# Mechanism of the Reaction Catalyzed by Staphylococcal Nuclease: Identification of the Rate-Determining Step†

Stephen P. Hale, Leslie B. Poole, and John A. Gerlt*

*Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742*

*Received January 15, 1993; Revised Manuscript Received March 30, 1993*

ABSTRACT: The hydrolysis of single-stranded DNA catalyzed by wild-type staphylococcal nuclease (SNase) and two mutants has been studied as a function of both pH and solvent viscosity. The $k_{cat}$ for wild-type SNase increases with pH; the slope of the plot of log $k_{cat}$ *vs* pH = 0.45 ± 0.01. The dependence of $k_{cat}/K_m$ on pH for wild-type SNase is biphasic with a break at pH ~8: for pH ≤8, the plot of log $k_{cat}$ *vs* pH is linear with a slope = 1.20 ± 0.06; for pH ≥8, the slope = 0.00 ± 0.04. The dependencies of both $k_{cat}$ and $k_{cat}/K_m$ on solvent viscosity are also pH-dependent: below pH 7.3, both kinetic parameters are independent of solvent viscosity; above pH 7.3, both are inversely proportional to solvent viscosity. Thus, at pH 9.5, where SNase is routinely assayed, the rate-determining steps for both $k_{cat}$ and $k_{cat}/K_m$ are external steps (product dissociation for $k_{cat}$ and substrate binding for $k_{cat}/K_m$) and not an internal step (e.g., hydrolysis of the phosphodiester bond). We have also studied the E43D mutant in which the putative active-site general basic catalyst Glu-43 is replaced with Asp. From pH 7.5 to pH 9.5, both log $k_{cat}$ and log ($k_{cat}/K_m$) are directly proportional to pH (slopes = 1.01 ± 0.03 and 0.95 ± 0.04, respectively) and independent of solvent viscosity. At pH 9.5, the rate-determining step is an internal step. Since the rate-determining steps differ at pH values ≥7.5, we conclude that comparisons of the numerical values of the kinetic parameters of active-site mutants of SNase with those of wild-type SNase are mechanistically uninformative at pH values where the $k_{cat}$s are maximal. We have begun to localize the structural elements responsible for the slow external step (product dissociation) in the $k_{cat}$ for wild-type SNase by characterizing a mutant (βVN ΔSNase) in which residues 44–49 of the Ω-loop (residues 43–52) have been deleted to generate a new solvent-exposed β-turn; in addition, Gly-50 and Val-51 have been replaced with Val and Asn, respectively. From pH 7 to pH 10, both log $k_{cat}$ and log ($k_{cat}/K_m$) are directly proportional to pH (slopes = 0.91 ± 0.02 and 0.78 ± 0.02, respectively) and independent of solvent viscosity, even though the $k_{cat}$ of this mutant approaches that of wild-type SNase at the highest pH values. This behavior suggests that residues in the Ω-loop may be responsible for the rate-determining external step that characterizes the $k_{cat}$ of wild-type SNase at high pH. The dependencies of $k_{cat}$ on pH for both wild-type SNase and βVN ΔSNase suggest that the hydrolysis reaction is not general base catalyzed by Glu-43.

Staphylococcal nuclease (SNase)[1] catalyzes the $Ca^{2+}$-dependent hydrolysis of the phosphodiester bonds in DNA at a rate that exceeds the rate of the nonenzymatic reaction by a factor estimated to be as large as $10^{16}$ (Serpersu et al., 1987). High-resolution X-ray structures of wild-type SNase and various site-directed mutants are available (Cotton et al., 1979; Loll & Lattman, 1989, 1990) as are the $^1H$, $^{13}C$, and $^{15}N$ NMR resonance assignments for wild-type SNase and several mutants (Torchia et al., 1989; Wang et al., 1990a,b; Baldisseri et al., 1991). Thus, this protein is an ideal phosphodiesterase/DNase for detailed mechanistic studies. In particular, an explanation for the large rate acceleration that characterizes the enzyme-catalyzed hydrolysis of phosphodiester bonds by this and other nucleases is an important problem in mechanistic enzymology.

For a number of years, this laboratory has addressed the potential involvement of Glu-43 as a general basic catalyst in the hydrolysis reaction. That Glu-43 might be involved as a general basic catalyst was initially postulated by Cotton and his co-workers when they reported the first high-resolution X-ray structure of wild-type SNase (Cotton et al., 1979). The active-site structure of SNase derived from a more recent high-resolution X-ray analysis (Loll & Lattman, 1989) is shown in Figure 1. The 5′-phosphate group of the competitive inhibitor thymidine 3′,5′-bisphosphate (pdTp) is coordinated to the essential $Ca^{2+}$ ion as well as to the guanidinium groups of Arg-35 and Arg-87. Although pdTp is not a substrate, the position of its 5′-O–P bond is thought to mimic that of the scissile bond in a substrate phosphodiester since the products of the enzymatic reaction are 3′-mononucleotides. The γ-carboxylate group of Glu-43 is hydrogen-bonded to two water molecules: one in the inner coordination sphere of the $Ca^{2+}$ ion and a second that is also hydrogen-bonded to the metal-liganding, 5′-phosphate group of pdTp. Support for the hypothesis that Glu-43 is involved in catalysis as a general base was provided by stereochemical experiments performed in this laboratory that demonstrated that the reaction proceeds with inversion of configuration at phosphorus (Mehdi & Gerlt, 1982). This stereochemical outcome is consistent with, but does not prove, the hypothesis that one of the water molecules hydrogen-bonded to the 5′-phosphate group of the competitive inhibitor may be appropriately located to act as the nucleophile when a substrate phosphodiester is bound and that the γ-carboxylate group of Glu-43 activates the nucleophilic water molecule for attack by general base catalysis. The attack of

† This work was supported by NIH NRSA Grant GM-13211 (to L.B.P.) and NIH Grant GM-34573 from the National Institutes of Health (to J.A.G.).

[1] Abbreviations: SNase, staphylococcal nuclease; E43D, mutant of SNase in which Glu-43 is changed to Asp; PIPES, sodium piperazine-*N*,*N*′-bis(2-ethanesulfonate); NMR, nuclear magnetic resonance; βGV ΔSNase, mutant of SNase in which residues 44–49 are deleted and residues 50 and 51 are Gly and Val, respectively; βVN ΔSNase, mutant of SNase in which residues 44–49 are deleted and residues 50 and 51 are Val and Asn, respectively; Ω-loop, residues E43–E52 of wild-type SNase.

0006-2960/93/0432-7479$04.00/0 

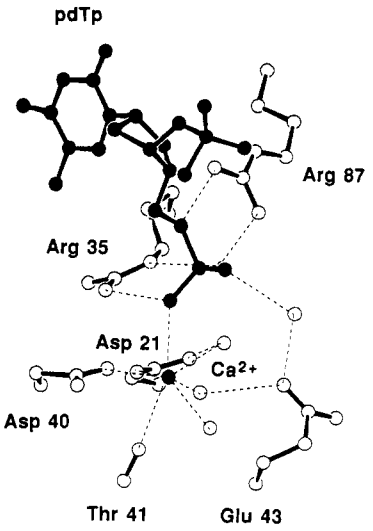



FIGURE 1: Active site of SNase as reported by Loll and Lattman (1989). The competitive inhibitor thymidine 3′,5′-bisphosphate is shown in black.

the incipient hydroxide ion is thought to be facilitated by three electrophilic catalysts; i.e., the $Ca^{2+}$ ion and the guanidinium groups of Arg-35 and Arg-87 would minimally function to neutralize the anionic charge of the phosphodiester substrate.

The role of Glu-43 in catalysis has been investigated by site-directed mutagenesis and structural analysis of mutant enzymes by both X-ray crystallography and multidimensional NMR analysis. In particular, at pH 9.5, the $k_{cat}$ for the E43D mutant was found to be 200-fold less than that of wild-type SNase (Hibler et al., 1987). However, this substitution was found to be structurally disruptive since small but significant changes in structure were observed throughout the structure of the protein by both NMR spectroscopy (Wilde et al., 1988) and X-ray crystallography (Loll & Lattman, 1990). Thus, while the reduction in activity caused by the Glu to Asp substitution might have been interpreted as evidence in support of Glu-43 acting as a general basic catalyst, this interpretation was regarded as equivocal given the widespread structural alterations in the mutant protein.

