Deletion of the Ω-Loop in the Active Site of Staphylococcal Nuclease. 1. Effect on Catalysis and Stability†

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ABSTRACT: The high-resolution X-ray structure of wild-type staphylococcal nuclease (E43 SNase) suggests that Glu 43 acts a general basic catalyst to assist the attack of water on a phosphodiester substrate [Loll, P., & Lattman, E. E. (1989) *Proteins: Struct., Funct., Genet. 5*, 183]. Glu 43 is located at the base of the solvent-exposed and conformationally mobile Ω-loop in the active site of E43 SNase having the sequence Glu_{43}-Thr_{44}-Lys_{45}-His_{46}-Pro_{47}-Lys_{48}-Lys_{49}-Gly_{50}-Val_{51}-Glu_{52}, where the γ-carboxylate of Glu 52 is hydrogen bonded to the amide hydrogen of Glu 43. With a metabolic selection for SNase activity produced in an *Escherichia coli* host, we detected an unexpected deletion of residues 44–49 of the Ω-loop of E43 SNase in cassette mutagenesis experiments designed to randomize codons 44 and 45 in the Ω-loop and increase the activity of the previously described E43D mutation (D43 SNase). A high-resolution X-ray structure of D43 SNase has revealed that the E43D substitution significantly changes the structure of the Ω-loop, reduces the interaction of the essential Ca^{2+} ion with its active-site ligands, and diminishes the network of hydrogen-bonded water molecules in the active site [Loll, P., & Lattman, E. E. (1990) *Biochemistry 29*, 6866]. This deletion of six amino acids from the Ω-loop generates a protein (E43 ΔSNase) having a partially solvent-exposed, surface β-turn with the sequence Glu_{43}-Gly_{50}-Val_{51}-Glu_{52}; the structure of this β-turn is addressed in the following article [Baldisseri et al. (1991) *Biochemistry* (following paper in this issue)]. The deletion of six amino acids from the Ω-loop starting with residue 43 is less damaging to catalysis than the excision of a single methylene group from residue 43 (in D43 SNase) as assessed by measurements of V_{max}. The deletion of the Ω-loop and the E43D substitution are nonadditive mutations, presumably the result of the amount of rate acceleration possible with general basic catalysis and a conformational alteration produced by the E43D substitution. The deletion of the Ω-loop increases the stability of the folded form of E43 ΔSNase relative to the E43 SNase by 2.5 kcal/mol. In contrast to the E43D substitution [Hibler, D. W., Stolowich, N. J., Reynolds, M. A., Gerlt, J. A., Wilde, J. A., & Bolton, P. H. (1987) *Biochemistry 26*, 6278], the changes in catalysis and stability induced by deletion of the Ω-loop are *not* accompanied by significant changes in either the chemical shifts or the interresidue nuclear Overhauser effects of aromatic and upfield-shifted aliphatic residues both in the hydrophobic core and near the base of the Ω-loop/β-turn.

The high-resolution X-ray structure of wild-type staphylococcal nuclease (E43 SNase[1]) in the presence of catalytically essential Ca^{2+} and the competitive inhibitor thymidine 3′,5′-bisphosphate (pdTp) (Cotton et al., 1979; Loll & Lattman, 1989) suggests that the γ-carboxylate group of Glu 43 acts as a general basic catalyst to assist the attack of water on a phosphodiester substrate coordinated to Ca^{2+} and the guanidinium groups of Arg 35 and Arg 87. We have reported on the effects of the replacement of Glu 43 with Asp (Hibler et al., 1987) and of Arg 35 and Arg 87 with Lys (Pourmotabbed et al., 1990); D43 SNase[1] (E43D), R35K SNase, and R87K SNase are significantly impaired catalytically ($V_{max}/K_m < 10^3$ that of wild-type SNase). In each case, thermal denaturation and ^1H NMR spectroscopy suggested that structural changes accompany the "conservative" amino acid substitutions (presumably of greatest magnitude in the vicinity of the substitution). In the case of D43 SNase, structural changes in the active site have also been observed by X-ray crystallography (Loll & Lattman, 1990). The structural information for D43 SNase reveals changes within the active site and especially in the solvent-accessible, conformationally mobile Ω-loop [a loop whose termini are 10 Å or less from one another; terminology of Leszczynski and Rose (1986)] that starts at residue 43 and ends at Glu 52; the sequence of the Ω-loop in

