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Random Mutagenesis of Staphylococcal Nuclease and Phage Display Selection

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Abstract—Multiple cycles of mutagenesis and phage display selection have been investigated as a method for obtaining enzymes with altered catalytic properties. A library of staphylococcal nuclease mutants displayed on phage was created by error-prone PCR mutagenesis and selected for binding to thymidine- or guanosine-containing substrate analogs. After discarding non-binders, the binding mutants were then subjected to further mutagenesis and selection rounds. After four mutagenesis and selection cycles, the catalytic properties of some of the resulting nucleases were studied and one nuclease with nine accumulated mutations was found to have a two-fold reduction in k_{cat} for DNA hydrolysis, but a two-fold increase in k_{cat}/K_m for hydrolysis of a thymidine containing small molecule substrate. The possibility of this technique for *in vitro* evolution of enzyme properties is discussed.

Introduction

Phage display is a powerful technique for selecting specific binding interactions from protein libraries. Many types of libraries have been examined, depending on the type of interaction sought. Some of the first libraries were randomized peptide epitopes, which were used to identify antibody ligands.[1] Phage display has also been used to clone antibodies from humans and mice.[2,3] In addition to natural sources, library diversity can be created *in vitro* by random mutagenesis of codons or entire genes, as demonstrated with antibodies,[4] hormones,[5,6] and zinc fingers.[7,8] Proteins thus obtained through phage display have been shown to have improved or altered specificity or both. Our goal therefore is to demonstrate that phage display and random mutagenesis can be used to obtain variants of the enzyme staphylococcal nuclease with new catalytic properties. Such mutants could provide mechanistic information as well as demonstrate a new enzyme engineering technique.

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Abbreviations: LB, Luria Broth; SB, super broth (Barbas *et al.*, 1991); TBD, toludine blue dye; NP-pdT, *p*-nitrophenyl thymidine-5'-phosphate; NP-pdG, *p*-nitrophenyl guanosine-5'-phosphate; Snase, staphylococcal nuclease; PCR, polymerase chain reaction; SOC, media (Sambrook *et al.*, 1989); carb^R, carbenicillin resistance; pfu, plaque forming units; TBS, 25 mM Tris–HCl, 150 mM NaCl, 2.5 mM KCl, pH 7.4; TBSC, TBS plus 10 mM $CaCl_2$; TEAB, triethylammonium bicarbonate; DMSO, dimethyl sulfoxide; WT, wild type; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis-(oxyethylenenitrilo)tetraacetic acid; CIP, calf intestinal alkaline phosphatase; dNTP, nucleoside triphosphates; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

The power of phage display arises from the ease with which binders can be selected from libraries of 10^{6-9} members. Library diversity originates in DNA sequences, which are then translated into protein. In the type 3 + 3 system[9] used here, the DNA of interest is fused to the C-terminal fragment of the filamentous phage minor coat protein gIII contained in a phagemid vector. Fusion proteins produced from such a vector in the presence of a helper phage are incorporated on to the surface of the phage virions and the phagemid vector is packaged within the phage. In this way phenotype (the displayed protein) and genotype (packaged vector DNA) are physically linked. Phage displaying the desired phenotype are selected from the library by binding to a target immobilized on a solid support, a process called panning. After washing away non-binding phage, bound phage are recovered and the identity of binding proteins easily determined from the DNA sequences. Entire libraries can be quickly screened in one tube, and the upper limit on library size is determined only by the transformation efficiency of DNA into the *Escherichia coli* host.

Staphylococcal nuclease was chosen as a test case for phage display for several reasons. It is a 149 amino acid, Ca^{2+}-dependent phosphodiesterase that accelerates the hydrolysis of DNA up to 10^{16} fold.[10] DNA is cleaved as shown in Figure 1 with a slight preference for sites on the 5' side of thymidine.[11,12] An extensive database of information about SNase has been accumulated,[12–14] including crystal structures,[15–17] and the effects of a large number of mutations on folding stability[18–21] and catalytic function.[10,22–29] Thus a basis for comparison of future mutants is already established and methods for kinetic analysis are well established. In addition, it has been shown that SNase can be expressed in the periplasm of *E. coli*,[30] a condition necessary for phage display.

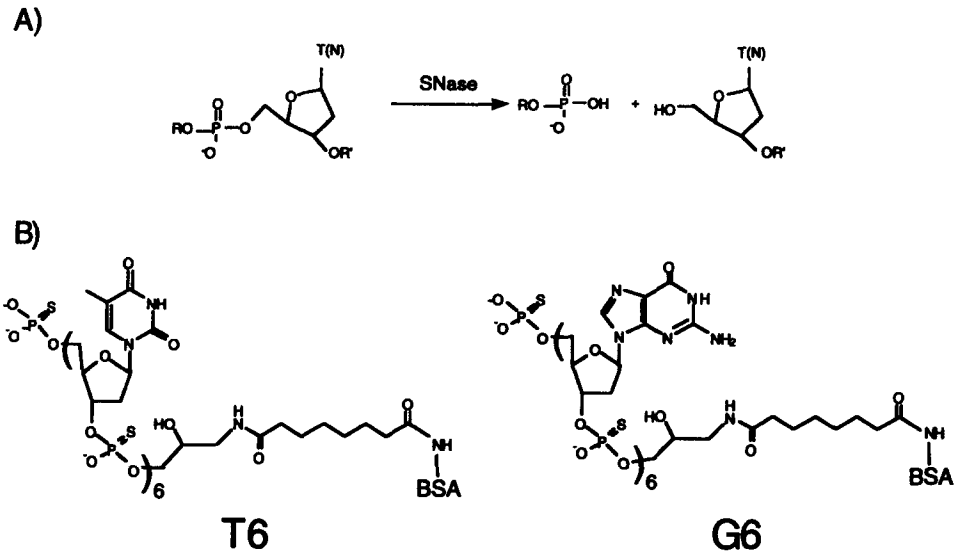

Figure 1. A) Hydrolysis of nucleotide substrates by SNase. B) Phosphorothioate substrate analogs used in panning selections. The substrate analogs were attached to BSA by the linker shown, to facilitate immobilization for panning.

While phage display of active enzymes has already been demonstrated for alkaline phosphatase,[31] trypsin,[32] β-lactamase,[33] and a catalytic antibody,[34] among others, in this work we will examine selection of SNase mutants by phage display from libraries created by error-prone PCR mutagenesis. We chose to use random mutagenesis to generate library diversity, rather than codon-directed mutagenesis, in order to minimize initial constraints on the solutions, which are mutants obtained from the selection process. Furthermore, rounds of mutagenesis and selection will be examined—an *in vitro* mimic of an evolutionary process. In this manner multiple point mutations can be obtained and examined. Two targets were chosen to investigate the role of substrate binding and catalysis, differing in the nucleoside bases. Phosphorothioate hexamer of thymidine (T_6) is expected to be a good inhibitor of wild type (WT) nuclease based upon its similarity to the well known inhibitor 5',3'-thymidine diphosphate. A second target has guanine as the nucleotide base, and is expected to bind less strongly to SNase based upon the known preference of SNase for thymidine.[35-37] A question that will be addressed is if selection of mutants that bind to the G_6 substrate analog will yield nucleases with altered substrate preferences and catalytic properties.