We have also used an analogous approach to investigate the importance of the guanidinium groups of both Arg-35 and Arg-87 in catalysis by studying the R35K and R87K mutants (Pourmotabbed et al., 1990). Again, major reductions in catalytic activity were observed, but the structures of the mutant enzymes were altered as revealed by NMR spectroscopy. Mildvan and Shortle and their co-workers have also studied a number of active-site mutants of SNase (Serpersu et al., 1986, 1987, 1988), but without detailed structural characterization, and attempted to account quantitatively for the $10^{16}$ rate acceleration by multiplying the reductions in $k_{cat}$ (at pH 9.5) associated with point mutations for a number of active-site residues (Serpersu et al., 1987).

Despite the application of sophisticated molecular biological and structural techniques to the study of the mechanism of the reaction catalyzed by SNase, few studies of the kinetics of the reaction have been reported. Early studies by Anfinsen and co-workers demonstrated that SNase has an alkaline pH optimum and that the activity decreases abruptly above pH 10.5 (Cuatrecasas et al., 1967). Mildvan and his co-workers reported kinetic and EPR studies of the E43S mutant of SNase (Serpersu et al., 1989), including a characterization of the dependence of $k_{cat}$ and $k_{cat}/K_m$ on pH. This mutant, like E43D, is less active (approximately 300-fold) than wild-type SNase at pH 9.5 (Hibler et al., 1987). They noted, but did not pursue, the observation that a plot of log $k_{cat}$ *vs* pH for E43S had a slope $\approx 1$ but that the slope of an analogous plot for wild-type SNase had a slope $\approx 0.6$. They pointed out that the dependence of $k_{cat}$ on pH for E43S was analogous to that reported for the D102N mutant of trypsin ($k_{cat}$ directly proportional to hydroxide ion concentration for D102N but independent of pH for wild-type trypsin; Sprang et al., 1987; Craik et al., 1987). In wild-type trypsin, Asp-102 participates in a catalytic triad that allows His-57 to act as a general basic catalyst; in the D102N mutant, the observed dependence of $k_{cat}$ on pH was used to suggest that general base catalysis by His-95 was no longer possible and that the hydrolysis reaction was specific base-catalyzed. Thus, the presumption was that general base catalysis by Glu-43 had been inactivated by the E43S mutation.

In this paper, we describe kinetic characterization of wild-type SNase and two mutants as a function of both pH and solvent viscosity. The identity of the rate-determining step for wild-type SNase is pH-dependent: at pH 9.5, where the kinetic properties of site-directed mutants of SNase routinely have been studied, the rate-determining steps for wild-type SNase are external steps (e.g., substrate binding for $k_{cat}/K_m$ and product dissociation for $k_{cat}$) and not an internal step (e.g., hydrolysis of the phosphodiester bond); at pH values below 7.5, the rate-determining step for wild-type SNase is an internal step. For the E43D mutant, the identity of the rate-determining step is independent of pH and associated with an internal step. Thus, even in the absence of the structural changes that have been observed for at least some active-site mutants (Wilde et al., 1988; Loll & Lattman, 1990; Pourmotabbed et al., 1990), the rate reductions associated with mutants of SNase cannot be readily interpreted in terms of the mechanism of the reaction catalyzed by wild-type SNase. The kinetics of the reaction catalyzed by a mutant that is missing a portion (residues 44–49) of the active-site Ω-loop (residues 43–52) suggest that this Ω-loop may be responsible for the viscosity dependence of the reaction catalyzed by wild-type SNase at high pH. The dependence of $k_{cat}$ on pH for wild-type SNase suggests that the hydrolysis reaction is not general base catalyzed by Glu-43.

## MATERIALS AND METHODS

*Plasmids.* We have previously described the expression plasmid pLBP1 (Poole et al., 1991) that directs the synthesis of a version of the E43D mutant of SNase in which the seven amino acid sequence Met-Asp-Pro-Thr-Val-Tyr-Ser is appended to the N-terminal Ala residue of wild-type SNase. This expression plasmid is a derivative of pCQV2 and has the gene for the elongated nuclease under the control of the bacteriophage λ $P_R$ promoter. We have also described a second expression plasmid, pLBP2, that directs the synthesis of the same N-terminal elongated version of the E43D mutant of SNase (Poole et al., 1991). pLBP2 is a derivative of pAS1 and has the gene for the elongated nuclease under the control of the bacteriophage λ $P_L$ promoter. The amount of the N-terminal extended version of E43D produced by host strains containing pLBP2 exceeds that produced by host strains containing pLBP1. Both pLBP1 and pLBP2 contain a unique *Spe*I site starting at codon 37 in the gene for SNase, a unique *Rsr*II site starting at codon 54, and a unique *Sal*I site downstream of the 3′-end of the gene for SNase.

The expression plasmid pLBP3 used in the present work was constructed from pLBP2 by deletion of the codons for the sequence Asp-Pro-Thr-Val-Tyr-Ser in the N-terminal extension and replacement of the codon for residue 43 (Asp-43) with that of wild-type SNase (Glu-43). pLBP2 contains two

sites for *Hin*dIII; the plasmid was digested with *Hin*dIII. The smaller 2152-bp *Hin*dIII–*Hin*dIII fragment that encodes the N-terminal extension as well as the first 102 codons of the gene for E43D was cloned into M13 mp18. The deletion mutagenesis was performed by the phosphorothioate method with the kit and instructions supplied by Amersham. The mutagenic primer was d(GTAATTTTTTAGTTGAAGT-TGC-Δ-CATATGTAAGTATTTC), where Δ denotes the position of the deleted codons, CAT is the complement of the initiating Met codon, and TGC is the complement of the N-terminal Ala codon of mature SNase. Following identification of a phage containing the desired deletion, the mutant 2134-bp *Hin*dIII–*Hin*dIII fragment was excised and ligated into the larger *Hin*dIII–*Hin*dIII fragment from pLBP2. The *Spe*I–*Rsr*II fragment containing the codon for Asp-43 was then excised and replaced with an *Spe*I–*Rsr*II fragment in which the codon for residue 43 had been mutated to encode Glu-43. This plasmid was designated pLBP3. The N-terminal sequence of SNase purified from extracts of *Escherichia coli* strain KL740 containing pLBP3 was determined in the Protein Nucleic Acid Laboratory, University of Maryland, College Park. This sequence (Ala-Thr-Ser-Thr) revealed that the N-terminal Met was removed by the *E. coli* host and that the N-terminal sequence was identical to that of mature SNase produced by *Staphylococcus aureus*.

We recently described a deletion mutant of SNase in which residues 44–49 of the active site Ω-loop (residues 43–52) had been deleted (Poole et al., 1991). As a result of this deletion, residues 43, 50, 51, and 52 constitute a new solvent-exposed β-turn (Baldisseri et al., 1991). In this paper, we designate this protein as βGV ΔSNase[1] to identify the residues in the $i + 1$ (Gly, residue 50) and $i + 2$ (Val, residue 51) positions of the new β-turn. Later in this paper we will describe the kinetic characterization of a sequence variant of βGV ΔSNase in which residues 50 and 51 are Val and Asn, respectively. We designate this protein as βVN ΔSNase.[1] The $k_{cat}$ of the βVN ΔSNase mutant is approximately 8-fold greater than that of βGV ΔSNase at pH 9.5. This mutant was identified by simultaneous random mutagenesis of codons 50 and 51 in βGV ΔSNase and selection for increased catalytic activity by the methods described by Poole et al. (1991) (S. P. Hale, L. B. Poole, and J. A. Gerlt, unpublished experiments). The pool of random mutants was constructed in the expression plasmid pLBP3 by using polymerase chain reaction methodology to generate a mixture of the smaller *Spe*I–*Sal*I fragment of pLBP3 containing the random substitutions for codons 50 and 51. The details of this construction and selection will be described elsewhere.

*Proteins.* Wild-type SNase, E43D, and βVN ΔSNase were isolated from extracts of *Escherichia coli* strain KL740 transformed with pLBP3 containing the coding sequence for the appropriate version of SNase (constructed as described in the previous section). The various SNases encoded by the various versions of pLBP3 were induced by temperature induction and purified by Bio-Rex chromatography as previously described (Poole et al., 1991), except that FPLC was substituted for the column chromatography procedures that had previously been performed by gravity elution. All three proteins were homogeneous as indicated by SDS–PAGE. Concentrations of samples of SNase were quantitated by assuming that $\epsilon_{280nm} = 0.93$ (mg/mL)$^{-1}$ cm$^{-1}$ and that the molecular weight of wild-type SNase is 16 800.

