent, at least for short DNA fragments such as M13−14.

The apparent K_M for the 14 base pair DNA fragment is 24-fold greater than that seen for M13 DNA and suggests that *Nae*I has an apparent 24-fold lower affinity for the 14 base pair DNA fragment than for M13 DNA. This result appears to be consistent with the DNAse I protection studies that indicate that *Nae*I has a footprint of 24 base pairs that symmetrically surrounds the recognition site (Baxter & Topal, 1993). The 14 base pair DNA fragment M13−14 may not completely fill the DNA binding cleft of *Nae*I and so may not utilize all the favorable DNA−protein contacts available for optimal binding.

Despite the large respective differences between k_{cat} and K_M for M13 DNA cleavage and k_{cat} and K_M for M13−14 DNA cleavage, the value for the apparent second-order rate constant k_{cat}/K_M for M13 is only about 2-fold larger than that for the 14-mer. Because the relative values of k_{cat}/K_M indicate the relative specificities of an enzyme for its substrates regardless of reaction mechanism pathways (Fersht, 1985), this result suggests that *Nae*I has similar specificities for M13 and the 14-mer, even though *Nae*I possesses an apparent 17-fold greater affinity for M13 than for the 14-mer as reflected in K_M.

*The Accuracy of DNA Cleavage by Nae*I. The accuracy of the enzymes involved in restriction-modification is important to the host: unwanted cleavage at a noncognate (improper) recognition site may kill the host organism. In addition, the mechanisms used to attain accuracy are important to our understanding of DNA−protein interactions. We characterized the role(s) of binding and catalysis in the accuracy of *Nae*I-mediated DNA cleavage by determining the relative binding of cognate and noncognate DNAs at the substrate and effector binding sites. The overall accuracy of DNA cleavage was determined from the relative values of k_{cat}/K_M for cleavage of cognate and noncognate sequences and expressed (eq 3) as a free energy difference in kcal/mol.

$$\Delta\Delta G_{cleavage} = -RT \ln[(k_{cat}/K_M)_{cognate}/(k_{cat}/K_M)_{noncognate}] \quad (3)$$

Ideally, this difference in specificity between cognate and noncognate sequences would be demonstrated directly. The examination of the cleavage of noncognate DNA sequences by activated *Nae*I, however, was already at the lower limits of our detection. The two-site model for *Nae*I interaction with DNA (Yang & Topal, 1992; Baxter & Topal, 1993) implies that the overall free-energy differences for discrimination can be measured by determining the free-energy differences for binding at the effector sites as well as the free-energy differences for cleavage at the substrate site. Measurements of specificity from separate contributions of binding and catalysis have been used successfully by others [e.g., Lesser et al. (1990)].

The ability was examined of the substrate DNA binding site of *Nae*I to discriminate between a cognate and noncognate (differing from cognate by a single base pair) recognition sequence. Three different 14-base pair DNA fragments were examined for their ability to competitively inhibit *Nae*I cleavage of M13 DNA. These DNA fragments contained the same noncognate *Nae*I recognition site with different flanking sequences. Values of K_I for these three noncognate DNA fragments varied from 11 to 15 μM (Table 4). These values of K_I (a measure of the affinity of a DNA fragment for the substrate binding site) indicate that flanking sequences do not significantly affect the affinity of *Nae*I for noncognate recognition sequences. The binding of cognate sequences, on the other hand, are subject to DNA context effects: values of K_I vary by over an order of magnitude depending upon sequences flanking cognate recognition site (Yang & Topal, 1992). Comparing K_I values for corresponding cognate and noncognate DNA fragments showed that the binding affinity at the *Nae*I substrate binding site varied for cognate vs noncognate from 40- to 500-fold ($\Delta\Delta G \approx 3$ kcal/mol).

The ability of the effector DNA binding site to discriminate cognate from noncognate sequences was also examined. Activation of *Nae*I cleavage of M13 DNA by noncognate vs cognate DNA fragments indicated that the affinity of the effector binding site for the noncognate recognition sequence was at least 1000-fold lower ($\Delta\Delta G \approx 5$ kcal/mol) than for the corresponding cognate recognition sequence (Table 4).

k_{cat} and K_M for cleavage of cognate and noncognate DNA fragments (Table 3) indicated that catalysis (k_{cat}) was significantly decreased for the noncognate recognition sequence in addition to its decreased affinity for the substrate binding site (measured through K_M). k_{cat} is 10^6-fold smaller for the noncognate sequence than for cognate. The specificity (in the sense of discrimination between cognate and noncognate sequences) of activated *Nae*I for cognate is 10^8-fold higher than for noncognate sequence as indicated by the ratio of cognate to noncognate k_{cat}/K_M (Table 3). This ratio amounts to an energetic difference of 11.3 kcal/mol at 37 °C (calculated as described above). When the difference in the affinity of the effector binding site for a noncognate sequence is taken into account (4000-fold difference in K_A for *Nae*I interaction with HA versus HA nc, Table 4), the specificity of *Nae*I for cleavage of cognate over noncognate sequences exceeds 11 orders of magnitude (4×10^{11}), or 16.5 kcal/mol.