Results

SNase display vector

A phagemid vector that allows SNase to be displayed on gIII of filamentous phage has been constructed and designated pS120+ (Fig. 2). Its most noteworthy features are highlighted here, namely: 1) the *ompA* leader sequence precedes the coding region of mature SNase;[44] 2) the C-terminus of the nuclease is fused to the coding sequence for a short TSGGGGS linker followed by C-terminal codons 198–406 of gIII of M13 filamentous phage; 3) the fusion construct is under control of the *trc* promoter, with strong transcriptional regulation by additional *lacI*^{q} gene in the phagemid; 4) a f1 origin allows production of single stranded phagemid DNA and packaging into phage; 5) phagemid vectors conveying carbenicillin (pS120+) and chloramphenicol resistance (pS122 and pS123) have been constructed. 6) Soluble expression vectors can be created by removing the gIII anchor by either Nhe I/Spe1 or BamH I/Bgl II digestion and self ligation. The sequence of pS120+ determined from construction and partial sequencing has been deposited in Genbank (to be deposited).

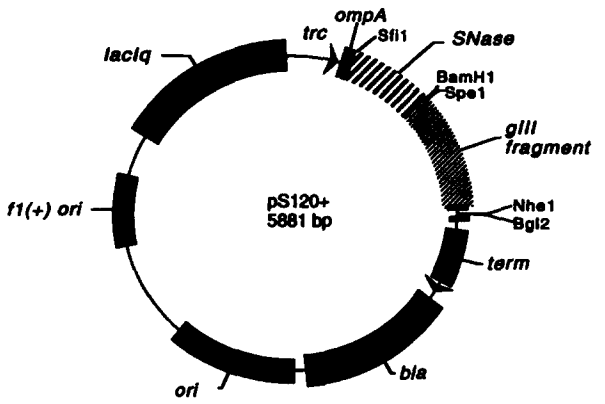

Figure 2. The staphylococcal nuclease display vector pS120+. *trc*, promoter; *ompA*-, *ompA* leader sequence with A(–6)P mutation; *Snase*, codons 1–148 of staphylococcal nuclease: *gIII fragment*, STSGGGGS linker followed by codons 198–406 of the filamentous phage gIII coat protein; *term*, transcription terminator: *bla*, β-lactamase: *ori*, plasmid origin of replication; *f1(+) ori*, filamentous phage origin of replication and packaging signal; *lacI*^{q}, repressor.

Display of SNase

Phage display of functional SNase was demonstrated in the following manner. As a negative control, pS120+ was converted to the soluble SNase expression vector

pS124 by excision of the gIII coding sequence. Phage were then produced from *E. coli* XL-1 Blue containing either helper phage R408 or VCSM13 and either pS120+ or pS124. At this point it was determined that overexpression of the SNase–gIII fusion by induction of pS120+ with IPTG was lethal to the host. However, gratuitous inducers present in the media allowed sufficient SNase–gIII fusion to accumulate and be incorporated into phage. The DNase activities of CsCl purified phage displaying SNase were compared to known SNase and phage expressed in the presence of SNase without the gIII anchor (Table 1). From the kinetic data, it was calculated that on average one of every 2–20 phage displayed active enzyme. Further evidence of SNase display on phage is seen in the following panning selection experiments.

Table 1. Nuclease activity[a] of CsCl purified phage

sample	Vector	rate
0.005 U SNase	–	0.048 A264 min^{-1}
SNase-phage/R408	pS120+	0.0081
SNase-phage/VCS-M13	pS120+	0.0038
phage + free SNase	pS124	<0.00004

[a]Activity was measured by the initial change in absorbance when 0.22 A_{260} of phage were added to 1.0 mL of pH 8.8 sodium borate, 10 mM $CaCl_2$, 50 μg mL^{-1} DNA, 23 °C.

Error prone PCR mutagenesis

Random mutations were introduced into the nuclease gene by error prone PCR.[38] The rate of mutagenesis was increased by performing PCR in three tubes with 10-fold dilutions of templates. The reactions were combined and ligated into the Sfi I/BamH I sites of pS120+ for panning selection. The rates and types of mutations were characterized by sequencing nine randomly picked clones from the first round of mutagensis. Figure 3 shows the locations of mutations in the nine sequences, which appear to be randomly distributed over the nuclease gene. Overall the mutation rate was 2.3% bp^{-1}.

However, the types of mutations that are summarized in Table 2 are not as random as those previously observed by other authors.[38] A definite bias exists in the mutation rates of individual bases, which was 3.5% in A and 2.4% in T, but only 0.5% in G and 1% in C, even after allowance for the 66.4% A–T content of the template. Also observed in the nine sequences (3780 bp sequenced) were three deletions, one insertion, two ochre stop codons (TAA), and one amber stop codon (TAG). Considering the amber suppressor function of the host, four of the nine clones could potentially express full length fusion proteins. The number of active nucleases in the initial library was 3%. Mutation in subsequent selection rounds was performed on the appropriate collection of plasmid DNA, as described later. The number of clones with nuclease activity was 0–5 out of 50 tested (0–10%) after each mutagensis, 3% average.

Panning selection

The randomized phage library was panned against T_6- and G_6-BSA conjugates immobilized on ELISA plates. After binding, the plates were washed 5–7 times, and the bound phage then eluted with acid. Staphylococcal nuclease is completely denatured below pH 3.5.[14] The eluted phage were titered, then amplified, and the panning repeated a second time, completing one selection round. After the second panning, plasmid DNA from the amplification was subjected to PCR mutagenesis, and the selection process repeated for a

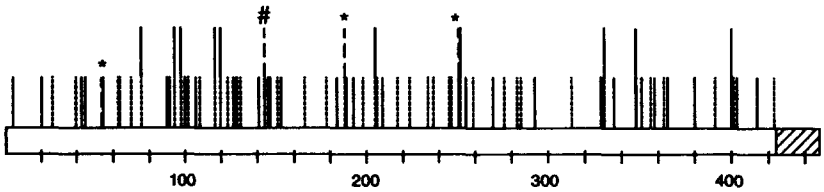

Figure 3. Location of mutations in initial nuclease library. Nine clones were sequenced, a total of 3780 base pairs, giving 86 mutations as follows: 40 transitions (solid lines), 46 transversions (dotted lines), 3 deletions (* and dashed line), and one deletion (# and dashed line). The hashed region indicates the PCR primer site.