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[1] In this and the following paper, we are concerned with the catalytic and structural properties of the full-length, wild-type staphylococcal nuclease (SNase) and a shortened version in which an active-site Ω-loop has been deleted. In the past our mutagenic studies of SNase focused only on comparative studies of the properties of wild-type enzyme and single-site mutants of active-site residues so that we might establish structure/function relationships in the wild-type protein by explaining the differences in kinetic properties that are induced by the substitution. Accordingly, our nomenclature for the Asp for Glu substitution at residue 43 was E43D in order to focus on the structural origin for the observed kinetic and structural differences. However, we now wish to expand our focus to the additional effect of the presence or absence of the active-site Ω-loop on the catalysis. In this pair of papers the focus is on both substitutions for residue 43 and the presence or absence of the Ω-loop; we believe that confusion can be avoided if the reader remembers that the wild-type residue is Glu and we always explicitly designate the identity of residue 43, e.g., E43 for Glu, D43 for Asp, and A43 for Ala. Additionally, the full-length protein will be designated by SNase and the shortened sequence with the Ω-loop deletion will be designated by ΔSNase. By this convention, wild-type SNase will now be designated E43 SNase and its shortened version without the Ω-loop will be designated E43 ΔSNase. An analogous pair of enzymes having Asp at residue 43 is designated D43 SNase and D43 ΔSNase, respectively. Other abbreviations: amp, ampicillin; NMR, nuclear magnetic resonance; pdTp, thymidine 3′,5′-bisphosphate; NPpdTp, thymidine 3′-phosphate 5′-[(4-nitrophenyl)phosphate]; NOESY, nuclear Overhauser effect correlation spectroscopy; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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FIGURE 1: α-Carbon backbone of Glu 43 SNase showing the conformationally mobile, solvent-accessible Ω-loop. The Ω-loop begins at Glu 43 and terminates at Glu 52; residues 44–51 are shown in bold.

E43 SNase is Glu_{43}-Thr_{44}-Lys_{45}-His_{46}-Pro_{47}-Lys_{48}-Lys_{49}-Gly_{50}-Val_{51}-Glu_{52}, with the γ-carboxylate group of Glu 52 hydrogen-bonded to the amide proton of residue 43 (Figure 1). In E43 SNase the γ-carboxylate group of Glu 43 is hydrogen-bonded to a water molecule in the first coordination sphere of the Ca^{2+} (Loll & Lattman, 1989), whereas in D43 SNase the β-carboxylate group of Asp 43 is hydrogen-bonded primarily to the imidazolium functional group of His 46 in the Ω-loop and points away from the Ca^{2+} (Loll & Lattman, 1990). On the basis of these structural differences between E43 SNase and D43 SNase, we concluded that quantitative assessment of the importance of Glu 43 in general basis catalysis was not possible.

In order that we might circumvent the potential kinetic effects of this structural change, we sought to determine whether we could revert the Ω-loop of D43 SNase to the conformation observed in Glu 43 SNase by replacing specific residues within the Ω-loop with all possible substitutions. Instead, using a metabolic selection for SNase activity, we identified an unexpected deletion of Thr 44–Lys 49 in E43 SNase (E43 ΔSNase) having a V_{max} 2.5% that of E43 SNase with single-stranded DNA as substrate. For comparison the V_{max} of D43 SNase is 1.2% that of E43 SNase. Thus, the deletion of six residues from the Ω-loop is less damaging to catalysis than excision of a single methylene group from Glu 43. Inspection of the structure of E43 SNase allows the prediction that this deletion converts the conformationally mobile, solvent-exposed Ω-loop in E43 SNase to a conformationally restricted, surface β-turn in E43 ΔSNase having the sequence Glu_{43}-Gly_{50}-Val_{51}-Glu_{52}. The stability of the folded state of E43 ΔSNase is greater than that of E43 SNase by approximately 2.5 kcal/mol.

We also compared the kinetic properties of D43 SNase with those of D43 ΔSNase, its Ω-loop deleted version. The V_{max} for D43 ΔSNase is reduced 6-fold relative to that of D43 SNase as compared to the 40-fold reduction in V_{max} associated with deletion of the Ω-loop in the Glu 43 context. We attribute the nonadditive effects of the Ω-loop deletion and E43D substitution to both the finite amount by which general basic catalysis is likely to accelerate the rate of the enzymatic reaction (as assessed by the kinetic properties of A43 SNase) and a conformational alteration produced by the E43D substitution. The deletions of the Ω-loops to produce E43 ΔSNase and D43 ΔSNase are not accompanied by significant changes in either chemical shifts or interresidue nuclear Overhauser effects involving the aromatic and upfield-shifted aliphatic protons such as those detected when E43 SNase was converted to D43 (Hibler et al., 1987) or when E43 ΔSNase is converted to D43 ΔSNase.

The use of three-dimensional NMR spectroscopy to compare the structure of E43 SNase with E43 ΔSNase and the structure of D43 SNase with D43 ΔSNase as well as to determine the structure of the β-turn generated by deletion of the Ω-loop is described in the following article (Baldisseri et al., 1991).

Materials and Methods

Restriction endonucleases *Rsr*II, *Sal*I, and *Spe*I and polynucleotide kinase from bacteriophage T4 were purchased from New England Biolabs. Restriction endonuclease *Bam*HI, DNA ligase from bacteriophage T4, and calf intestinal phosphatase were purchased from Boehringer Mannheim. All enzymes were used according to the supplier's instructions. Site-directed mutagenesis was performed by the phosphorothioate method with the kit and instructions supplied by Amersham. DNA sequence analysis was performed with the Sequenase kit and instructions supplied by U.S. Biochemicals. Synthetic oligonucleotides were purchased from the Protein Nucleic Acid Laboratory, University of Maryland at College Park, and purified by reverse-phase HPLC on a C_{18} column using a triethylammoniuum acetate/acetonitrile solvent system. Bio-Rex-70, DNA-grade hydroxyapatite, and Bio-Gel P-60 gel filtration resins were obtained from Bio-Rad Laboratories. Standard techniques were employed for isolating plasmid DNA and DNA fragments, cloning and transformation, and resolving protein and DNA samples by electrophoresis.