*Substrate DNA.* Salmon sperm DNA (type III from Sigma) denatured as described by Cuatrecasas et al. (1967) was sonicated with the microtip of a Branson sonicator at an 80% duty cycle, level 4 setting, for 10 min while being cooled in an ice bath. The sonicated samples were then heated to 100 °C for 10 min followed by rapid cooling on ice. The DNA prepared by this method had a size distribution between 200 and 500 bases as determined by agarose gel electrophoresis. DNA concentrations were quantitated by assuming that $\epsilon_{260nm} = 25$ (mg/mL)$^{-1}$ cm$^{-1}$. A molecular weight of 330 was used to convert DNA concentrations from micrograms per milliliter to molarity of internucleotide bonds.

*Other Materials.* The viscogenic agents, sucrose (99+% pure; DNase and RNase free), poly(ethylene glycol) 8000 (DNase and RNase free), and Ficoll-70, were used as purchased from Sigma. The buffers sodium acetate, glycylglycine, and glycine were obtained from Sigma. The buffer PIPES was obtained from Research Organics. Restriction endonucleases and "Vent" polymerase were obtained from New England Biolabs. Polynucleotide kinase from bacteriophage T4 was purchased from Boehringer Mannheim. DNA sequencing kits were from U.S. Biochemicals. Phosphorothioate mutagenesis kits were obtained from Amersham. Synthetic oligonucleotide primers for mutagenesis and DNA sequence analysis were purchased from Oligos Etc (Wilsonville, OR). All other chemicals used were the best grades commercially available.

*Kinetic Measurements.* The activities of the wild-type SNase, E43D, and βVN ΔSNase were quantitated at 25 °C with a Gilford Response II spectrophotometer using sonicated, single-stranded DNA as substrate. Substrate DNA concentrations varied from 2 to 300 μg/mL (6–900 μM in internucleotide bonds), with the exact choice of substrate concentrations being dependent upon the $K_m$ observed under each set of reaction conditions (pH and solvent viscosity). Assays were performed at a minimum of three substrate concentrations both below and above the value of $K_m$ being determined. All assays were performed in duplicate.

Samples of SNase were diluted in Eppendorf centrifuge tubes, and appropriate dilutions were assayed in silanized cuvettes to minimize the adsorption of enzyme to the cuvette. The concentration of enzyme was chosen to produce a $\Delta OD_{260}$/min ≤ 0.1. For assays of wild-type SNase, the enzyme concentration decreased from 1 μM at pH 5.5 to 0.6 nM at pH 10. For assays of E43D, the enzyme concentration decreased from 4 μM at pH 7.5 to 40 nM at pH 9.5. For assays of βVN ΔSNase, the enzyme concentration decreased from 2 μM at pH 7.0 to 2 nM at pH 10.

The buffers used were PIPES ($pK_a = 6.6$), pH 5.5–7.5; glycylglycine ($pK_a = 8.25$), pH 7.5–9.0; and glycine ($pK_a = 9.8$), pH 9.0–10. Values of pH were measured at 25 °C with a Radiometer PHM26 pH meter equipped with a Fisher combination electrode and calibrated with Fisher standard buffer solutions. The rates of the enzymatic reactions were judged to be independent of the identity of the buffer by performing assays with both buffers in the transition region between their buffering ranges. The concentration of $Ca^{2+}$ used in the assays (10 mM) was observed to produce maximal activity at each of the pHs used in the kinetic studies described in this paper.

The hyperchromicity produced by DNA hydrolysis was used to convert the observed velocities measured as $\Delta OD_{260}$/min to $k_{cat}$ units of s$^{-1}$. We have determined that within error the extinction coefficient of single-stranded DNA at 260 nm is independent of pH from pH 5.5 to 10. We have measured the pH dependence of the percent increase in absorbance associated with the complete hydrolysis of single-stranded DNA (hyperchromicity) by wild-type SNase. From pH 5.5 to 9.5, the hyperchromicity was observed to be constant at 29%; at pH 10, the hyperchromicity was slightly smaller, 26%.

Changes in hyperchromicity occur when polymeric DNA is degraded into oligonucleotides smaller than 10 nucloetides in length (Brahms et al., 1966). Since the increase in hyperchromicity is approximately proportional to the decrease in chain length from 10 to 1 nucleotide units (Brahms et al., 1966), we assume that the changes in hyperchromicity can be used to quantitate the amount of internucleotide bond hydrolysis. The changes in hyperchromicity were observed to be independent of the presence of sucrose, the microviscogenic agent used in the studies described in this article.

*Preparation of Viscogens.* Stock solutions of the viscogens were prepared at twice the concentrations to be used in enzyme assays by appropriate dissolution of the viscogenic agents in deionized water.

*Viscosity Determinations.* The viscosities of 1:1 mixtures of stock viscogens and assay buffers (at twice the desired final concentration) were determined at 25 °C with an Ostwald viscometer. After dilution, the final concentrations of sucrose used in the assays were 13.3, 15.6, 21.3, 26.7, 30.0, and 32.0 wt %; these were found to yield relative viscosities, $\eta_{rel}$, of 1.2, 1.5, 1.8, 2.1, 2.4, and 2.9, respectively, as compared to the viscosity measured in the absence of sucrose.

*Kinetic Measurements as a Function of Solvent Viscosity.* Viscosities of the assay mixtures were increased by the addition of the appropriate stock solution of viscogen while maintaining constant ionic strength and buffer concentration. A typical assay at 25 °C contained 500 μL of viscogen stock solution, 25 mM buffer, 10 mM $CaCl_2$, heat-denatured, single-stranded DNA, and enzyme in a total volume of 1 mL.

*Data Analysis.* The kinetic parameters $k_{cat}$ and $k_{cat}/K_m$ and their associated errors were determined with the program HYPER as described by Cleland (1979).

The slopes of plots of $k_{cat}$ *vs* pH and $k_{cat}/K_m$ *vs* pH (Figures 2 and 3) were determined by weighted least-squares regression analysis using the program SYSTAT 5.0 (SYSTAT, Inc., Evanston, IL). The values of $k_{cat}$ and of $k_{cat}/K_m$ used in these calculations were weighted inversely to the errors obtained from HYPER.

The pH dependence of $k_{cat}$ predicted by the kinetic model (Scheme I) for the reaction catalyzed by wild-type SNase (Figure 6) was calculated using the program MacII-MATLAB (Mathworks, Inc., Natick, MA).

## RESULTS AND DISCUSSION

*Choice of Substrate.* The kinetic analyses described in this paper utilized single-stranded (heat denatured) salmon sperm DNA as substrate. This DNA was sonicated to reduce its length to 200–500 base pairs as assessed by gel electrophoresis since we observed that this procedure enhanced the reproducibility of velocity measurements. While we recognize that this substrate is heterogeneous and that the enzyme does prefer to hydrolyze on the 5′-side of A and T residues, this substrate offers the advantage that its is easy to obtain in large quantities and that it is rapidly hydrolyzed. At present, no synthetic substrate is available that is hydrolyzed at rates comparable to those observed for single-stranded DNA.

The $k_{cat}$s for mononucleotide synthetic substrates [$\approx$0.2 $s^{-1}$ at pH 9.5 for both thymidine 5′-[(4-nitrophenyl)phosphate) and thymidine 3′-phosphate 5′-[(4-nitrophenyl)phosphate]; Cuatrecasas et al., 1969] are $\geq$1000-fold less than those observed for single-stranded DNA at pH 9.5. In preliminary experiments, we have observed that this rate difference can be associated with a change in the nature of the rate-determining steps; i.e., the rate-determining step of the reaction using thymidine 3′-phosphate 5′-[(4-nitrophenyl)phosphate] as substrate is an internal step as assessed by its independence

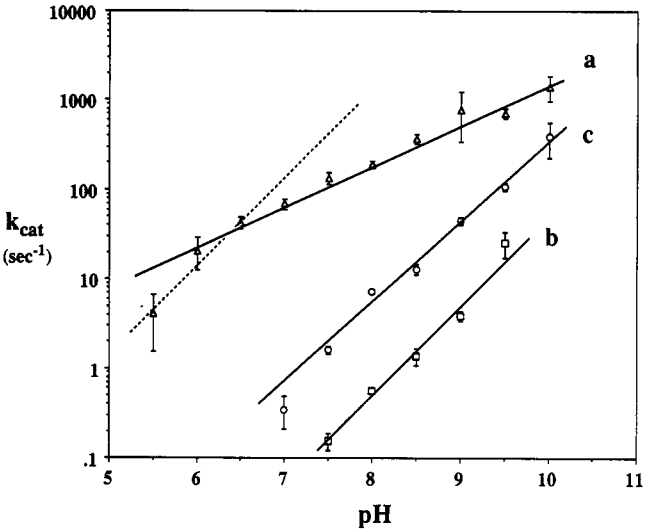



FIGURE 2: Dependence of log $k_{cat}$ on pH for (a) wild-type SNase (△), (b) E43D (□), and (c) βVN ΔSNase (○). The assay conditions are described in the text. For (a), the slope = 0.45 ± 0.01; for (b), the slope = 1.01 ± 0.03; and for (c), the slope = 0.91 ± 0.02. The values of $k_{cat}$ used in the calculations of the slopes were weighted inversely to the errors obtained from the program HYPER. The dotted line has a slope = 1.0.

of solvent viscosity at pH 9.5 (S. Hale and J. A. Gerlt, unpubished experiments) whereas that with DNA as substrate at the same pH is an external step as assessed by its inverse proportionality to solvent viscosity at pH 9.5 (*vide infra*). We believe that it is important to understand the difference in reactivities of mononucleotide substrates and polymeric substrates, and in this article, we characterize the kinetics of the reaction with single-stranded DNA as sustrate.