Table 2. Mutations observed in 9 clones after initial round of PCR mutagenesis

		Number times base changed to:					
Starting base	Number of base	A	C	G	T	Total times base changed	mutation rate in base
A	1572	–	5	27	23	55	3.5%
C	519	1	–	0	4	5	1
G	753	2	1	–	1	4	0.5
T	936	9	7	6	–	22	2.4
Total	3780	12	13	33	28	86	2.3
		0.54%	0.40%	1.1%	1.0%		

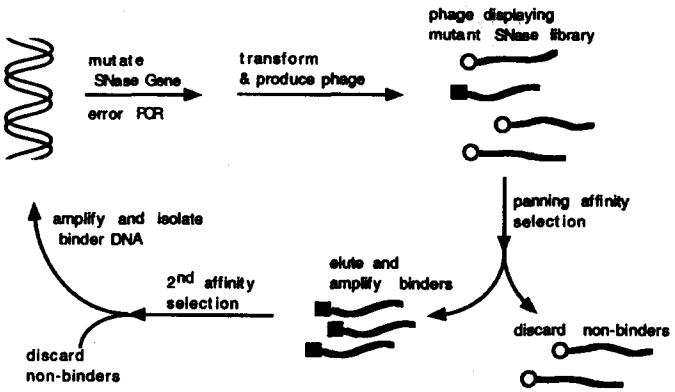

Figure 4. Mutation and selection scheme used to obtain nucleases with altered catalytic activity. The SNase gene was subjected to error prone PCR mutagenesis and cloned into the phage display vector, giving a mutant nuclease library displayed on phage after transformation and phage production. Mutant nuclease that could bind to the substrate analogs T_6 and G_6 were selected by panning, and non-binders discarded. After a second panning selection, the library was amplified and collected as DNA, which was then subjected to mutagenesis, and the scheme repeated for a total of four cycles.

total of four rounds. Figure 4 summarizes the mutagenesis and selection process. After each mutagenesis and panning round, 50 randomly picked clones were tested for nuclease activity. Figure 5 summarizes the results of the selection process in terms of library size or panning output and per cent of clones with strong and weak nuclease activity at each stage of selection. After four selection rounds against T_6-BSA, 50/50 of the clones tested positive for nuclease activity. Sixteen clones were randomly picked and their sequences determined (Table 3). Thirteen of the 16 were the same clone G1. In addition, three other clones were recovered; H1, I1, and J12.

For the selection against G_6-BSA, a drop in the number of clones with nuclease activity was seen in the second panning of the fourth selection round, leaving only 4/50 (8%) nuclease active clones. Therefore additional clones were tested until eight active clones were found. These were sequenced, giving two different clones E6 and F14. In addition, eight of the inactive clones were randomly picked and found to be the same sequence K16 (Table 3).

Protein expression and purification.

Initial attempts at expression of the WT and mutant nucleases were attempted using the soluble expression version of the gIII display vector pS120+. However, only low yields of less than 1–2 mg L^{-1} could be obtained, and the nucleases appeared to be contaminated by significant amounts of unprocessed *ompA* leader–nuclease fusions, as judged by SDS-PAGE analysis. Therefore, the WT and mutant nucleases were subcloned into a T7 expression vector without the leader sequence. The possibility that the nucleases from this system have an N-terminal methionine has not been investigated. Although these vectors allowed protein expression to levels greater than 50% of total cell proteins, purified yields after ion exchange chromatography were 12–60 mg L^{-1}. Figure 6 shows an SDS-PAGE of 10 μg of each purified nuclease. The purity of each nuclease was judged to be better than 95%.

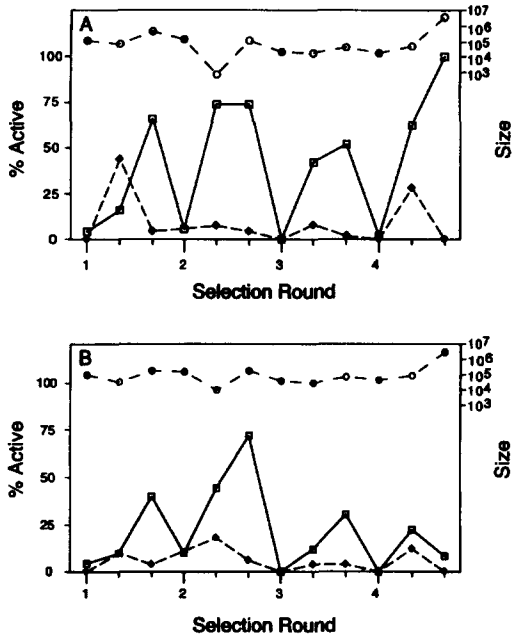

Figure 5. Population size and percentage with nuclease activity during selection experiment. A) Selection against T_6-BSA. B) Selection against G_6-BSA. Four selection rounds were performed, each consisting of PCR mutagenesis (numbered) and 2 rounds of panning (intervening hash marks). Size of library after mutagenesis (closed circle), phage output from panning (open circle), percentage with strong activity greater than 10% of WT activity (open square), percentage with weak activity less than 10% of WT activity (open diamond).

With the clones panned against G_6-BSA, two well resolved peaks were obtained for each clone E6, F14, and K16. These peaks, designated early and late, were collected separately, and found to be the same size by SDS-PAGE. WT SNase gave one peak corresponding to the early peak. It was concluded that these peaks arose from a process that was slow on the chromatography time scale. Indeed, it was found that the late peak could be converted to the early form by heating. The

Table 3. Sequences of nucleases after 4 selection rounds

clone		10	20	30	40	50	60	70	80
SNase		ATSTK	KLHKE	PATLI	KAIDG	DTVKL	MYKGQ	PMTFR	LLLVD	TPETK	HPKKG	VEKYG	PEASA	FTKKM	VENAK	KIEVE	FDKGQ
G1		.....	E....	.....	.....	.....	..R.R	.....	...•.	.....	.....	.....	.....	..R..	..D..	.....	.....
H1		...•.	E....	.•...	....•	.....	..R.R	.....	.....	.....	...•.	.....	.....	.....	..D..	.....	.....
I3		...○.	E....	.....	•.•..	.....	..R.R	.....	...•.	.....	.....	.....	.....	...I.	•.**D**..	.....	.....
J12		....•	E....	.....	.....	.....	..R.R	.....	.....	.•...	.....	.....	.....	..?..	..D..	I....	Y.R..
E6		.....	.....	.V...	....•	.....	.....	.....	...•.	A..•.	.....	.....	.....	.M•..	.....	.•...	•...H
F14		.....	.....	.V...	....•	.....	....R	.....	...•.	A..•.	.....	.....	.....	.....	.....	.....	....H
K16	(-)	....N	.....	V	RT...	.M.R.	K...R	.....	.....	.....	.....	...C.	.....	.....	...•.	.....	....R

clone		90	100	110	120	130	140			
SNase		RTDKY	GRGLA	YIYAD	GKMVN	EALVR	QGLAK	VAYVY	KPNNT	HEQHL	RKSEA	QAKKE	KLNIW	*SEDNA*	*DSGS*	freq.	Δcharge
G1		.....	.....	.....	.....	..•..	L....	.....	.....	.....	...•.	.....	.....	.....	.•..	(13/16)	-2
H1		.....	.....	.....	.....	.•...	L....	.....	..S..	.....	...•.	.....	.....	.....	....	(1/16)	-2
I3		.....	..S..	.....	.....	.....	L....	.....	...D.	.....	.E•..	.....	.....	.....	....	(1/16)	-6
J12		.....	.....	.....	.....	.....	L....	.....	.....	.....	...•.	.....	.....	.....	....	(1/16)	-3
E6		...R.	.....	.....	.....	..•..	.....	.....	...Y.	•....	.....	.....	.....	.....	....	(2/8)	+1
F14		.....	.....	.....	.....	..•..	.....	.....	...Y.	.....	.R...	.....	.....	.....	....	(6/8)	+2
K16	(-)	.....	.....	.....	.**I**...	.....	.....	.....	.....	.....	.....	L.•..	.....	.....	....	(8/8)	+1

italics, primer or restriction site overlap
outline, double mutation in codon
small period, no change
large period, silent mutation
?, codon not determined
freq., number of times clone recovered
Δcharge, net change in charge compared to WT. Charge = number of H, K, and R minus number of D and E