Site-Directed Mutagenesis. The 794-bp *Bam*HI–*Sal*I restriction fragment from pNJS (Hibler et al., 1987) containing the gene for D43 SNase as well a 5′-flanking sequence encoding the 7 amino acid N-terminal extension Met-Asp-Pro-Thr-Val-Tyr-Ser was cloned in M13mp19. The primers directing introduction of the recognition site for *Spe*I starting at codon, 37, d(GACTATTACTAGTTGATAC) and of the recognition site for *Rsr*II starting at codon 54, d-(GAGAAATACGGTCCGGAAGCAAG), where the positions of the mutagenic mismatches are underlined, were used sequentially in the phosphorothioate method of site-directed mutagenesis. Following sequence analysis of the entire gene containing the unique *Spe*I and *Rsr*II sites, the *Bam*HI–*Sal*I restriction fragment containing the silently mutated gene for

D43 SNase was excised and ligated into the expression plasmid pCQV2; the plasmid so constructed is designated pLBP1. This expression plasmid utilizes the bacteriophage λ P_R promoter and also encodes the cI857 temperature-sensitive mutation of the bacteriophage λ repressor so that it can be used in most strains of *E. coli*.

Mutageneses to substitute Asp and Ala for Glu 43 in ΔSNase utilized the primers d(CACCCGACGGTGTAGAG) and d(GATACACCCGCAGGTGTAGAG), respectively.

Cassette Mutagenesis and Metabolic Selection for Altered Levels of Nuclease Activity. The following synthetic restriction fragment was prepared for cassette mutagenesis to randomize residues 44 and 45 in D43 SNase:

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5'CTAGTTGATACACCCGACNNSNNSCATCCTAAAAAAGGTGTAGAGAAATACG3'
   3' AACTATGTGGGCTGIIIIIIGTAGGATTTTTTCCACATCTCTTTATGCCAG5'
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N represents an equimolar mixture of A, C, G, and T, and S represents an equimolar mixture of C and G. The underlined base designates a silent base change in the codon for Pro 42 (CCC) so that mutations derived from the cassette could be distinguished from wild-type contaminants.

This synthetic restriction fragment was annealed and ligated with the large *Spe*I–*Rsr*II fragment of pLBP1. The ligation mixture was used to transform *E. coli* strain CR34 (*thyA*⁻) obtained from Dr. Barbara Bachmann, *E. coli* Genetic Stock Center, Yale University. The transformation mixture was plated on LB plates containing 40 μg/mL ampicillin (LB/amp) to select for transformants. After growth overnight at 30 °C, the colonies were replicate plated on minimal medium plates (100 mM sodium glycinate, pH 8.5, containing 1 mM KH_2PO_4, 18.7 mM NH_4Cl, 51 mM NaCl, 22 mM KCl, 0.2% glucose, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 μg/mL thiamine hydrochloride, 40 μg/mL ampicillin, 1% casamino acids, and 1.5% agar) containing 500 μg/mL sonicated salmon sperm DNA (Sigma) as the sole source of thymidine (selection plates). Colonies that grew well after 1–2 days at 39 °C were purified by streaking on LB/amp plates and then grown in liquid culture (4 mL of LB/amp medium inoculated with 200 μL of an overnight culture, grown 2 h at 30 °C and then 2 h at 42 °C). The cells were pelleted by centrifugation and lysed by sonication in 200 mL of 25 mM Tris-HCl buffer, pH 8.0, containing 50 mM glucose and 10 mM EDTA. Aliquots were used for assays with single-stranded DNA as substrate, measurement of protein concentration, and analysis by SDS–PAGE to assess level of SNase induction.

Isolation of Mutant Nucleases. High-level expression of E43 ΔSNase was accomplished following ligation of the *Bam*HI–*Sal*I fragment encoding the entire mutant gene from the pCQV2-derived plasmid identified by the selection procedure into the same unique restriction sites in the expression plasmid pASI, which utilizes the bacteriophage λ P_L promoter.

Following site-directed mutagenesis to generate the coding sequences for D43 ΔSNase and A43 ΔSNase, *Spe*I–*Rsr*II restriction fragments were excised from the M13mp19 replicative form and ligated in the pAS1 expression plasmid.

E43 SNase and D43 SNase were isolated by a modification of the previously published procedure (Calderon et al., 1985). After chromatography of cell extracts on Bio-Rex-70 and analysis of the fractions containing enzymatic activity by SDS–PAGE, those fractions containing pure protein were dialyzed against three changes of deionized water containing Chelex-100. No further purification was necessary for these proteins.

E43 ΔSNase, D43 ΔSNase, and A43 ΔSNase eluted from the Bio-Rex-70 column at a significantly lower concentration of NaCl and required gel filtration to remove high molecular weight contaminants. Following dialysis of the Bio-Rex-70 fractions containing enzymatic activity and lyophilization, the residue was dissolved in 50 mM ammonium acetate buffer, pH 7, and gel-filtered through a 1.5 × 30 cm column of Bio-Gel P-60. The purity of the fractions was assessed by SDS–PAGE, and those fractions containing pure protein were combined and lyophilized. Residual ammonium acetate was removed by dissolving the residue in water and lyophilizing; this procedure was repeated three times.