*Kinetic Behavior of Wild-Type SNase.* The dependence of log $k_{cat}$ on pH for wild-type SNase is displayed in Figure 2, line a. This plot is unusual since $k_{cat}$ is neither pH independent nor described by the ionization of distinct acidic or basic functional groups (ascending or descending limbs to/from pH-independent plateaus). Within the errors in the measurements, a straight line with slope = 0.45 ± 0.01 (solid line) can be drawn through the points. As noted in the introduction, a fractional dependence of log $k_{cat}$ on pH was noted in an earlier analysis of the kinetic behavior of the E43S mutant (Serpersu et al., 1989), although no definitive explanation was then provided for this behavior.

Inspection of the data points describing line a in Figure 2 suggests that as the pH decreases, the slope of the dependence of log $k_{cat}$ on pH *may* be increasing and perhaps approaching unity (the dotted line in Figure 2 has a slope of 1). The errors associated with using polymeric DNA as substrate make accurate quantitation of the dependence of $k_{cat}$ on pH difficult as the pH decreases below 7. However, over the limited range of pH from 5.5 to 7.0, the slope = 0.67 ± 0.06 (and from pH 7.0 to 9.5, the slope = 0.47 ± 0.02). It is impossible to measure $k_{cat}$ at pH values $\leq$5. However, in multiple, independent determinations of the dependence of $k_{cat}$ on pH, the same trend toward unit slope is always observed at pH values $\leq$7, although the errors are also always as large as those shown in Figure 2. A potential reason to suspect a change in slope in this plot is described later in this section.

The dependence of log ($k_{cat}/K_m$) on pH for wild-type SNase is illustrated in Figure 3, line a. Above pH values $\geq$8.0, $k_{cat}/K_m$ is independent of pH since the data are described by a slope = 0.00 ± 0.04. At these pH values, the value of $k_{cat}/K_m$ reaches a maximum value of $10^7$ $M^{-1}$ $s^{-1}$ which is appropriate for the diffusion-controlled binding of the DNA substrate to

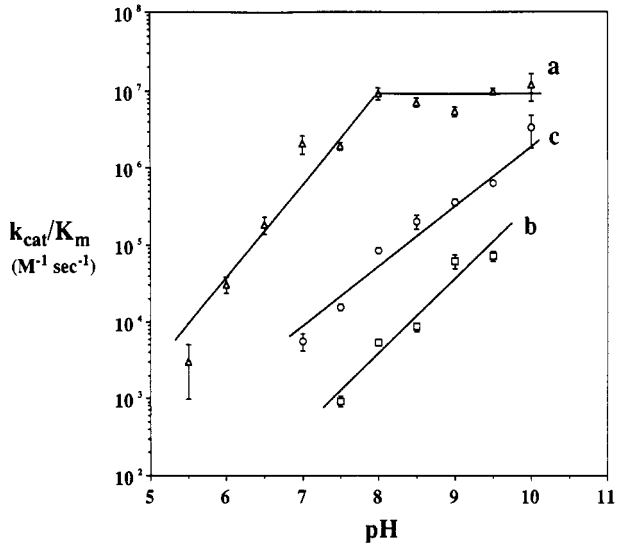



FIGURE 3: Dependence of log ($k_{cat}/K_m$) on pH for (a) wild-type SNase (△), (b) E43D (□), and (c) βVN ΔSNase (○). The assay conditions are described in the text. For (a), the slope = 1.20 ± 0.06 for pH values ≤8.0 and slope = 0.00 ± 0.04 for pH values ≥8; for (b), the slope = 0.95 ± 0.04; and for (c), the slope = 0.78 ± 0.02. The values of $k_{cat}/K_m$ used in the calculations of the slopes were weighted inversely to the errors obtained from the program HYPER.

the enzyme. This observation suggests that the rate-determining transition state for $k_{cat}/K_m$ at pH values ≥8.0 is that for substrate binding. In contrast to the uncertain dependence of $k_{cat}$ on pH as the pH decreases below 7.0, an ascending limb can clearly be recognized for $k_{cat}/K_m$ at pH values ≤8.0. The slope = 1.20 ± 0.06 using the data points from pH 5.5 to 8.0, suggesting that $k_{cat}/K_m$ is proportional to hydroxide ion concentration.

The dependencies of both $k_{cat}$ and $k_{cat}/K_m$ on solvent viscosity were examined as a function of pH to determine whether the obvious change in the dependence of log ($k_{cat}/K_m$) on pH (Figure 3, line a) and the less certain change in the dependence of log $k_{cat}$ on pH (Figure 2, line a) at pH ~7 could be due to changes in the nature of the rate-determining step as the pH is varied. The rates of substrate binding to and product dissociation from the enzyme [external steps using the terminology of Albery and Knowles (1976)] are expected to be inversely proportional to solvent microviscosity. The rates of processes which occur within enzyme–substrate complexes (internal steps; Albery & Knowles, 1976) are expected to be independent of solvent microviscosity (Brouwer & Kirsch, 1982; Blacklow et al., 1988; Caldwell & Raushel, 1991). Macroviscogens, including poly(ethylene glycol) 8000 and Ficoll-70, were found to have no effect on the kinetics of the SNase-catalyzed reaction. While the detailed studies described in this paper were performed with sucrose as the microviscogen, similar results were obtained under selected conditions of pH and solvent viscosity when glycerol was used as the microviscogen, thereby supporting the interpretations we make in this article.

The dependence of $k_{cat}$ on solvent viscosity, $\eta$, for wild-type SNase was determined at a number of pH values from pH 5.5 to pH 10. In Figure 4, we display the relative $k_{cat}$ ($k_{cat}^{\circ}/k_{cat}^{\eta}$) as a function of relative solvent viscosity ($\eta/\eta^{\circ}$) at pHs 6.5, 7.3, and 9.5 (where $k_{cat}^{\eta}$ is the $k_{cat}$ in buffer containing microviscogen, $k_{cat}^{\circ}$ is the $k_{cat}$ in buffer without microviscogen, $\eta$ is the viscosity of the buffer containing microviscogen, and $\eta^{\circ}$ is the viscosity of the same buffer without microviscogen). At pH 6.5, $k_{cat}^{\circ}/k_{cat}^{\eta}$ is independent of $\eta/\eta^{\circ}$, indicating that diffusive processes are not involved in the rate-determining step; i.e., the rate-determining step is an internal step. At pH

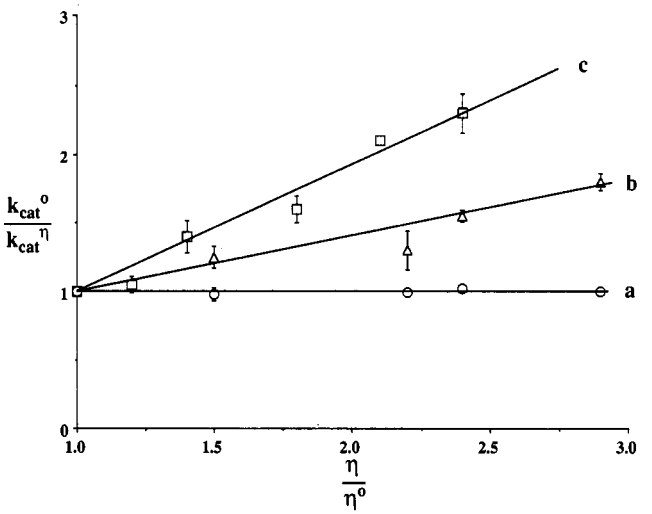



FIGURE 4: Dependence of $k_{cat}^{\circ}/k_{cat}^{\eta}$ on relative solvent viscosity, $\eta/\eta^{\circ}$, for wild-type SNase at (a) pH 6.5 (○), (b) pH 7.3 (△), and (c) pH 9.5 (□). The concentrations of the microviscogen and the assay conditions are described in the text.