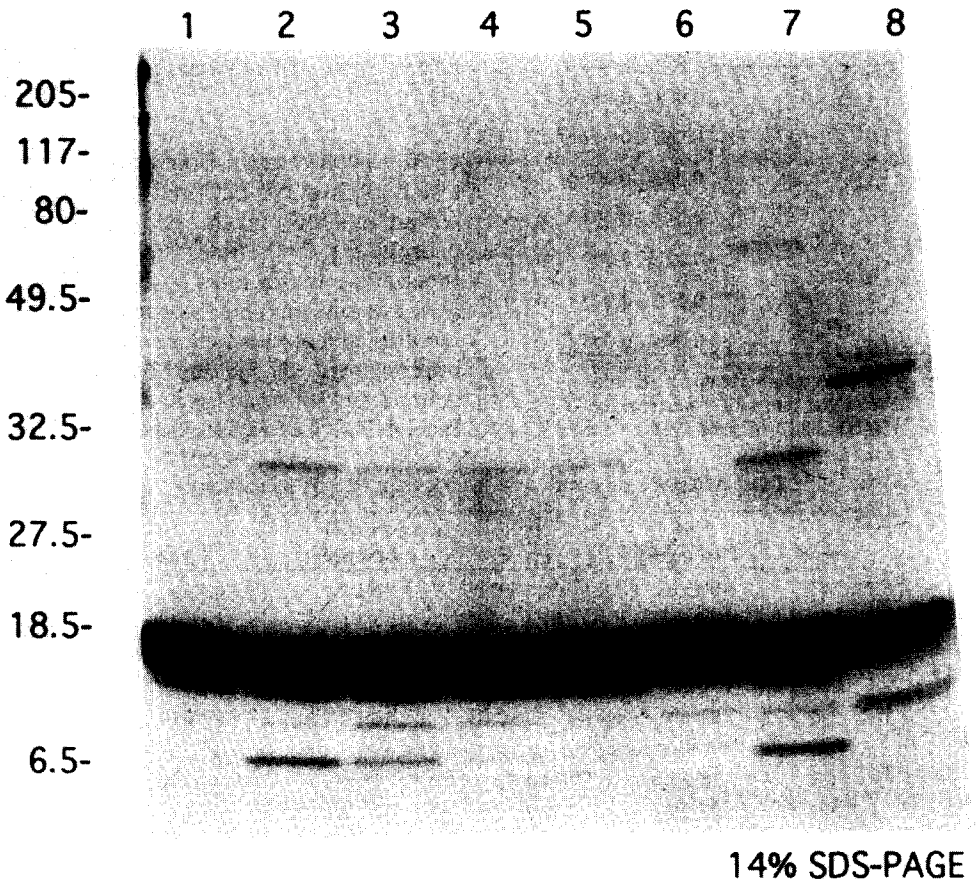

Figure 6. SDS-PAGE gel of purified nucleases. 10 μg of each purified protein was run on a 14% acrylamide gel and stained with coomassie blue according to standard methods (Sambrook *et al.*, 1989). Lane 1) WT SNase expressed in this experiment; 2) E6; 3) F14; 4) G1; 5) H1; 6) I3; 7) J12; 8) SNase purchased from Sigma.

conversion was slow at 4 °C (half life of weeks), but could be completed within 48 h at 50 °C at 0.5 mg mL^{-1}. Gel filtration chromatography gave calculated molecular sizes of 17.5 kD for the early peak and 35 kD for the late peak of clone F14. Thus it appears that the early and late peaks from ion exchange correspond to nuclease monomer and dimers, respectively. These preliminary observations indicating that some of the mutant nucleases become kinetically trapped as a dimer during folding during overexpression will be the subject of further study. For this paper, all kinetic measurements were performed with protein obtained from the monomeric forms of the nucleases.

Kinetic analysis

The activities of the purified nuclease against DNA and chromogenic substrates were measured under conditions that matched the panning conditions in ionic strength and pH. DNase activity was measured by the initial hyperchromicity change at 264 nm[36] on an HP diode array spectrophotometer with manual mixing. However, the apparent K_m for the DNA substrate was much lower than the DNA concentrations that could be followed spectrophotometrically. Thus, only accurate calculations of V_{max} could be made. The maximum rates observed for each clone are summarized in Table 4. For the clones selected against T_6-BSA, the rates are identical within experimental error, except that I3 has half the activity against DNA at pH 7.6. Even slower are the clones that were selected against G_6-BSA, which were three to four times slower than WT SNase for DNA hydrolysis.

Table 4. Maximum DNA hydrolysis rates[a] of selected nucleases

clone	V_{max}
WT	0.037 ± 0.010
G1	0.043 ± 0.008
H1	0.029 ± 0.006
I3	0.017 ± 0.004
J12	0.027 ± 0.003
E6	0.012 ± 0.003
F14	0.015 ± 0.005

[a]From Lineweaver–Burk plots of initial rates measured by hyperchromicity change at 264 nm with 1 nm enzyme in pH 7.4 10 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$.

Colorimetric substrates

In order to better characterize the effect of the selection process on specificity of SNase, the hydrolysis of two well characterized small substrates was studied. NP-pdT is known to be a good substrate of WT SNase.[39] In addition, NP-pdG was also tested with clones that were selected against G_6-BSA and compared to WT enzyme. In each case the nuclease was allowed to cleave the phosphodiester bond, giving *p*-nitrophenylphosphate and free nucleoside. After inhibiting SNase activity with excess EGTA, the amount of *p*-nitrophenylphosphate could be quantitated by a colorimetric assay with CIP.

All nucleases were tested against NP-pdT (Table 5). The largest differences are seen in clones I3, E6 and F14, when compared to SNase. Using k_{cat}/K_m as a measure of catalytic efficiency, clone I3, which was two times less effective against DNA, is now 2.2 times more effective than SNase against the small substrate NP-pdT. Clones E6 and F14, which were selected against the guanosine-containing analog, are about 10 times less effective than WT. The other clones that were also selected against the thymidine containing target, show 20% decreases (G1, H1) or are the same (J12).

Clones panned against the guanosine substrate G_6 were tested against NP-pdG and compared to WT nuclease. NP-pdG is a much poorer substrate for SNase, hydrolyzing 500 times more slowly than NP-pdT. Indeed, at these rates (~2 × 10^{-4} min^{-1}) a 10-fold increase in enzyme concentration to 50 μm was required along with longer reaction times. However, no improvement of E6 and F14 against the guanosine containing substrate was seen as compared to WT. Indeed F14 was eight times slower, and E6 was even

Table 5. Kinetic parameters[a] for hydrolysis of NP-pdT by nucleases

clone	k_{cat} (10^{-3} min^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} min^{-1})	relative
WT	90 ± 7	6.1 ± 0.5	15 ± 2	1.0
G1	91 ± 7	7.4 ± 0.9	12 ± 2	0.80[c]
H1	98 ± 7	8.0 ± 1.0	12 ± 2	0.80[c]
I3	136 ± 5	4.3 ± 0.3	32 ± 2	2.1
J12	92 ± 4	5.7 ±0.3	16 ± 1	1.1[c]
E6	16 ± 2	11 ± 1	1.5 ± 0.3	0.10
F14	23 ± 2	13 ± 1	1.7 ± 0.2	0.11
K16	<0.1[b]	-	-	-

[a]From Lineweaver–Burk plots with 5 mM enzyme in pH 7.6 100 mM Tris–HCl, 150 mM NaCl, 10 mM $CaCl_2$, at 37 °C.
[b]No activity was measurable for K16.
[c]Relative rate same as WT within 95% confidence limit.

slower at 1/17 of WT rate. In each case the reduced catalytic efficiency was a result of decreases in k_{cat} and increases in K_m. The rates are shown in Table 6.