The lyophilized, purified proteins were stored at −20 °C.

Characterization of Mutant Nucleases. The activities of the various proteins were measured spectrophotometrically at 25 °C with single-stranded DNA (Hibler et al., 1987) and thymidine 3′-phosphate 5′-[(4-nitrophenyl)phosphate] (NPpdTp; Cuatrecasas et al., 1969) as previously described. Kinetic data were analyzed by using the program HYPER described by Cleland (1979). Fluorescence studies of the binding of thymidine 3′,5′-bisphosphate (pdTp) were conducted at 22 °C as described by Cuatrecasas et al. (1967). Fluorescence studies of denaturation by guanidine hydrochloride were conducted exactly as described by Shortle (1986).

One-dimensional ^1H NMR spectra and two-dimensional ^1H–^1H homonuclear nuclear Overhauser effect correlation (NOESY) spectra were obtained at 30 °C on a Varian XL-400 NMR spectrometer as previously described (Hibler et al., 1987).

Results and Discussion

Cassette Mutagenesis and Metabolic Selection for SNase. Shortle has described a colorimetric screen for SNase activity in *E. coli* cells transformed with plasmids encoding mutant versions of SNase (Shortle, 1983). In our hands, this screen gave false positives when small increases in catalytic activity were sought following random chemical mutagenesis of the entire gene for D43 SNase. Accordingly, we have now devised a *metabolic* selection for SNase that is more reliable for identifying activity variants of SNase.

We reasoned that active SNase variants should be able to degrade DNA to small oligonucleotides and/or mononucleotides, with these hydrolysis products potentially complementing an auxotrophy in the biosynthesis of a nucleotide. We investigated whether a strain auxotrophic for thymidine, by virtue of a disruption in the gene for thymidylate synthase (*thyA*), could serve as an appropriate host for the proposed metabolic selection. The genes for E43 SNase and D43 SNase were subcloned in the expression vector pCQV2 to place them under the control of the bacteriophage λ P_R promoter; denaturation of the plasmid-encoded temperature-sensitive cI857 bacteriophage λ repressor leads to the intracellular accumulation of SNase. These were then transformed into *E. coli* strain CR34, which requires 2 μg/mL thymidine for growth. As a control, pBR322 was also transformed into CR34.

Evaluation of the success of the metabolic selection was performed by plating 10-μL aliquots of cultures of CR34/E43 SNase, CR34/D43 SNase, and CR34/pBR322 grown overnight in LB/amp medium at 30 °C on selection plates. Note that our selection medium (see Materials and Methods) contains less inorganic phosphate and more Ca^{2+} than standard minimal medium; in addition, the pH is higher than that of standard minimal medium. These conditions were chosen to optimize the activity of SNase, which requires Ca^{2+} and has a alkaline pH optimum. CR34/pBR322 was unable to grow on selection plates at 39 °C, CR34/D43 SNase grew poorly over a 3-day period at 39 °C, and CR34/E43 SNase grew to confluence overnight.[2]

Silent mutations were introduced into the gene encoding D43 SNase to place unique restriction sites for *Spe*I and *Rsr*II at codons 37 and 54, respectively. A synthetic *Spe*I–*Rsr*II restriction fragment encoding Asp at residue 43 (GAC) was prepared with one strand containing equimolar mixtures of A, C, G, and T in the first and second bases and of C and G in the third base of codons 44 and 45 (Thr and Lys, respectively, in SNase); the complementary strand was prepared with I in all three positions of these codons. Assuming an equal distribution of bases in the mixed positions, this restriction fragment encodes 32 × 32 or 1024 possible two-codon substitutions. (Despite the presence of I in the complementary strand, we have not observed A or T in the third base on any mutant genes we have generated by this approach.) The need for replicate plating in our present metabolic selection[2] imposes the practical limit of randomizing only two codons.

Isolation of the Protein Containing the Deletion of the Ω-Loop in E43 SNase (E43 ΔSNase). The library of synthetic *Spe*I–*Rsr*II restriction fragments was ligated with the large *Spe*I–*Rsr*II fragment of pLBP1. After transformation into strain CR34 and plating on LB/amp plates, the transformants were replicate plated on selection plates. Plasmids were isolated from several colonies that grew better than D43 SNase on the selection plates and were subjected to DNA sequence analysis. In one plasmid, designated pE43 ΔSNase, and DNA sequence encoding the Ω-loop was GAA-GGT-GTA-GAG corresponding to the amino acid sequence Glu_{43}-Gly_{50}-Val_{51}-Glu_{52}. Thus, Thr_{44}-Lys_{45}-His_{46}-Pro_{47}-Lys_{48}-Lys_{49} had been deleted. The codon for Pro 42 was the CCC directed by the synthetic restriction fragment, indicating that the deletion of the Ω-loop was the result of the cassette mutagenesis. Other than a single base change in the codon for residue 43 encoding Glu (GAA) rather than Asp (GAC), no unexpected mutations were found when the entire gene was sequenced. The sequences of plasmids in other transformants did, in fact, encode Pro 42 (CCC), Asp 43 (GAC), the randomized substitutions at codons 44 and 45, and the correct sequence for the remainder of the intact Ω-loop.