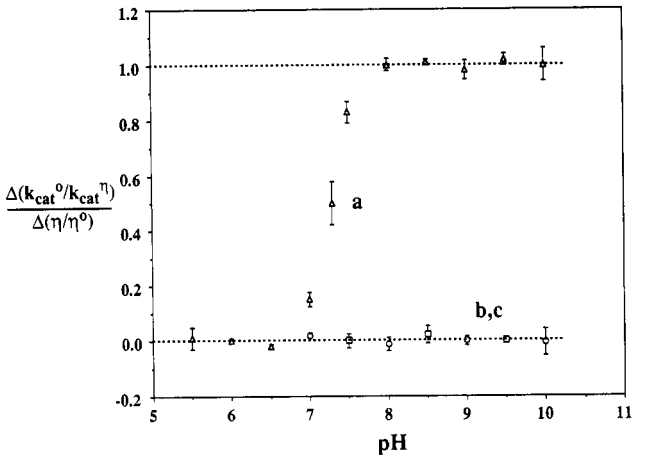



FIGURE 5: Plots of $\Delta(k_{cat}^{\circ}/k_{cat}^{\eta})/\Delta(\eta/\eta^{\circ})$ (the slopes of plots such as those shown in Figure 4) as a function of pH for (a) wild-type SNase (△), (b) E43D (□), and (c) βVN ΔSNase (○). The assay conditions are described in the text.

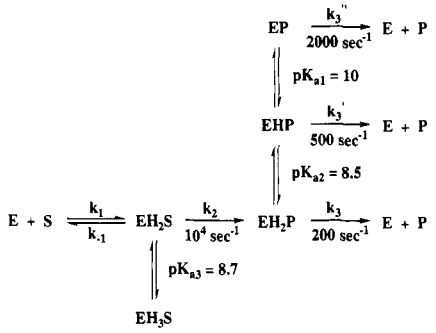
7.3, $k_{cat}^{\circ}/k_{cat}^{\eta}$ is partially dependent on $\eta/\eta^{\circ}$ since the plot has a slope of 0.5; this suggests that both external and internal steps are comparable in rate. At pH 9.5, $k_{cat}^{\circ}/k_{cat}^{\eta}$ is directly proportional to $\eta/\eta^{\circ}$, indicating that diffusive processes are rate-determining; i.e., the rate-determining step is an external step.

In Figure 5, line a, we display the slopes of plots of $k_{cat}^{\circ}/k_{cat}^{\eta}$ vs $\eta/\eta^{\circ}$ [$\Delta(k_{cat}^{\circ}/k_{cat}^{\eta})/\Delta(\eta/\eta^{\circ})$] determined as a function of pH from pH 5.5 to 10. Since $K_m$ was observed to be independent of solvent viscosity, the dependence of $\Delta\{(k_{cat}/K_m)^{\circ}/(k_{cat}/K_m)^{\eta}\}/\Delta(\eta/\eta^{\circ})$ on pH is the same as the dependence of $\Delta(k_{cat}^{\circ}/k_{cat}^{\eta})/\Delta(\eta/\eta^{\circ})$ on pH shown in Figure 5.

From Figure 5, line a, we conclude that *the nature of the rate-determining step in both $k_{cat}$ and $k_{cat}/K_m$ is pH-dependent for wild-type SNase*, with an internal step being rate-determining below pH 7.3 and external steps being rate-determining at higher pH, including pH 9.5 where SNase and its mutants are routinely assayed.

While these data do not prove that nucleophilic attack of water on the substrate phosphodiester is rate-determining below pH 7.3, that $k_{cat}/K_m$ (and, perhaps, $k_{cat}$) may be proportional to hydroxide ion concentration below pH 7.3 does suggest that the hydrolysis step is rate-determining. If a proton must be removed from a water molecule in order for the hydrolysis reaction to occur (e.g., by either general or

Scheme I




specific base catalysis) and this step is rate-determining, the observed dependence of $k_{cat}/K_m$ (and, perhaps, $k_{cat}$) on increasing hydroxide ion concentration at pH values below 7.3 can be explained. It is for this reason that we earlier speculated that the plot of log $k_{cat}$ *vs* pH (Figure 2, line a) may be biphasic, with the slope approaching unity as the pH decreases below 7. The origin of the pH dependence of the rate of the internal step on hydroxide ion concentration is considered later in this article.

The conclusion that the rate-determining step in $k_{cat}/K_m$ is an external step at pH values ≥7.3 is in accord with our earlier conclusion that the rate-determining, pH-independent transition state for $k_{cat}/K_m$ is substrate binding to the enzyme. The conclusion that the rate-determining step in $k_{cat}$ is also an external step at pH values ≥7.3 suggests that the rate-determining transition state for $k_{cat}$ is product dissociation from the enzyme. That the energy difference between this transition state and the lowest energy E·S or E·P complex appears to be pH-dependent suggests that the rate of product dissociation can be influenced by ionizable groups in or immediately adjacent to the active site. Thus, we conclude that different external transition states appear to be rate-determining for $k_{cat}$ and $k_{cat}/K_m$ at pH values ≥7.3.

*Kinetic Model for the pH Dependence of $k_{cat}$ for Wild-Type SNase.* While the interpretation of the dependence of log ($k_{cat}/K_m$) on pH may be relatively straightforward, e.g., rate-determining hydrolysis of the phosphodiester bond below pH 7.3 and substrate binding above pH 7.3, the dependence of log $k_{cat}$ on pH is more difficult to rationalize since $k_{cat}$ as controlled by an external step (product dissociation) continues to increase with pH but with a fractional dependence on hydroxide ion concentration.

Examination of the high-resolution X-ray structure for wild-type SNase reveals the presence of the previously mentioned Ω-loop immediately adjacent to the active site. This Ω-loop contains three lysine residues, Lys-45, Lys-48, and Lys-49. In addition, a fourth lysine residue, Lys-84, and a tyrosine residue, Tyr-85, are also adjacent to the active site, although they are located on the major globular domain of the nuclease molecule and physically removed from the lysine residues of the Ω-loop. Thus, multiple amino acid side chains with ionizable groups likely to have p$K_a$s ≥ 8 are adjacent to the active site, and the function of these may be to interact with the phosphodiester linkages of the DNA substrate and the products of hydrolysis and influence the rate of product dissociation from the enzyme.

In Scheme I, we describe a simple kinetic model that can describe the observed fractional dependence of $k_{cat}$ on hydroxide ion concentration. In this kinetic model, we propose that DNA binds to the enzyme and that hydrolysis of the phosphodiester bond (conversion of $EH_2S$ to $EH_2P$) is dependent on the ionization of a group in the active site (e.g., an active-site general basic catalyst or the nucleophilic water molecule). The dissociation of the product from $EH_2P$ occurs

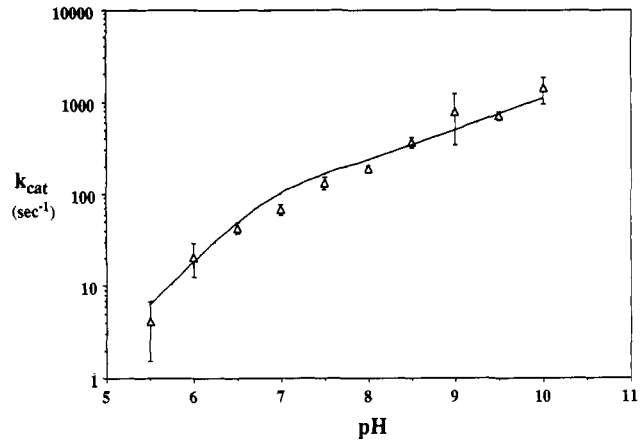



FIGURE 6: Fit of the kinetic model in Scheme I to the measured dependence of log $k_{cat}$ on pH for wild-type SNase (Δ). The line was calculated using MacII-MatLab programmed with the rate equation derived from a steady-state treatment of the kinetic model and using the values shown in Scheme I.

at a rate that is determined by at least two ionizable groups with p$K_a$s > 8, with proton removal as the pH is increased being associated with an increased rate of product dissociation.