Discussion

Random mutagenesis and phage display selection have been used to generate new mutants of SNase with multiple point mutations and altered catalytic properties. The use of phage display to connect phenotype and genotype allows *in vitro* evolution to be applied to proteins and peptides, as has been applied to RNA.[40] In this case the mutations are generated *in vitro* at the DNA level, and then translated into proteins displayed on phage. These proteins can be selected for binding to immobilized targets, and the associated DNA also recovered. This DNA can be easily amplified and used to identify the sequence of the displayed proteins or as a starting point for further mutations. In a sense the amplification can be thought of as 'reproduction', and the panning as 'natural selection', completing the *in vitro* evolution process.

Mutations were achieved by error-prone PCR. Under optimal conditions,[38] mutation without strong preference for the type of base substitution can be achieved. However, in this work a definite bias in the types of base substitutions was observed. The mutational bias was most likely a result of three factors: 1) different reaction buffer; 2) lesser amount of template; 3) uneven base composition in the template, which is 66.4% AT. Template composition can be expected to have a large effect in the concentrations of nucleoside triphosphates as the PCR progresses. During PCR of an AT rich template, depletion of dATP and dTTP compared to dCTP and dGTP will occur, and would be expected to increase the rate of mutations in AT base pairs compared to GC base pairs (Table 1, Ref. 38). While it should be possible to adjust the PCR conditions to obtain a more even distribution of base substitutions, we felt that the unoptimized conditions with a mutation rate of 2% per base pair were sufficient to test the phage display selection method.

Coupling a phage display selection process to multiple rounds of mutagenesis enriches the population for the desired activity. Without selection, four rounds of mutagenesis would give a final population with 1 in 10^6 (33^4) active nucleases, assuming that each round returns one in 33 (3%) active clones. Finding active clones in such a library would require extensive screening. However, when phage display is used to enrich for active clones at each step, the final number of active clones was 8 and 100% for panning against T_6 and G_6, respectively. However, duplications do arise in the amplification steps, so there were only two unique out of eight clones sequenced in the population selected against G_6 and four unique out of 16 selected against T_6. After the fourth round, nuclease active mutants had accumulated between five and nine coding and three and eight silent mutations. Thus, the phage display selection allowed the accumulation of multiple point mutations without extensive screening to find them.

Several aspects of the selection process illustrate the enrichment for activity in the panning process. One sign of successful panning is that the number of output phage increases with each panning step, as the library is enriched for better binders. Indeed, this enrichment behavior is seen in every selection round for both targets: the panning outputs are always greater for the second panning than the first. The number of active clones in the library also shows enrichment with each panning step. In all but one selection round (the fourth round against G_6), the percentage of active clones increased with each panning step (Fig. 5). Furthermore, the panning also selected for more active clones at the expense of clones with weaker activity. In all rounds the number of clones with weak activity (less than 10% relative to WT) decreased from the first to the second round of panning.

It should be noted that the selection process does not just select for higher affinity binding. The selection process also includes assembly of SNase–phage, mutagenesis and amplification. Assembly of SNase–phage includes host functions such as plasmid replication, translation of fusion protein, translocation to the periplasm, as well as helper phage functions such as replication of phagemid DNA, and packaging into phagemid particles. Any sequences that have deleterious effects on any of these processes would be selected against.

The fourth panning round against G_6 illustrates one of the potential problems with the selection process. In this round, the number of active clones in the library dropped from the first to the second panning steps, where in all other rounds it increased. This drop highlights one limitation of the selection process used

Table 6. Kinetic parameters[a] for hydrolysis of NP-pdG by SNase and mutants selected against a guanine substrate analog.

clone	k_{cat} (10^{-5} min^{-1})	K_m (mM)	k_{cat}/K_m (10^{-3} min^{-1} M^{-1})	relative
wt	20 ± 10	10 ± 6	21 ± 3	1.0
B6	8 ± 6	70 ± 30	1.2 ± 0.2	0.058
F14	5 ± 2	20 ± 5	2.5 ± 0.3	0.12

[a]From Lineweaver–Burk plots with 5 mM enzyme in pH 7.6 100 mM Tris–HCl, 150 mM NaCl, 10 mM $CaCl_2$, at 37 °C.

here, namely that binding to a substrate analog rather than catalytic activity is the selection. Thus better binders that are not necessarily catalysts can take over the library, as seen in the fourth panning round against G_6, giving the enzymatically inactive K16 clone. This clone has no measurable activity against DNA or NP-pdT or NP-pdG, being more than 100 times slower for mononucleotide substrates and 10^6 times slower against DNA.

Types and location of mutations

After four selection rounds, mutant nucleases were recovered that contained 9–16 mutations. Aside from the silent mutations, these changes must be responsible for the altered catalytic properties seen in I3, E6, F14 and K16. Yet not every mutation must contribute to the changes in kinetic parameters, since it is likely that mutations will accumulate that do not affect the selection process. Even though the clones G1, H1 and J12 have mutations, their catalytic properties were not found to differ from WT under the conditions tested. Mutations that are neutral with respect to catalysis have been selected. Noting that the selection was for binding affinity only, it has not yet been determined if these mutations improve binding ability of these clones to their respective panning targets.

Similarly, even in clones I3, E6, F14 and K16, some of the mutations could be neutral spectators in the selection process, occurring coincidentally with positively selected mutations. Thus, an accurate assessment of the effects of each mutation will require careful comparison to other mutants as well as future site-directed mutagenesis and structural studies.

Inspection of the crystal structure of SNase[16,17] shows the bulk of the accumulated mutations to be in solvent exposed side chains, as opposed to buried hydrophobic side chains, which would be expected to be more sensitive to mutations that affect side chain packing. Indeed, the changes accumulated in charged residues lead to changes in the overall charge of the mutants, calculated in Table 3.

When panning against G_6-BSA, a selection for a residue with a positive charge at position 80 of SNase is seen. In the two active clones E6 and F14 this residue changed from a glutamine to histidine, and to an arginine in the inactive clone K16. Selection for a positively charged side chain is seen starting with the first selection round. Even though the same initial library was panned against T_6, 8/8 clones selected against G_6 and sequenced had Arg in position 80 after the first selection, but 8/8 selected against T_6 had no change after the first or fourth selection rounds. In the WT crystal structure, Q80 is located in the pyrimidine binding pocket (6–7 Å from the pyrimidine). The Q80R mutation may allow favorable H-bonding interactions with the guanine base of G_6.

The T41A mutations seen in E6 and F14 also have interesting mechanistic implications. In WT SNase, the carbonyl of T41 is a ligand of the Ca^{2+} metal cofactor. It is known that a T41P mutation reduces the V_{max} for DNA hydrolysis by 37-fold.[10] Perturbation of this residue would be expected to affect the environment around the catalytic center.