When single-stranded DNA was used to measure the nuclease activity in extracts of CR43 transformed with pE43 ΔSNase, the specific activity was severalfold higher than that measured for extracts of CR34/D43 SNase. Accordingly, the protein lacking the complete Ω-loop (E43 ΔSNase) was isolated from cell extracts. An additional gel-filtration step was required for purification of E43 ΔSNase to homogeneity since the deletion of the three Lys residues present in the Ω-loop causes E43 ΔSNase (and other variants of ΔSNase at residue 43) to elute from the Bio-Rex-70 column at lower concentrations of NaCl than that used to purify E43 SNase. By SDS–PAGE, E43 ΔSNase has a mobility indistinguishable from the mature SNase encoded by pONF1 (149 amino acids; Takahara et al., 1985) but greater than that of E43 SNase (156 amino acids; Hibler et al., 1987). This is consistent with the length of the E43 ΔSNase deduced from the DNA sequence (150 amino acids).

Table I: Comparison of Kinetic Properties for SNase Mutants[a]

enzyme	V_{max} [ΔOD_{260}/(min·mg)]	K_m (μg/mL)	rel V_{max}
Single-Stranded (Heat-Denatured) DNA (Substrate)			
Glu 43 SNase	5440 ± 570	17 ± 4	100
Glu 43 ΔSNase	135 ± 30	60 ± 22	2.5
Asp 43 SNase	63 ± 5	29 ± 5	1.2
Asp 43 ΔSNase	11 ± 2	74 ± 21	0.2
Ala 43 ΔSNase	21 ± 3	94 ± 19	0.4
NPpdTp (Substrate) and pdTp (Inhibitor)[b]			
enzyme	V_{max} [nmol/(min·mg)]	K_m (μM)	rel V_{max}	K_D[c] (nM)
Glu 43 SNase	920 ± 11	50 ± 2	100	120
Glu 43 ΔSNase	12 ± 0.4	122 ± 11	1.2	290
Asp 43 SNase[d]	0.12	64 ± 9	0.01	340
Asp 43 ΔSNase	2.6 ± 0.2	482 ± 49	0.3	580

[a] Assays using both single-stranded DNA and NPpdTp were performed at 25 °C in 25 mM sodium glycinate buffer, pH 9.50, containing 10 mM $CaCl_2$. Assays were performed in duplicate at five concentrations of substrate bracketed around the value for K_m. Enzymes were diluted in 0.1% DNase-free bovine serum albumin. Data were analyzed by using Cleland's program HYPER (Cleland, 1979). [b] Fluorescence measurements for quantitating the binding of pdTp were made at 22 °C in 10 mM Tris-HCl buffer, pH 7.0, containing 100 mM NaCl, 10 mM $CaCl_2$, and 2.2 μM protein. The excitation wavelength was 280 nm, and the emission wavelength was 295 nm. Each titration experiment was performed in triplicate. [c] Binding constants for pdTp. [d] The values for V_{max} and K_m are approximate since the enzyme concentration was comparable to the substrate concentrations used in the assays.

The distance between the α-carbons of Glu 43 and Glu 52 in E43 SNase is 5.5 Å (Loll & Lattman, 1989), which suggests that a β-turn having the sequence Glu_{43}-Gly_{50}-Val_{51}-Glu_{52} can be accommodated without significant structural distortion. Modeling and energy minimization studies support this prediction (J. Moult, personal communication). The three-dimensional NMR studies reported in the following article (Baldisseri et al., 1991) allow description of the structure of the β-turn that, in fact, results from the deletion.

Preparation and Isolation of D43 ΔSNase and A43 ΔSNase. Since our original intention was to improve the catalytic activity of D43 SNase by random mutagenesis of residues that might compensate for the conformational disruption present in this mutant, we introduced the Glu to Asp substitution into E43 ΔSNase by site-directed mutagenesis to generate D43 ΔSNase. We also generated A43 ΔSNase so that we could determine the catalytic efficiency of the ΔSNase structure in the absence of a general basic functional group at residue 43.

Comparison of the Kinetic Properties of E43 SNase, E43 ΔSNase, D43 SNase, D43 ΔSNase, and A43 ΔSNase. The values of V_{max} and K_m for E43 SNase, E43 ΔSNase, D43 SNase, and D43 ΔSNase were compared with both heat-denatured DNA and NPpdTp as substrates; the values of V_{max} and K_m for A43 ΔSNase were determined with only heat-denatured DNA as substrate. The kinetic parameters are compiled in Table I. [The concentration dependence for activation by Ca^{2+} was determined for E43 SNase, E43 ΔSNase, and D43 ΔSNase at DNA concentrations 4-fold greater than the K_m values reported in Table I. The values so determined did not significantly differ from one another (0.99 ± 0.14 mM, 1.11 ± 0.33 mM, 0.81 ± 0.16 mM, and 1.52 ± 0.17 mM for E43 SNase, E43 ΔSNase, D43 SNase, and D43 ΔSNase, respectively), so the reported differences

[2] While this selection does discriminate between E43 SNase and D43 SNase, we believe that the basis for its success is more subtle than our original hypothesis. When directly plated on selection plates, neither CR34/E43 SNase nor CR34/D43 SNase grows. Instead, transformants must be first plated on LB/amp plates and selected by antibiotic resistance. Subsequent replicate plating of these colonies on selection plates produces viable colonies whose sizes and growth rates are indicative of SNase activity. Alternatively, individual transformants can be grown overnight in liquid medium, and an aliquot spotted on selection plates; the growth rate of the colonies is again indicative of SNase activity. Our interpretation is that both colonies and stationary liquid phase cultures *contain lysed cells* that have released the SNase produced intracellularly so that it is available to degrade the DNA present in the selection plates. Once the DNA is degraded to oligo- or mononucleotides, thymidine is apparently available to the intact, viable cells for growth.

in V_{max} are not artifacts of the Ca^{2+} concentration used in the assays (10 mM).]