Two classes of type II restriction endonucleases have been characterized based on the kinetic properties of their substrate

recognition and cleavage. The first of these derives its specificity from both binding and from catalysis (type IIi). DNA binding specificity for these enzymes is independent of the magnesium ion cofactor. *Eco*RI (Halford & Johnson, 1980; McLaughlin et al., 1987; Lesser et al., 1990) and *Rsr*I (Aiken et al., 1991b) are examples of this subclass that have been examined in detail. The second subclass derives its specificity predominantly at the catalytic level: enzymes from this subclass possess nominally similar affinities for cognate and random DNA sequences (type IId) and are dependent on the presence of magnesium to derive their specificity. Examples of these include *Eco*RV (Taylor & Halford, 1989) and *Taq*I (Zebala et al., 1992b). *Nae*I represents yet another subclass, type IIe, that requires the binding of an effector DNA element for cleavage of substrate to occur. The results presented here indicate that both DNA binding and catalysis play significant roles in the derivation of specificity for *Nae*I. The requirement for two DNA recognition elements results in a specificity for cognate versus noncognate that exceeds 16 kcals/mol. This energetic difference significantly exceeds the specificities for other type II restriction endonucleases for which specificity has been measured.

The specificity of the type IIi restriction endonuclease *Eco*RI as described by the ratios of k_{cat}/K_M for cognate and noncognate sequences is 5×10^7 in λ DNA (Halford & Johnson, 1980) and $\geq 10^6$-fold for 16 base-pair DNA fragments (Thielking et al., 1990). Two orders of magnitude of this specificity come from catalysis, and the remaining five orders of magnitude come from DNA binding. The specificity in binding is not affected by Mg^{2+}. In the absence of Mg^{2+}, *Eco*RI binds short DNA fragments containing cognate DNA sequences up to 10^4-fold tighter than the same fragments containing noncognate sequences (Lesser et al., 1990; Thielking et al., 1990). In addition, the separate contributions to specificity of *Eco*RI DNA cleavage from both binding and catalysis are evident in the glutamate-111 to glycine mutant of *Eco*RI. This mutation reduced the rate constants for DNA strand cleavage by $>10^4$-fold but did not change the protein's affinity for its recognition sequence (King et al., 1989). Like *Eco*RI, the *Nae*I substrate site possesses specificity contributions from both catalysis and binding. *Nae*I and *Eco*RI, however, differ in the relative contributions of binding and catalysis toward specificity. For activated *Nae*I, the majority of the contribution to substrate specificity comes from catalysis as reflected in the difference in the values of k_{cat} between cognate and noncognate recognition sites (six orders of magnitude, Table 3). The remaining two orders of magnitude come from DNA binding (K_M).

The specificity (k_{cat}/K_M) of the type IId restriction endonuclease *Eco*RV is 10^6 (Taylor & Halford, 1989). The mechanism of this specificity, however, differs from that of *Eco*RI: *Eco*RV binds all DNA sequences with equal affinity in the absence of Mg^{2+} (Taylor et al., 1991). These results implied that Mg^{2+} is required for DNA binding specificity and catalysis of DNA cleavage by *Eco*RV. Mutation of catalytically essential aspartic acid-90 to alanine abolished cleavage, but in the presence of Mg^{2+} the enzyme bound cognate recognition sequence at least 4000-fold tighter than nonspecific DNA (Thielking et al., 1992).

In summary, we have purified *Nae*I to $\geq 97\%$ and demonstrated that product release is rate limiting for turnover of this protein when cleaving M13 DNA. The specificity of *Nae*I cleavage of cognate versus noncognate DNA sequences was studied: in the presence of cognate effector sequences (i.e., independent of the effector site) this specificity is 10^8. This value is similar to the specificities observed for *Eco*RI of 5×10^7 (Halford & Johnson, 1980) and for *Eco*RV of 1×10^6 (Taylor & Halford, 1989) and corresponds to an energetic difference of 11.3 kcal/mol. When the required recognition of cognate sequences at the effector DNA binding site of *Nae*I is taken into account, the specificity for *Nae*I is 4×10^{11}, corresponding to an energetic difference of 16.4 kcal/mol. These results demonstrate that the effective specificity of *Nae*I for cognate recognition sequences is significantly increased over those observed for *Eco*RI and *Eco*RV because *Nae*I must bind two recognition sequences for cleavage to proceed. The accuracy for *Nae*I-mediated cleavage of a noncognate site is approximately 10^4-fold larger than for any other protein recognizing a similar size recognition sequence, but requiring only a single bound sequence for function.

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