Kinetic properties

The kinetic properties of the selected mutants for hydrolysis of DNA under the same ionic strength and pH as the panning conditions were measured. Since the apparent K_ms were much less than the lowest concentration of DNA that could be used with the UV assay, the K_m values were not calculated. However, accurate values for V_{max} could be obtained and are listed in Table 4. Clone G1, H1 and J12 have maximum rates the same as WT SNase within experimental error. Clone I3, on the other hand, is half as fast against DNA. This change may be a result of the overall –6 net charge change in I3 compared to WT, which might decrease its interaction with polyanionic DNA.

For the clones selected against G_6-BSA, a larger difference is seen. First, K16 has no measurable activity against DNA even when tested on the highly sensitive TBD plates.[26] Mutants E6 and F14 show a 2.5–3.0 decrease in V_{max} for DNA hydrolysis. Given the number of total mutations, picking exactly which changes are responsible for this rate decrease is not possible without further study such as site-directed mutagenesis of the mutated residues back to the WT and comparison of the kinetic properties. A likely place to start is the T41A mutation, which is a ligand for the Ca^{2+} cofactor.

The kinetic activity against two small molecule substrates was also tested, in part to avoid the heterogeneity of DNA, and to test the specificity requirements of the nucleases. Again the hydrolysis reactions were performed under conditions that matched the panning conditions. Again, the nuclease selected against T_6-BSA, G1 and H1, have catalytic efficiencies k_{cat}/K_m 20% less than WT SNase, but in this case I3 is twice as good as WT SNase for the hydrolysis of NP-pdT. This rate increase comes from a slight increase in k_{cat} and a decrease in K_m. Thus, one of the four thymidine selected clones shows a two-fold increase against the T-containing substrate, and the other three clones show 0–20% decreases.

The nucleases selected by panning against G_6-BSA show a greater decrease in their ability to catalyze the hydrolysis of NP-pdT. Now E6 and F14 are 8–10 times slower than WT SNase. The decrease in catalytic power comes from 4–5-fold decrease in k_{cat}, close to the 2.5–3-fold decrease in k_{cat} seen against DNA, and two-fold increases in K_m. Selection against guanosine decreased affinity for the thymidine-containing substrate.

These G_6-panned clones and WT SNase were then tested against NP-pdG. NP-pdG is a much poorer substrate of SNase than the thymidine substrate NP-pdT, with a k_{cat}/K_m 500-fold slower. E6 and F14 are 17 and eight times, respectively, less effective against NP-pdG than WT nuclease. For both E6 and F14, the K_m values are greater than WT K_m, indicating weaker binding in a catalytically productive mode.

The role of the selection process in the properties of the mutants is not clearly demonstrated by the kinetic evaluations. Only one clone, I3, showed an improvement for hydrolysis of the small substrate NP-pdT, but not DNA. All other clones examined showed equal or reduced efficiency. Furthermore, clones selected for binding to the guanine-containing substrate analog did not have a measurable improvement for hydrolysis of the guanine-containing substrate, even though there appeared to be a selection for a positively charged residue at position 80. Catalytic activity was also lost in the clone K16, emphasizing the limitations of selecting for binding over activity. Considering the size of the gene randomized, 140 codons, the number of potential solutions 140^{20} (10^{42}) is far greater than the number tested in the selection scheme 10^6 round^{-1}. Of course, not all of the possible sequences would be expected to be able to fold into a catalytically competent form. Given these considerations, it would seem that the major role of the selection for binding in this experiment is to throw away garbage mutations that prevent folding and recognition of the substrate analog. The changes in catalytic ability appear to be coincidental, yet the enrichment allowed them to be discovered with a minimal amount of screening (only 16 clones in each scheme were examined and tested).

Phage display selection of an enzyme as described in this paper could be used to engineer physical properties of enzymes (proteins) without the structural knowledge required by traditional site directed mutagenesis approaches. Instead, random mutations can be selected from libraries of 10^6 to 10^9 members by phage display. For example, the temperature of the panning step could be increased in each selection round, selecting for higher thermal stability. Likewise, solution properties such as pH, ionic strength, or resistance to denaturants could be used to select for global enzyme properties. In these cases the selection would be for specific binding of folded enzyme (protein) to an appropriate target like a competitive inhibitor. Methods to select for catalytic activity of displayed enzymes have been demonstrated[33,34] and are under further development in this lab.

Conclusion

Phage display and random mutagenesis have been combined to mimic *in vitro* evolution of an enzyme. In this experiment, selection for catalysis was coincidental to selection for binding to a substrate analog, making the role of the selection to be the removal of mutations that prevent substrate recognition through misfolding or other mechanisms. Selecting for mutations based upon binding ability translated equivocally into catalytic abilities. Mutant nucleases showed all three possible outcomes: no change, reduced, or increased catalytic activity. A more effective approach would be to directly select for catalytic mechanism. With the development of phage display selections based upon catalytic function, the phage display method promises to be a powerful tool for the study and manipulation of enzyme properties.

Experimental

Escherichia coli XL-1 Blue, helper phages R408[41] and VCS-M13, and pBCSK(–) were obtained from Stratagene (La Jolla, CA). *Staphylococcus aureus,* strain Foggi, was obtained from the American Type Culture Collection (ATCC #27735, Rockville, MD). BL21(λDE3)pLysS and pET-20b(+) were obtained from Novagen (Madison, WI), pSE380 from Invitrogen (La Jolla, CA). Chemicals and NP-pdT were purchased from Sigma Chemical Co. (St Louis, MO) or Aldrich (Milwaukee, WI). NP-pdG, sodium salt, was synthesized by the method of Borden and Smith.[42] Restriction enzymes, T4 kinase, Vent polymerase were from New England Biolabs (Beverly, MA). Magic or Wizard miniprep kits, dNTPs, and Taq DNA polymerase from Promega (Madison, WI). LB broth, LB agar and T4 ligase and buffer were from GIBCO-BRL (Grand Island, NY). TBD plates (nuclease assay plates) have been described.[43,44] Oligonucleotides were synthesized on a Pharmacia gene synthesizer by the phosphoramidite method or were purchased from Operon Technologies (Alameda, CA). DNA was sequenced using the Sequenase 2.0 kit of United States Biochemical (Cleveland, OH) using oS9, oS21, oS24, T7pro, or T7ter as primers.

Oligonucleotides

oSl 5'-GGGCTCATGAAAAAGACAGCTATCGCGATT GCAGTGGCACTGGCTGGTTTCGCTACCGTGGCCA GGCGGCCACTTCAACTAAAAAATTACATAAAGAA C-3'
oS2 5'-GGCCGGGATCCTGAATCAGCGTTGT CT-3'
oS7 5'-CTTAAGATCTG-3'
oS8 5'-CTAGCAGATCTTAAGAGCT-3'
oS9 5'-CATCCGGCTCGTATAATG-3'
oS2l 5'-GGTCCTGAAGCAAGTGCA-3'
oS23 5'-ATCAGACCGCTTCTGCGT-3'
oS24 5'-GGGCAGTGGCATATGGCCACTTCAACTAA A-3'
oS25 5'-GTCAGGCTCGAGTTACTGTCCTGAATCAGC GTTGTC-3'
T7Pro 5'-ATTAATACGACTCACTATAGG-3'
T7Ter 5'-GCTAGTTATTGCTCAGCGG-3'

Vector construction

The SNase–phage display vector pS120+ was constructed in the expression vector pSE380 (Invitrogen

Corp., La Jolla, CA) by standard methods.[45] It contains the gIII anchor from pComb3,[46] the f1 origin from pBCSK(–) (Stratagene Inc., La Jolla, CA), and the SNase gene from *Staphylococcus aureus,* Foggi strain. Briefly, the synthetic linkers oS7 and oS8 were ligated to pComb3 digested with Nhe I/Sac I to give pS108. The small BamH I/Sac I fragment of pS108 was exchanged into pSE380 cut BamH I/Sac I, giving pS104. Undesired sites in pS104 were removed by digestion with Sac I/Hind 3, blunting the overhangs with T4 polymerase, and ligation to give pS109. However, later it was discovered by sequencing that the 3' overhang of the Sac I sited was retained in pS109.