Deletion of the Ω-loop to produce E43 ΔSNase decreases V_{max} 40-fold with single-stranded DNA as substrate. However, this level of activity is 2-fold greater than that observed for D43 SNase with the same substrate. Thus, the adventitious deletion of six amino acids from the active-site Ω-loop is less damaging to catalysis than the planned excision of a single methylene group from E43 SNase to generate D43 SNase.

When the Glu to Asp substitution and Ω-loop deletion are combined in the same protein (D43 ΔSNase), the V_{max} with single-stranded DNA as substrate is reduced only 6-fold relative to that observed for D43 SNase (or, equivalently, 12-fold relative to E43 ΔSNase). Thus, the effects of the Glu to Asp substitution and of the Ω-loop deletion on catalysis are not additive. This result can be rationalized if (1) a lower limit exists for the rate acceleration that can be obtained from general basic catalysis and/or (2) either single mutation is accompanied by a conformational alteration in the protein molecule. We believe that both of these explanations may be at least partially true on the basis of the activity of A43 ΔSNase (vide infra) and the results of NMR-based structural studies of E43 SNase, E43 ΔSNase, D43 SNase, and D43 ΔSNase described both later in this article and in the following article (Baldisseri et al., 1991).

With single-stranded DNA as substrate, A43 ΔSNase has a V_{max} nearly identical with (twice) that of D43 ΔSNase (Table I). Since the Glu to Ala substitution may allow either hydroxide ion or buffer access to the active site, the catalytic activity of A43 ΔSNase may be a measure of the upper limit of the rate acceleration possible without participation by an active-site functional group.

When NPpdTp is used as substrate, we observe that the relative activities of E43 SNase, E43 ΔSNase, and D43 ΔSNase parallel those found with the polymeric substrate. However, the activitty of D43 SNase with NPpdTp is barely detectable. This observation suggests that the Ω-loop is directly involved in binding polymeric substrates. The extremely low activity of D43 SNase with NPpdTp may reflect the significantly altered active-site geometry observed in the presence of the monomeric competitive inhibitor pdTp (Loll & Lattman, 1990). However, in the presence of DNA the conformational alteration induced by the single amino acid substitution may be at least partially prevented by the binding energy made available by the interaction between the polymeric substrate and the Ω-loop, including its three Lys residues.

We have also measured the affinity of the various enzymes for pdTp, using quenching of tyrosine fluorescence as the spectroscopic probe (Table I). The absence of the Ω-loop does not significantly diminish the affinity for pdTp. This observation is in agreement with our recent conclusion (A. Kohlekar, M. A. Reynolds, P. H. Bolton, and J. A. Gerlt, unpublished observations) that the interaction of the 3′-phosphate group of pdTp with the enzyme is mediated primarily by both Lys 84 and Tyr 85 and not by residues in the Ω-loop. We assume that the increases in the values for K_m for DNA observed for all three ΔSNases reflect the role of the Ω-loop in substrate binding.[3]

Stability of ΔSNases. Whereas we previously used thermal denaturation to evaluate the relative stabilities of E43 SNase and various site-directed mutants thereof, including D43 SNase (Calderon et al., 1985; Hibler et al., 1987; Pourmotabbed et al., 1990), we have now used denaturation by guanidine hydrochloride to quantitatively compare the stabilities of E43 SNase, D43 SNase, E43 ΔSNase, and D43 ΔSNase. As demonstrated by Shortle (1986), the relatively low concentration of the denaturant necessary to unfold E43 SNase can permit extrapolation of the denaturation curves to 0 M denaturant so that the free energy for unfolding can be obtained and the stabilities of sequence variants can be compared.

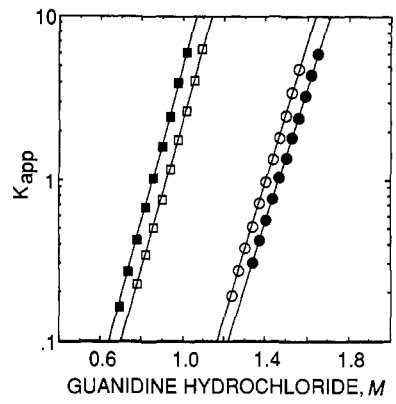

FIGURE 2: Logarithm of the apparent equilibrium constant, K_{app}, as a function of guanidine hydrochloride concentration. All titrations were performed at 20 °C in potassium phosphate buffer, pH 7, containing 100 mM NaCl and 50 μg of the appropriate protein. The excitation wavelength was 295 nm, and the emission wavelength was 325 nm. Data shown represent the average of three titrations each for E43 SNase (closed squares), D43 SNase (open squares), E43 ΔSNase (closed circles), and D43 ΔSNase (open circles).