Using the values for the various rate constants and p$K_a$s shown in Scheme I, the observed dependence of log $k_{cat}$ on pH can be described (Figure 6). We note, however, that the values for the rate constants and p$K_a$s shown in the scheme do not represent a unique description of the observed dependence of $k_{cat}$ on pH. While the observed increase of $k_{cat}$ with pH imposes a lower limit of ~8.7 on p$K_{a3}$ as specified in Scheme I, if p$K_{a3}$ > 8.7, the values of $k_2$ and of p$K_{a3}$ become interdependent and, therefore, indeterminate. For example, if the values of $k_2$ and of p$K_{a3}$ are $10^6$ s$^{-1}$ and 10.7, respectively, the model would equivalently describe the observed dependence of $k_{cat}$ on pH within the observed experimental errors.

Despite this limitation, our model illustrates the important point that the fractional dependence of $k_{cat}$ on hydroxide ion concentration (at pH values ≥7.3) can be explained by the rate of product dissociation being determined by the state of protonation of *multiple* functional groups within and/or adjacent to the active site. We emphasize that the participation of *at least two* ionizable groups in determining the rate of product dissociation is required to explain the observed fractional dependence of $k_{cat}$ on pH (limiting slope at high pH = 0.4). At present, we cannot assign the ionizable groups that influence the rate of product dissociation to specific amino acid residues in or adjacent to the active site.

If the observed values for $k_{cat}$ at pH <7 do, in fact, approach a linear dependence on hydroxide ion concentration (Figure 2), as shown in Figure 6 the kinetic model described in Scheme I can describe this behavior. The kinetic model predicts the observed transition from an internal step being rate-determining at pH <7.3 to an external step being rate-determining at pH >7.3 (Figure 5, line a). We note, however, that the p$K_a$ of 7.3 that can be obtained from the data in Figure 5, line a, is likely to be an apparent kinetic p$K_a$ that cannot be associated with any of the microscopic p$K_a$s in the model. Instead, this kinetic p$K_a$ results from the particular values of the rate constants and p$K_a$s used to define the model.

An important consequence of the kinetic model is that the rate for the internal (chemical) step must exceed that of the external (product dissociation) step when the latter is rate-determining (pH ≥7.3). In other words, since the rate of the external step increases in proportion to $[OH^-]^{0.4}$, the rate of the internal step must be increasing more rapidly with hydroxide ion concentration so that the external step can

remain rate-determining as the pH is increased. We conclude that the rate of the internal (chemical) step must continue to increase with increasing hydroxide ion concentration when the rate-determining step is the external (product dissociation) step.

This analysis and our conclusion that the rate-determining step for wild-type SNase at high pH is product dissociation rather than an internal (chemical) step require that the rate acceleration for the enzyme-catalyzed P–O bond cleavage reaction be greater than the factor of $10^{16}$ estimated previously (Serpersu et al., 1987), since this factor was based upon a value for $k_{cat}$ which we now recognize is associated with an external rather than an internal (chemical) step.

*Kinetic Behavior of E43D.* The experiments described in the previous section for wild-type SNase were repeated with E43D. In Figure 2, line b, we show the dependence of log $k_{cat}$ on pH for E43D. The slope of the linear plot = 1.01 ± 0.03, indicating that $k_{cat}$ is directly proportional to hydroxide ion concentration.

In Figure 3, line b, we display the dependence of log ($k_{cat}/K_m$) on pH for E43D. The slope of the linear plot = 0.95 ± 0.04, indicating that $k_{cat}/K_m$ is also directly proportional to hydroxide ion concentration. In contrast to the behavior of wild-type SNase (lines a in Figure 2 and 3), no breaks in the plots of $k_{cat}$ and $k_{cat}/K_m$ as a function of pH are observed (lines b in both Figures 2 and 3).

In Figure 5, line b, we display the slopes of plots of $k_{cat}^{o}/k_{cat}^{\eta}$ *vs* $\eta/\eta^{o}$ [$\Delta(k_{cat}^{o}/k_{cat}^{\eta})/\Delta(\eta/\eta^{o})$] determined for E43D from pH 7.5 to 9.5. The values for $k_{cat}$ are independent of solvent viscosity, demonstrating that an internal step is rate-determining. No dependence of $K_m$ on solvent microvsicosity was observed at any value of pH for E43D; i.e., $(k_{cat}/K_m)^{o}/(k_{cat}/K_m)^{\eta}$ is also independent of $\eta/\eta^{o}$ at all pH values examined. Thus, an internal step is also rate-determining for $k_{cat}/K_m$.

The observed dependence of log $k_{cat}$ on pH for E43D can be understood starting with the kinetic model proposed for wild-type SNase in Scheme I. At pH 7.5, the $k_{cat}$ for E43D is ~2000-fold ($10^{3.3}$) less than that of wild-type SNase. If p$K_{a3}$ is increased to ≥10 from the value of 8.7 shown in Scheme I (≥1.3 units) and log $k_2$ is decreased by ≤2.0 units (so that $\Delta pK_{a3} - \Delta \log k_2 = 3.3$, where Δ refers to E43D–wild type) and the remaining rate constants and p$K_a$s are unchanged, log $k_{cat}$ for E43D is predicted to be directly proportional to pH from pH 7.5 to 9.5. [Since the value for p$K_{a3}$ may, in fact, be ≥10 for wild-type SNase (as noted previously, the values for $k_2$ and p$K_{a3}$ cannot be uniquely determined from our data), the decreased $k_{cat}$ for E43D may be solely the result of a decrease in $k_2$. This uncertainty does not alter the conclusion that the kinetic model in Scheme I can be used to describe the behavior of E43D.] With these altered values for $k_2$ and/or p$K_{a3}$, the rate of the conversion of $EH_2S$ to $EH_2P$ does not exceed the rate of product dissociation that is determined by the values for $k_3$, $k_3'$, and $k_3''$, so $k_{cat}$ is predicted to be independent of solvent viscosity (Figure 5, line b). Structural explanations for decreases in $k_2$ and/or increases in p$K_{a3}$ are not possible, although structural analyses (Wilde et al., 1988; Loll & Lattman, 1990) revealed that the E43D substitution is structurally disruptive.

*Thus, for E43D an internal step is rate-determining, irrespective of pH.*

*Validity of Comparisons of the Kinetic Parameters for Wild-Type and Mutant Versions of SNase.* The data presented in Figures 2, 3, and 5, lines a and b, reveal that mechanistically informative comparisons of kinetic parameters determined for wild-type SNase and E43D and, presumably, other seriously catalytically impaired active-site mutants [R35K, R35G, R87K, and R87G, to name a few other mutants that have been studied in this laboratory (Hibler et al., 1987; Pourmotabbed et al., 1990), and others (Serpersu et al., 1987)] cannot be made over the pH range where the mutant proteins are sufficiently active to yield reliable kinetic data. For the mutant enzymes, the rate-determining step will be an internal step; for wild-type SNase, the rate-determining step is an external step. Even when the structural changes that are caused by the E43D (Wilde et al., 1988; Loll & Lattman, 1990), R35K, and R87K (Pourmotabbed et al., 1990) mutants are disregarded, a comparison of the kinetic properties of these enzymes with those of wild-type SNase is clearly an "apples and oranges" situation, i.e., mechanistically uninformative. At a minimum, an quantitative comparisons of $k_{cat}$ and $k_{cat}/K_m$ that are made *underestimate* the difference in the rates of the internal step.

*Evidence for the Importance of the Active-Site Ω-Loop in the Rate-Determining External Step for Wild-Type SNase: Kinetic Behavior of βVN ΔSNase.* Both the X-ray crystallographic (Loll & Lattman, 1989) and high-resolution NMR spectroscopic (Torchia et al., 1989) determinations of the structure of wild-type SNase revealed that the Ω-loop immediately adjacent to the active site (residues 43–52) is highly disordered. Neither the X-ray nor the NMR studies were able to define the conformation of this Ω-loop. Since this loop is expected to be cationic at even moderately alkaline pH by virtue of the presence of three lysine residues (Lys-45, Lys-48, and Lys-49), a logical hypothesis is that this loop may be directly involved in the substrate binding and product dissociation that are now known to be rate-determining for $k_{cat}/K_m$ and $k_{cat}$, respectively. Interaction of the Ω-loop with bound substrate may also be indirectly involved in determining the rate constant for hydrolysis of the phosphodiester bond.