The SNase gene was cloned by PCR on chromosomal DNA template[47] from *S. aureus* ATCC #27735 using the following conditions: 6.4 μg template, 0.1 μM each oS1 and oS2, 200 μM each dNTP, 3.5 mM $MgCl_2$, 2 U Vent DNA polymerase in 100 μL of 1X Vent buffer. The reaction was heated to 94 °C for 30 s, and cycled 94 °C 15 s—52 °C 15 s—72 °C 27 s for 25 times, and completed with a 10 min 72 °C incubation. After gel purification, the 521 bp fragment was digested with BspH I/BamH I and ligated into Nco I/BamH I digested pSE380. A clone expressing active nuclease was recovered by replica screening colonies from LB agar on to TBD agar, with an active clone selected as pS114. Finally, the small Sph I/BamH I fragment of pS114 and the large fragment of Sph I/BamH I digested pS109 were ligated to give the SNase display vector pS120+.

Other vectors

The SNase display pS120+ vector was modified to pS124, which expresses free SNase, by self ligation after removal of the BamH I/Bgl II fragment of pS120+, which contains the gIII anchor sequence.

The SNase display vectors that convey chloramphenicol resistance were constructed in two steps. First, pBCSK(–) (Stratagene, Inc., La Jolla, CA) was shortened by digestion with Afl III and Bgl I, followed by blunt ending with T4 polymerase and self ligation, giving pS117. Finally the BspH I fragment of pS117 was exchanged into the respective sites in pS120+, giving pS122 (sense) and pS123 (antisense), which differ only in the orientation of the chloramphenicol marker with respect to the nuclease gene.

T7 Expression vectors

The SNase mutants were cloned into the T7 expression vector pET-20b(+) by PCR. For PCR, the 100 μL reactions contained 1 μg ssDNA template, 0.4 μM each oS24 and oS25, 200 μM each dNTP, 2 mM $MgCl_2$, 3 U Vent polymerase, and 1X Vent buffer. The reactions were heated to 94 °C for 30 s, and then cycled 94 °C 15 s—48 °C 15 s—72 °C 30 s 25 times, and completed with a 10 min 72 °C incubation. Inserts and vector pET-20b(+) were cut with Nde I/Xho I and ligated. Vectors containing inserts were resequenced to confirm the sequences of the mutants in the expression vectors.

PCR Mutagenesis

Random mutations were introduced in the nuclease gene by error-prone PCR[38] with the following modifications. Three dilutions of template were used in separate reactions, which were combined after PCR, and the manufacturer's buffer was used. Each 100 μL reaction contained either 3, 0.3 or 0.03 pmol plasmid template, and 50 mM KCl, 10 mM Tris–HCl pH 8.8 at 25 °C, 0.1% Triton X-100, 7 mM $MgCl_2$, 0.2 mM dATP and dGTP, 1.0 mM dCTP and dTTP, 0.3 μM each oS2 and oS9, 0.5 mM $MnCl_2$ (added next to last), and 5 U Taq polymerase. The reactions were heated to 94 °C for 30 s, and then cycled 94 °C 15 s—50 °C 15 s—72 °C 60 s 30 times, and completed with a 10 min 72 °C incubation. Reactions on the same template were combined, and purified using Wizard PCR preps, then digested with Sfi I, BamH I, and the inserts gel purified.

Initial library construction

Mutated inserts (~100 ng) were ligated into cut, unmutated parent vector pS120+ (~1 μg) in 200 μL total volume at room temperature overnight. The ligations were extracted with phenol:chloroform, precipitated with sodium acetate:ethanol, and resuspended in 20 μL water for transformation into 200 μL electrocompetent XL-1 Blue using a Bio Rad Gene Pulser electroporator set at 25 μF and 2.5 kV, equipped with a 200 Ω external resistance and a 0.2 cm gap cuvette. Electrocompetent bacteria were previously prepared as described in the electroporator manual. The culture was allowed to recover for 1 h at 37 °C in 3 mL of SOC, then diluted to 10 mL with SB (100 μg mL^{-1} carbenicillin, 10 μg mL^{-1} tetracycline) and titered for $carb^R$. After further 1–2 h incubation at 37 °C, the culture was added to 100 mL SB with the same antibiotics and 1×10^{10} pfu of R408, and incubation continued overnight, 14–16 h. Cells were removed from 3 mL of culture by centrifugation for 1–2 min at 14,000 *g*, and plasmid DNA recovered. To the supernatants, 1/5 volume of 20% PEG-8000, 2.5 M NaCl was added and the mixture chilled on ice for 30 min to precipitate the phage. Phage were collected at 14,000 *g* 15 min 4 °C, and resuspended in 100 μL TBS.

Panning selection process

ELISA plates (#3690, Costar Corp., Cambridge, MA) were coated with 25 μL of target T_6- or G_6-BSA overnight at 4 °C, and blocked for 1–2 h with 1% BSA in TBS at 37 °C. Phage (25 μL + 25 μL 1% BSA 20 mM $CaCl_2$ in TBS) were allowed to bind for 1 h, and then nonspecific phage removed with 5–7 washes of 200 μL of 10 mM $CaCl_2$, 1% BSA, 0.05% Tween-20 over 1 h. Bound phage were eluted in 50 μL of 0.1 M glycine–HCl pH 2.2 for 10 min and neutralized with 4 μL of 2 M Tris. For amplification, 25 μL of neutralized phage was added to 1 mL of XL-1 Blue grown on LB

(10 μg mL^{-1} tetracycline) to A_{600} = 0.6–0.8. After 20 min at room temperature, 10 mL of SB (100 μg mL^{-1} carbenicillin, 10 μg mL^{-1} tetracycline) was added and the panning output size determined by the $carb^R$ titer. The culture was incubated for 1 h at 37 °C, before adding R408. Phage were grown and recovered as described above for the next round of panning, and plasmid DNA recovered for mutagenesis.

CsCl purification of SNase–phage

SNase–phage were produced using either the R408 or VCS-M13 helper phages. XL-1 Blue/pS120+ were infected with a 10-fold excess of each helper phage separately and grown overnight in 100 mL SB with carbenicillin and tetracycline. The cells were separated and the phage precipitated with 1/5 vol. 20% PEG-8000, 2.5M NaCl on ice for 30 min, and then pelleted at 27,000 *g* for 15 min and the supernatants removed. The pellets were resuspended in 1/10 volume TBSC, and shaken for 2 h 37 °C. Debris and aggregated phage were removed by centrifugation at 27,000 *g* for 15 min, and then the phage reprecipitated and resuspended as before. The phage were further purified on a CsCl gradient, average density 1.3 g mL^{-1}, for 48 h at 220,000 *g* . The phage bands were removed, diluted with 10 vol. TBSC, and the phage pelleted 48 h at 220,000 *g*. The pellets were softened by soaking in 2 mL TBSC overnight at 4 °C, shaken for 4 h, repelleted for 2 h at 239,000 *g*, and finally resuspended in 1 mL TBSC as before, giving 20–24 A_{260} of phage.