As shown in Figure 2, at 20 °C the midpoint for denaturation of E43 SNase is 0.85 M guanidine hydrochloride; the midpoint for denaturation of D43 SNase is approximately the same, 0.92 M. The midpoints for the denaturation of both E43 ΔSNase and D43 ΔSNase are significantly elevated to 1.47 and 1.40 M, respectively. The slopes of the denaturation curves, designated *m*, are similar but not identical (D43 SNase, E43 ΔSNase, and D43 ΔSNase are 0.96, 0.85, and 0.90, respectively, relative to that of E43 SNase); we have not detected any deviations from linearity. Linear extrapolation of the data to 0 M guanidine hydrochloride suggests that the relative stabilities of the folded states of E43 ΔSNase and D43 ΔSNase, both 8.0 kcal/mol, are approximately 2.5 kcal/mol greater than those of E43 SNase (5.5 kcal/mol) and D43 SNase (5.7 kcal/mol).

By Shortle's classification, D43 SNase, E43 ΔSNase, and D43 ΔSNase are all class I stability mutants since the measured values of *m* are less than that observed for E43 SNase (Shortle & Meeker, 1986). Thus, the interpretation of the relative *m* values for the various mutants is that the interaction of the denaturant with the protein is decreased in the denatured state relative to that of E43 SNase, presumably the result of a decrease in the volume or denaturant accessible area of the denatured states of the mutant proteins (Shortle et al., 1988; Shortle, 1989). If effects in the folded state were responsible for the observed class I behavior, the volume or surface area would have to increase in the folded states of the mutant proteins. While such an increase is not expected in E43 ΔSNase and D43 ΔSNase, it could, at least in part, be responsible for the slightly smaller value for *m* observed for D43 SNase.

The increase in stability of the folded state that accompanies deletion of the Ω-loop apparently exceeds that achieved when two point mutations that are global suppressors of stability

[3] The measured values for V_{max} and particularly K_m are sensitive to the particular sample of heat-denatured DNA that is used as substrate. For example, with another sample of DNA, the K_m of Glu 43 ΔSNase for DNA (29 μg/mL) was 12-fold higher than that of Glu 43 SNase (2.5 μg/mL), and the K_m of Asp 43 ΔSNase (54 μg/mL) was 22-fold higher than that of Glu 43 SNase.

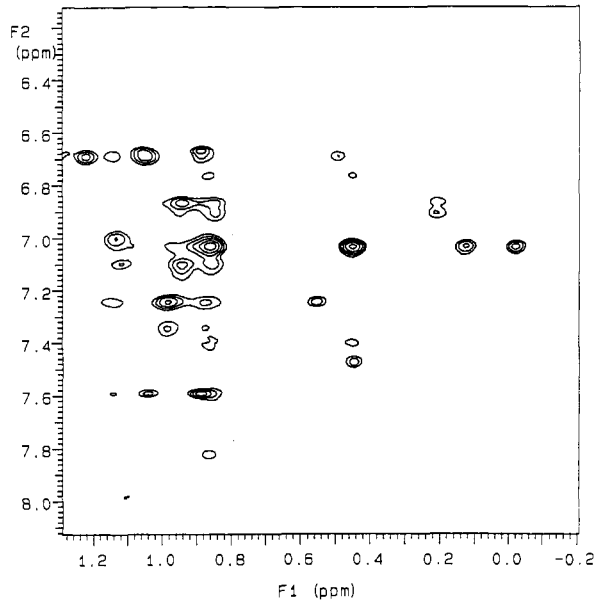

FIGURE 3: Aromatic–upfield-shifted region of the 400-MHz ^1H–^1H NOESY spectrum of E43 SNase.

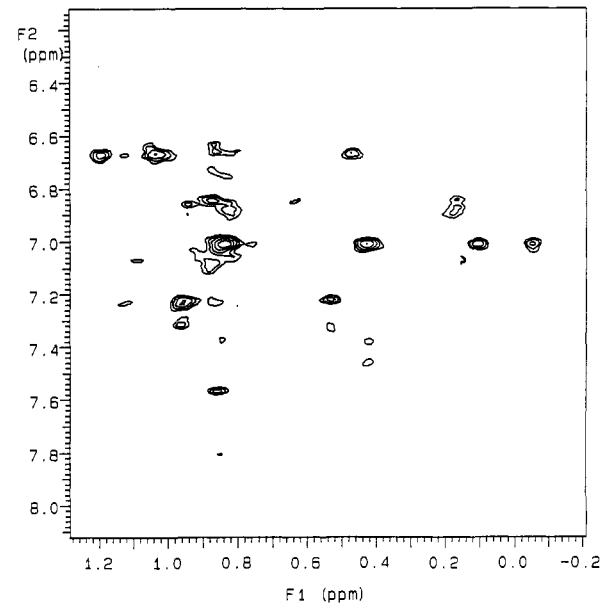

FIGURE 4: Aromatic–upfield-shifted region of the 400-MHz ^1H–^1H NOESY spectrum of E43 ΔSNase.

mutants were combined (H124L and V66L) (Shortle, 1985; Shortle & Meeker, 1986). The extremely elevated midpoints for denaturation we observed for E43 ΔSNase and D43 ΔSNase require extrapolation over a larger concentration range to assess the stability in the absence of denaturant; in addition, as noted in the previous paragraph, the slopes of the denaturation curves are not identical. While our estimates of stability may, therefore, be somewhat in error, our conclusion that deletion of the Ω-loop significantly increases the stability of the folded state must be qualitatively correct. The significant increases in stability presumably result from a decrease in the conformational entropy of the denatured state that results from the loss of degrees of freedom associated with the six deleted residues. We have not yet experimentally determined the enthalpy and entropy contributions to the observed increase in stability.