Knowles and his co-workers recently described studies of an Ω-loop adjacent to the active site of triosephosphate isomerase that appears both to bind the enediol intermediate in a conformation that prevents its chemical decomposition as well as to be responsible for the approach of the active-site general basic catalyst Glu-165 to the bound substrate to initiate the tautomerization reaction catalyzed by the isomerase (Sampson & Knowles, 1992a,b). We have begun to investigate whether the Ω-loop adjacent to the active site of SNase may be involved in the external processes that are rate-determining at pH values greater than 7.3 and also in hydrolysis of the phosphodiester bond.

We recently described a deletion mutant of wild-type SNase in which six residues of the Ω-loop immediately adjacent to the active site (residues 44–49) have been deleted (Poole et al., 1991; Baldisseri et al., 1991). The result of this deletion was to transform the Ω-loop into a β-turn, where Gly-50 and Val-51 are located in the $i + 1$ and $i + 2$ positions of the new solvent-exposed β-turn. In this article, we designate this protein as βGV ΔSNase. Glu-43 is retained in βGV ΔSNase. We previously reported that, *at pH 9.5*, the $k_{cat}$ for βGV ΔSNase was reduced 40-fold from that observed for the intact wild-type SNase, as compared to an 80-fold reduction in $k_{cat}$ measured for E43D under identical conditions (Poole et al., 1991). While deletion of the active-site Ω-loop impaired catalysis, the impact was concluded to be less than that caused by the Glu to Asp substitution at residue 43.

In work that will be described in detail elsewhere (L. B. Poole, S. P. Hale, and J. A. Gerlt, in preparation), by random mutagenesis and subsequent metabolic selection we have identified sequence variants of the β-turn in βGV ΔSNase for which the reduction in $k_{cat}$ relative to wild-type SNase *at pH*

*9.5* is less than that observed for βGV ΔSNase. In one such mutant, Gly-50 and Val-51 are changed to Val and Asn, respectively; we designate this protein as βVN ΔSNase. By two-dimensional $^1H$–$^{15}N$ chemical shift correlation NMR spectroscopy, the chemical shifts of the N–H groups in the polypeptide backbone of βVN ΔSNase are unchanged from those we have previously reported for βGV ΔSNase (Baldisseri et al., 1991) with the exception of residues within and in close proximity to the β-turn containing residues 50 and 51 (L. B. Poole, S. P. Hale, and J. A. Gerlt, unpublished results). We have subjected βVN ΔSNase to the same kinetic characterization that we described for wild-type SNase and E43D in the preceding sections of this article so that we might begin to assess the effects of the residues in the Ω-loop on the dependence of both $k_{cat}$ and $k_{cat}/K_m$ on pH.

In Figure 2, line c, we show the dependence of log $k_{cat}$ on pH for βVN ΔSNase. The slope of this plot = 0.91 ± 0.02, indicating that $k_{cat}$ is directly proportional to hydroxide ion concentration. At the highest value of pH at which measurements can be reliably made without denaturation of both enzymes (pH 10), the $k_{cat}$ for βVN ΔSNase is ~3-fold less than that for wild-type SNase (as compared to ~200-fold less below pH 7.5). In fact, extrapolation of line a for wild-type SNase and of line c for βVN ΔSNase predicts an intersection point at approximately pH 10.6, if both wild-type SNase and βVN ΔSNase could be assayed at this pH without denaturation.

In Figure 3, line c, we display the dependence of log ($k_{cat}/K_m$) on pH for βVN ΔSNase. The slope of this plot = 0.78 ± 0.02, indicating that $k_{cat}/K_m$ is also directly proportional to hydroxide ion concentration.

In Figure 5, line c, we display the slopes of plots of $k_{cat}^{\circ}/k_{cat}^{\eta}$ *vs* $\eta/\eta^{\circ}$ determined for βVN ΔSNase determined as a function of pH from 7 to 10. The values for $k_{cat}$ are independent of solvent viscosity, demonstrating that an internal step is rate-determining. No dependence of $K_m$ on solvent microviscosity was observed at any value of pH; i.e., both $K_m^{\circ}/K_m^{\eta}$ and $(k_{cat}/K_m)^{\circ}/(k_{cat}/K_m)^{\eta}$ are independent of $\eta/\eta^{\circ}$. Therefore, an internal step is also rate-determining for $k_{cat}/K_m$.

At the highest pH values accessible for wild-type SNase and βVN ΔSNase without denaturation (pH 10), i.e., when the rates of the reaction are nearly the same, the rate-determining step in $k_{cat}$ for wild-type SNase is an external step while the rate-determining step in $k_{cat}$ for βVN ΔSNase is an internal step (Figure 5). Despite this difference in rate-determining steps, the $k_{cat}$ for βVN ΔSNase can approach that of wild-type SNase because $k_{cat}$ is directly proportional to hydroxide ion concentration for βVN ΔSNase rather than fractionally proportional to hydroxide ion concentration for wild-type SNase. Thus, the kinetic impairment induced by deletion of the Ω-loop in βVN ΔSNase (as evidenced by the 200-fold decreased activity at pHs ≤7.5 relative to that of wild-type SNase) can be almost completely overcome by increasing the pH, since the rate-determining steps for the reactions catalyzed by wild-type SNase and βVN ΔSNase and their dependencies on hydroxide ion concentration are different.

That the $k_{cat}$ for βVN ΔSNase shows no detectable evidence for viscosity dependence when the $k_{cat}$ is only ~3-fold less than that of wild-type SNase (pH 10; line c, Figure 5) suggests, but does not prove, that the residues of the Ω-loop and/or movement of the Ω-loop are involved in the rate-determining external step that characterizes wild-type SNase. If βVN ΔSNase contained the structural feature(s) that is (are) responsible for the slow external step in the $k_{cat}$ for wild-type SNase, the 3-fold increase in solvent viscosity used in our experiments should have slowed the rate of the external step such that it would approach or even equal that of the viscosity-independent, internal step that is rate-determining for βVN ΔSNase. If this were the case, the $k_{cat}$ for the reaction catalyzed by βVN ΔSNase should have been partially influenced by solvent viscosity rather than being independent of solvent viscosity as observed. If two reactions are equally rate-determining in a multistep reaction, i.e., their transition states have the same energy, the rate of the overall reaction is determined by both transition states.

In terms of the kinetic model in Scheme I, at pH values ≤7.5 the ~200-fold ($10^{2.3}$) decrease in $k_{cat}$ for βVN ΔSNase relative to that for wild-type SNase can be associated with a 2.3 log unit increase in $pK_{a3}$, a $10^{2.3}$-fold decrease in $k_2$, or a combination of both effects, i.e., $\Delta pK_{a3} - \Delta \log k_2 = 2.3$, where Δ refers to βVN ΔSNase – wild type. (Again, since the value for $pK_{a3}$ may, in fact, be ≥10 for wild-type SNase, the decreased $k_{cat}$ for βVN ΔSNase may be solely the result of a decrease in $k_2$; this uncertainty does not alter the conclusion that the kinetic model in Scheme I can be used to describe the behavior of βVN ΔSNase.) A structural explanation for these changes in kinetic parameters for the internal step is not yet available, but that these changes occur suggests that the Ω-loop may be indirectly involved in the hydrolysis of the phosphodiester bond. These changes in the kinetic parameters are in addition to the presumed increase in the rate at which product dissociates from the active site of βVN ΔSNase. For example, if the lysine residues present in the Ω-loop are responsible for the rate-determining release of product from the active site of wild-type SNase (i.e., $k_3$, $k_3'$, and $k_3''$), we predict that their absence in βVN ΔSNase should significantly increase the rate of product dissociation, thereby allowing the internal step to be cleanly rate-determining, even at high pH (Figure 5, line c).

We recognize that the viscosity independence of the $k_{cat}$ for βVN ΔSNase does not constitute rigorous proof that residues in the Ω-loop are responsible for the external step that is rate-determining in $k_{cat}$ for wild-type SNase. However, we are pursuing this hypothesis with site-directed substitutions for selected residues in the Ω-loop. For example, mutation of the three Lys residues in the Ω-loop (Lys-45, -48, and -49) to eliminate their cationic charge, either individually or in combination, may provide evidence in support of the involvement of residues in the Ω-loop by eliminating the viscosity dependence of the reaction.