The SNase activity of the purified phage was compared to known SNase (Sigma). Phage stocks were diluted to 4.3 A_{260} mL^{-1}, and 50 μL of phage added to 1 mL of 10 mM $CaCl_2$, 10 mM sodium borate, 50 μg mL^{-1} DNA pH 8.8 and the initial rate of absorbance change at 23 °C measured.

T_6- and G_6-BSA

The phosphorothioate oligonucleotides T_6 (25.1 A_{260}, 900 μg) and G_6 (27.4 A_{260}, 920 μg) were obtained from Oligos Etc. (Wilsonville, OR). The oligonucleotides were purified by: 1) absorption to a C-18 Sep pack reverse phase column (Millipore, Bedford, MA) equilibrated with 25 mM TEAB pH 7; 2) elution into 30% CH_3CN in 25 mM TEAB; 3) lyophilization. To couple to BSA, either 10.5 A_{260} of T_6, or 5.3 A_{260} of G_6, or no oligo (negative control), each in 100 μL of water was added to 200 μL of 17 mg mL^{-1} BSA (Fraction V, Sigma) in 0.1 M pH 8.8 borate buffer, followed by 840 μg BS^3 (Pierce, Rockford, IL) in 24 μL DMSO. After an overnight incubation at room temperature, the conjugates were purified by size exclusion chromatography on PD-10 columns (Pharmacia, Piscataway, NJ) and recovered in a final volume of 1.5 mL TBS.

The protein concentrations were estimated to be 1 mg mL^{-1} in the purified fractions with an average of 4.0 T_6 or 4.6 G_6 haptens/BSA molecule by UV spectrometry.

Enzyme overexpression and purification

Overexpression of WT and mutant nucleases was achieved according to the directions from Novagen in a T7 system. Briefly, a fresh overnight colony of BL21 (λDE3)pLysS containing the appropriate expression vector was grown to A_{600} of 0.6–0.8 in 10 mL LB with 100 μg mL^{-1} carbenicillin and 30 μg mL^{-1} chloramphenicol, and then stored at 4 °C overnight. The next morning, the cells were transferred into fresh media and used to inoculate 1 L LB (same antibiotics). When the cell density reached A_{600} 0.6–0.7, IPTG was added to 0.4 mM. Time course experiments showed maximal expression after 4 h at 37 °C. The cells were collected at 3000 *g* for 15 min, resuspended in 50 mL 0.2 M Tris–HCl, 2 mM EDTA pH 7.6, and frozen. Upon thawing, the cells lysed completely. If an inactive nuclease was expressed, the lysate was sonicated 5 × 10 s until it was no longer viscous. Cell debris was removed at 34,000 *g* for 2 h at 4 °C. The clear supernatant was additionally filtered though a 0.2 μm glass fiber/nylon filter before chromatography.

Chromatography

Crude proteins were purified by ion exchange chromatography[25,28] on BioRex-70 (BioRad Laboratories, Hercules, CA). A 7.5 × 2 cm column of BioRex-70 was washed with 120 mL 0.5 N NaOH, 120 mL water, then 120 mL 1 M Tris–HCl pH 6, and finally with 120 mL 0.2 M Tris–HCl pH 7.6 (buffer A). The lysate was loaded at 0.5 mL min^{-1}, and then washed with 220 mL buffer A at 2.0 mL min^{-1}. The bound nuclease was eluted with a 400 mL linear gradient from 0 to 0.5 M NaCl in 0.2 M Tris–HCl pH 7.6. Finally, the column was regenerated by washing with 240 mL 1.0 M NaCl in 0.2 M Tris–HCl pH 7.6, before the next use. Peaks containing fractions pure by SDS-PAGE were combined and dialyzed extensively against 10 mM Tris–HCl pH 7.6 over 3 days, during which small amounts of white precipitate formed and were later discarded. The purified nucleases were concentrated by ultrafiltration against an 8 K MWCO Omega membrane (Filtron Technology Corp., Northborough, MA) to concentrations of 1–6 mg mL^{-1}, then passed through a 0.2 μm filter, quickly frozen in aliquots and stored at –80 °C for later use. The WT enzyme concentrations were calculated assuming ε_{280} = 0.93 mL mg^{-1} CM^{-1}.[48] The concentration of mutant enzymes was determined using the protein BCA assay (Pierce) and WT nuclease as the standard.

Apparent molecular weights were calculated by gel filtration chromatography on a Pharmacia S-100HR column in 500 mM NaCl, 25 mM Tris–HCl, 1 mM EDTA pH 7.6 and comparison to known calibration standards.

Kinetics of DNA hydrolysis

DNA hydrolysis was followed by the hyperchromicity change that occurs as polymeric DNA is cleaved to

oligonucleotides.[22,36] Denatured sonicated salmon sperm DNA was prepared as described,[22] and concentrations were calculated assuming ε_{260} = 25 mL mg^{-1} cm^{-1}. Rates were measured for the first 10% of the total absorbance change at 260 nm when 1 nM nuclease was added to 1 mL of TBS pH 7.4 containing 10 mM $CaCl_2$ and 2–42 μg DNA in a silanized cuvette. Enzymes were diluted in 1% BSA in TBS. Under these conditions, the Lineweaver–Burk plots were flat, indicating that the apparent K_m for the DNA substrate is low, much lower than the DNA concentrations that could be monitored spectrophotometrically. Hence only the maximum hydrolysis rates could be determined. Instrument sensitivity precluded experiments with lower DNA concentrations.

Hydrolysis of NP-pdT

The hydrolysis of NP-pdT was measured in two steps. First, NP-pdT (1–10 mg mL^{-1}) was incubated at 37 °C with 5 μM nuclease in 0.1 M Tris–HCl, 10 mM $CaCl_2$, 150 mM NaCl pH 7.6 in a total volume of 40 μL in an ELISA plate previously blocked with BSA. At the appropriate times (1–6 h), the reactions were quenched with 10 μL of 80 mM EGTA. The amount of *p*-nitrophenyl phosphate was then determined by a colorimetric assay using calf intestinal alkaline phosphatase, CIP and calibration against a known standard series as follows: 50 μL of 1 M triethanolamine, 12 mM $MgCl_2$, 1 U mL^{-1} CIP pH 9.8 was added, and the absorbance at 405 nm measured after 45 min. No nuclease activity could be detected after quenching with EGTA, the endpoints were stable > 24 h. The standard response was linear from 0.016 to 0.25 mg mL^{-1} *p*-nitrophenyl phosphate, and the rates linearly dependent on enzyme from 1 to 10 μM.

Hydrolysis of NP-pdG

The hydrolysis of NP-pdG was measured in a similar manner, but with the following changes. The amount of enzyme was increased to 50 μM, and the incubation times were 1–5 days. After quenching with EGTA, 25 μL of 1 M triethanolamine pH 9.8 was added and the amount of *p*-nitrophenol produced in the competing reaction measured. After this background was established, 25 μL of 1 M triethanolamine, 24 mM $MgCl_2$, 1 U mL^{-1} CIP pH 9.8 was added, and the amount of *p*-nitrophenylphosphate then determined as before.

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