Effects of the Ω-Loop Deletion on the Chemical Shifts of Aromatic and Upfield-Shifted Amino Acid Residues. We previously have discussed the effect of the E43D substitution on the structure of the intact protein as evidenced by changes in the chemical shifts and interresidue nuclear Overhauser effects of the relatively well-resolved aromatic and upfield-shifted aliphatic residues (Hibler et al., 1987; Wilde et al., 1988). We assigned the observed changes to residues in the hydrophobic core, Val 23, Leu 25, Tyr 27, Phe 34, and Phe 76, and to residues near the base of the Ω-loop, Tyr 54, Ile 139, and Trp 140. Since the hydrophobic core is removed from residue 43 and the Ω-loop by as much as 25 Å, we argued that the E43D substitution produced a conformational change in the active site that was propagated throughout the structure. The crystal structure of D43 SNase has verified the anticipated significant change in the structure of the active site, including the Ω-loop, although the long-range conformational effects were small but observable (Loll & Lattman, 1990). Accordingly, we have used this same two-dimensional NMR approach to examine the effect of the Ω-loop deletion on the structures of both E43 SNase and D43 SNase. [The chemical shift changes observed in the three-dimensional NMR studies reported in the following article (Baldisseri et al., 1991) are also consistent with small changes throughout the structure of D43 SNase when compared with E43 SNase, in agreement with the crystallographic data.]

The aromatic–upfield-shifted sections of the NOESY spectra of E43 SNase and of E43 ΔSNase are presented in Figures 3 and 4; the aromatic–aromatic sections the NOESY spectra of E43 SNase and of E43 and both the aromatic–upfield-shifted and aromatic–aromatic regions of the spectra of D43 SNase and of D43 have been placed in the supplementary material. The spectra of E43 SNase and E43 ΔSNase are essentially identical, as are the spectra of D43 SNase and D43 ΔSNase.

Thus, in contrast to our earlier conclusion regarding the effect of the E43D substitution in intact SNase, the same NMR criteria suggest that deletion of the Ω-loop in either E43 SNase or D43 SNase causes minimal perturbations in the environments of the aromatic residues. Certainly, the long-range effects are much less than those observed when E43 SNase is compared to D43 SNase.

A more detailed description of the effect of the Ω-loop deletion on the structure of both E43 SNase and D43 SNase evaluated by two- and three-dimensional NMR spectroscopy is presented in the following article (Baldisseri et al., 1991). In both cases, the deletion reduces the Ω-loop to a type II′ β-turn with no apparent influence on the structure of the remainder of the protein. In agreement with the crystallographic studies (Loll & Lattman, 1990), the NMR studies suggest structural differences between E43 SNase and D43 SNase; these differences are maintained in E43 ΔSNase and D43 ΔSNase.

Conclusions. Sondek and Shortle (1990) recently described random insertions of Gly and Ala residues in E43 SNase and observed that such insertions in loops *as well as* in α-helics and β-sheets often can be accommodated with little influence on catalytic activity, although the stabilities of the mutant proteins are significantly reduced relative to that of the wild-type protein. Our observations on E43 ΔSNase demonstrate that amino acid deletions as well as insertions represent genetic mutations that are complementary to site-directed substitutions in establishing structure/function relationships in proteins.

Most reports of deleting or swapping loops and turns in proteins (Fetrow et al., 1989; Haynes et al., 1989; Pompliano et al., 1990) either have involved regions of proteins remote from their active sites or have produced very large defects in

catalytic activities. One exception to this generalization is a five-residue deletion from phospholipase A_2, which was shown to enhance catalytic activity and alter substrate specificity (Kuipers et al., 1989). We have now deleted the solvent exposed and disordered Ω-loop in E43 SNase to produce a β-turn with less impact on catalysis than replacing the putative general basic Glu 43 with its homologue Asp. The resulting protein, E43 ΔSNase, is significantly more stable than E43 SNase despite the insensitivity of the conformation of the folded state to the deletion.

Two- and three-dimensional NMR studies of the structures of E43 ΔSNase and D43 ΔSNase are described in the following article (Baldisseri et al., 1991).

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Supplementary Material Available

Six figures showing the aromatic–aromatic regions of the ^1H–^1H NOESY spectra of both E43 SNase and E43 ΔSNase and the aromatic–upfield-shifted and aromatic–aromatic regions of the ^1H–^1H NOESY spectra of both D43 SNase and D43 ΔSNase (6 pages). Ordering information is given on any current masthead page.

Registry No. pdTp, 2863-04-9; NPpdTp, 24418-11-9; L-Asp, 56-84-8; L-Glu, 56-86-0; L-Ala, 56-41-7; staphylococcal nuclease, 9013-53-0.

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