*On the Importance of General Base Catalysis in the Reaction Catalyzed by Wild-Type SNase.* While the "working hypothesis" for SNase has long been that Glu-43 participates in catalysis as a general base to assist the attack of water on the charge-neutralized phosphodiester, the data presented in Figures 2 and 3 *do not* support this hypothesis. If Glu-43 were participating in catalysis as a general base, the dependence of log $k_{cat}$ on pH should be described by an ascending limb that reaches a limiting, pH-independent plateau value above the $pK_a$ of Glu-43. Although the $pK_a$ of Glu-43 has not yet been measured, the close proximity of Glu-43 to the $Ca^{2+}$ ion in the highly polar active site might be expected to *decrease*, not increase, the $pK_a$ of Glu-43 from its unperturbed value ($pK_a$ = 4.3). That the rate of the internal step for wild-type SNase must continue to increase with pH above pH 7.5 (with a $pK_a \geq 8.7$ as concluded from the kinetic model in Scheme I and the successful fit of this model to the observed dependence of $k_{cat}$ on pH in Figure 6) suggests, but does not prove, that general basic catalysis by Glu-43 is unimportant.

In support of this interpretation is the observation that the $k_{cat}$ for βVN ΔSNase approaches that of wild-type SNase at pH 10. Since βVN ΔSNase retains Glu-43 and the $k_{cat}$ for βVN ΔSNase is directly proportional to hydroxide ion concentration even at pH 10, it is unlikely that Glu-43 can be functioning as a general basic catalyst in this protein. While, in principle, it may be possible that another functional group in the active site of βVN ΔSNase may act as a general basic catalyst in place of Glu-43 (e.g., Arg-35 or Arg-87 since the $pK_a$s of these residues presumably exceed 10), that both wild-type SNase and βVN ΔSNase are comparably efficient catalysts makes this complication unlikely.

If Glu-43 is not a general basic catalyst, one potential solution to the problem of how a water molecule is activated for nucleophilic attack on the substrate is that the $Ca^{2+}$ ion may lower the $pK_a$ of a water molecule in its inner coordination sphere, thereby increasing the effective concentration of hydroxide ion in the vicinity of the charge-neutralized phosphodiester substrate. The $Ca^{2+}$ ion then can be viewed as being equivalent to a general basic catalyst since it would facilitate the generation of hydroxide ion at pHs below the $pK_a$ of water. By this mechanism, the increase in the rate of the internal step with increasing pH may simply reflect titration of this $Ca^{2+}$-coordinated water molecule. In the case of hydroxide ion coordinated to $Co^{3+}$, the nucleophilicity of a metal-coordinated hydroxide ion is diminished only 100-fold (Jones et al., 1983); this suggests that a decrease in the $pK_a$ of water bound to $Ca^{2+}$ would be catalytically beneficial. An analogous role for an essential divalent metal ion in generating hydroxide ion in an active site in the absence of an amino acid general basic catalyst was recently proposed for the 3′ to 5′ exonuclease activity of DNA polymerase I (Beese & Steitz, 1991). This function of the $Ca^{2+}$ ion in the active site of SNase would be in addition to its presumed role in neutralizing the negative charge of the anionic phosphodiester substrate; the guanidinium groups of both Arg-35 and Arg-87 may also participate in this charge neutralization.

This mechanism does not specify a role for Glu-43 in catalysis. In the present absence of structural information for an SNase–inhibitor complex that allows both the geometry of the active site and the involvement of the Ω-loop in catalysis to be defined without significant structural disorder, the most persuasive evidence that Glu-43 is involved in catalysis is the large decrease in $k_{cat}$ that accompanies any of several substitutions (Hibler et al., 1987). Perhaps Glu-43 is involved in the attainment and maintenance of the proper active-site structure for catalysis [recall that the E43D substitution disrupts both the active-site structure and also the global conformation of the protein (Wilde et al., 1988; Loll & Lattman, 1990)]. For example, Glu-43 may be involved in controlling or facilitating movement of the active-site Ω-loop by hydrogen-bonding interactions with backbone peptide N–H bonds, thereby stabilizing a closed conformation that allows catalysis to proceed, or perhaps the γ-carboxylate of Glu-43 participates in catalysis by shuttling a proton from the water coordinated to the $Ca^{2+}$ ion to bulk solvent. These issues can be addressed only by additional structural and mechanistic studies.

*Conclusions.* A study of the dependence of the kinetic parameters ($k_{cat}$ and $k_{cat}/K_m$) for the reaction catalyzed by SNase on both pH and solvent viscosity has provided new and important information about the nature of the rate-determining step. While our results emphasize potential problems associated with using differences in the kinetic properties of wild-type and mutant enzymes to deduce the chemical mechanism of the reaction, i.e., the rate-determining steps need not be the same, they also reveal potential pitfalls in interpretations of the kinetic parameters for reactions catalyzed by wild-type enzymes. For those enzymes that utilize nucleic acids as substrates, e.g., DNases, RNases, and polymerases, the interpretation of kinetic parameters is likely to be particularly susceptible to the difficulties uncovered by our studies; i.e., substrate binding and/or product dissociation steps rather than bond-making and/or -breaking steps may be rate-limiting.

## REFERENCES


Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5631.

Baldisseri, D. M., Torchia, D. A., Poole, L. B., & Gerlt, J. A. (1991) *Biochemistry 30*, 3628.

Beese, L. S., & Steitz, T. A. (1991) *EMBO J. 10*, 25.

Blacklow, S. C., Raines, R. T., Lim, W. A., Zamore, P. D., & Knowles, J. R. (1988) *Biochemistry 27*, 1158.

Brahms, J., Michelson, A. M., & Van Holde, K. E. (1966) *J. Mol. Biol. 15*, 467.

Brouwer, A. C., & Kirsch, J. F. (1982) *Biochemistry 21*, 1302.

Caldwell, S. R., Newcomb, J. R., Schlecht, K. A., & Raushel, F. M. (1991) *Biochemistry 30*, 7438.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.

Cotton, F. A., Hazen, E. E., & Legg, M. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2551.

Craik, C. S., Roczniak, S., Largman, C., & Rutter, W. J. (1987) *Science 237*, 909.

Cuatrecasas, P., Fuchs, S., & Anfinsen, C. B. (1967) *J. Biol. Chem. 242*, 1541.

Cuatrecasas, P., Wilchek, M., & Anfinsen, C. B. (1969) *Biochemistry 8*, 2277.

Hibler, D. W., Stolowich, N. J., Reynolds, M. A., Gerlt, J. A., Wilde, J. A., & Bolton, P. H. (1987) *Biochemistry 26*, 6278.

Jones, D. R., Lindoy, L. F., & Sargeson, A. M. (1983) *J. Am. Chem. Soc. 105*, 7327.

Loll, P., & Lattman, E. E. (1989) *Proteins: Struct., Funct., Genet. 5*, 183.

Loll, P., & Lattman, E. E. (1990) *Biochemistry 29*, 6866.

Mehdi, S., & Gerlt, J. A. (1982) *J. Am. Chem. Soc. 104*, 3223.

Poole, L. B., Loveys, D. A., Hale, S. P., Gerlt, J. A., Stanczyk, S. M., & Bolton, P. H. (1991) *Biochemistry 30*, 3621.

Pourmotabbed, T., Dell'Acqua, M., Gerlt, J. A., Stanczyk, S. M., & Bolton, P. H. (1990) *Biochemistry 29*, 3677.

Sampson, N. S., & Knowles, J. R. (1992a) *Biochemistry 31*, 8482.

Sampson, N. S., & Knowles, J. R. (1992b) *Biochemistry 31*, 8488.

Serpersu, E. H., Shortle, D., & Mildvan, A. S. (1986) *Biochemistry 25*, 68.

Serpersu, E. H., Shortle, D., & Mildvan, A. S. (1987) *Biochemistry 26*, 1289.

Serpersu, E. H., McCracken, J., Peisach, J., & Mildvan, A. S. (1988) *Biochemistry 27*, 8034.

Serpersu, E. H., Hibler, D. W., Gerlt, J. A., & Mildvan, A. S. (1989) *Biochemistry 28*, 1539.

Sprang, S., Standing, T., Fletterick, R. J., Stroud, R. M., Finer-Moore, J., Xuong, N.-H., Hamlin, R., Rutter, W. J., & Craik, C. S. (1987) *Science 237*, 905.

Torchia, D. A., Sparks, S. W., & Bax, A. (1989) *Biochemistry 28*, 5509.

Wang, J., LeMaster, D. M., & Markley, J. M. (1990a) *Biochemistry 29*, 88.

Wang, J., Hinck, A. P., Loh, S. N., & Markley, J. M. (1990b) *Biochemistry 29*, 102.

Wilde, J. A., Bolton, P. H., Dell'Acqua, M., Hibler, D. W., Pourmotabbed, T., & Gerlt, J. A. *Biochemistry 27*, 4127.