Comparison of Conformational Features of Staphylococcal Nuclease in Ternary Complexes with pdTp, pdGp, and Nitrophenyl-pdTp†

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Received January 21, 1992

ABSTRACT: The conformations of wild-type staphylococcal nuclease (SNase) in the ternary complexes with thymidine 3′,5′-bisphosphate (pdTp), 2′-deoxyguanine 3′,5′-bisphosphate (pdGp), and thymidine 3′-phosphate 5′-(*p*-nitrophenylphosphate) (NpdTp) with Ca^{2+} were examined by two-dimensional NMR NOESY and ROESY experiments. The results of these experiments indicate that the conformational features of the SNase are quite similar in the three ternary complexes. This suggests that the conformational features of SNase, in these ternary complexes, are not strongly dependent on whether the 5′-phosphate is a mono- or diester. This is in contrast to our prior studies on substitutions of active site charged amino acids which indicated that the conformational features of SNase in the ternary complex are quite sensitive to substitutions for active site charged amino acids (Hibler et al., 1987; Wilde et al., 1988; Pourmotabbed et al., 1990). The similarity of the SNase conformational features in the ternary complexes with pdTp and pdGp indicates that the features of the nucleotide bound at the active site are not strong determinants of the enzyme conformation in the ternary complexes. These conclusions are in general agreement with the results on pdApdT ternary complexes with SNase which suggested that it is the conformational features of the bound nucleic acid which determine the differences in catalysis observed for SNase with different substrates (Weber et al., 1991), more so than the conformational features of the enzyme.

The accepted mechanism for the hydrolysis of DNA catalyzed by staphylococcal nuclease (SNase) is largely based upon the high-resolution X-ray crystallographic studies performed first at 1.5 Å by Cotton (Cotton et al., 1979) and his coworkers and more recently at 1.65 Å by Loll and Lattman (1989). These structural studies were performed with both the catalytically essential Ca^{2+} and the competitive inhibitor thymidine 3′,5′-bisphosphate (pdTp) bound at the active site. The observed positions of the Ca^{2+} and pdTp in this experimentally accessible nonproductive complex have been assumed to be representative of those of Ca^{2+} and substrate in the catalytically productive complex. The 5′-phosphate group of the competitive inhibitor is in close proximity to the Ca^{2+} ion and the guanidinium groups of both Arg 35 and Arg 87, suggesting that neutralization of the anionic charge of a phosphodiester substrate contributes significantly to the observed 10^{16} rate enhancement relative to the uncatalyzed reaction. In addition, the γ-carboxylate group of Glu 43 is hydrogen bonded to two water molecules, one in the inner coordination sphere of the Ca^{2+} and the second also hydrogen bonded to the 5′-phosphate group of the pdTp. This geometry suggests that Glu 43 functions as a general basic catalyst to assist the attack of water on the charge electrophilic phosphate group of a substrate bond in the active site (Hibler et al., 1987).

We previously have used this structural information as the basis for site-directed mutagenesis experiments investigating the roles of the putative general basic catalyst Glu 43 (Hibler et al., 1987; Wilde et al., 1988) and the electrophilic catalysts Arg 35 and Arg 87 (Pourmotabbed et al. 1990) in the hydrolysis of DNA. In all three cases, we observed that "conservative" substitution of these residues with Asp and Arg residues, respectively, significantly decreased both k_{cat} and k_{cat}/K_m. Various criteria, including reversible thermal denaturation and ^1H NMR spectroscopy, have shown that these substitutions are accompanied by conformational changes that are propagated into the hydrophobic core of the protein (Hibler et al., 1987; Wilde et al., 1988; Pourmotabbed et al., 1990). These and other results led us to hypothesize that these conformational changes resulted, at least in part, from alterations in the positions of a charged group buried in an active site which binds the anionic polyanionic substrate (DNA) by virtue of interactions with a large number of cationic residues (Hibler et al., 1987; Wilde et al., 1988; Pourmotabbed et al., 1990). The crystal structures of the ternary complexes of wild-type and E43D proteins with Ca^{2+} and pdTp have been found to be different, which supports the basic conclusions of the NMR and other studies (Loll & Lattman, 1990). By way of contrast, more recent investigations have indicated that the conversion of an Ω loop at the active site into a β turn has only small structural consequences (Baldesseri et al., 1991; Poole et al., 1991).

Since the substitution of charged amino acids at the active site, as well as the sizes of the amino acids at the active site, can have structural consequences (Hibler et al., 1987; Wilde et al., 1988; Pourmotabbed et al., 1990; Serpersu et al., 1986, 1987, 1988, 1989; Loll & Lattman, 1990), we began to reconsider whether the ternary complex of the active site inhibitor pdTp with SNase and calcium is a good model of the productive complex of SNase. The natural substrates of SNase are DNA and RNA, both of which have phosphate diesters at the 3′ and 5′ positions (Anfinsen et al., 1971; Cuatrecasas et al., 1967a–c; Mikulski et al., 1969). The hydrolysis reaction occurs at the 5′ position, and the charge at this position could have significant effects on the structure of the enzyme complex since the substitution of either of the arginines at positions 35 and 87, which neutralize the 5′ charge, has both structural and kinetic consequences. In particular, the structure of a ternary

† This research was supported, in part, by Grant DMB-9105003 from the National Science Foundation.

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0006-2960/92/0431-6396$03.00/0

complex in which the 5′ position is a diester has a formal −1 charge could be different from the structure of a ternary complex in which the 5′ position is a monoester and the 5′ position has a formal −2 charge. If a significant structural difference is found between ternary complexes containing 5′ diesters and those containing 5′ monoesters, then the relevance of the structural information obtained on the SNase–calcium–pdTp complex to that of the productive complex and hence the enzymatic reaction might be reduced. It is noted that SNase can hydrolyze a wide range of 5′ diesters.

SNase has a rather weak sequence specificity and will hydrolyze the 5′ diester of dA, dG, dC, and dT residues (Anfinsen et al., 1971; Cuatrecasas et al., 1967a–c; Mikulski et al., 1969). Therefore, it was also of interest to investigate whether the structure of the ternary complex depends on which nucleotide base is present in the complex. Structural differences which arise from changing the base in the ternary complex cannot have profound catalytic consequences since the enzyme has essentially the same activity with all sequences. The impact of the net charge at the 5′ position and the nucleotide base in the ternary complex on the conformation of SNase can be monitored by the NMR-based methods we have previously used to monitor the structural effects of amino acid substitutions.

To examine the effects of the charge at the 5′ position on the conformation of the ternary complex, we have utilized the 5′ diester of pdTp with the 5′ position substituted with *p*-nitrophenyl (NpdTp): thymidine 3′-phosphate 5′-(*p*-nitrophenylphosphate). One comparison made in this study is between the NMR properties of the pdTp–SNase–Ca^{2+} complex and that of the corresponding NpdTp ternary complex.

To examine the effect of the nature of the base on the structure of the complex, we have also obtained and compared the NMR results obtained on the pdTp ternary complex with those obtained on the 2′-deoxyguanine 3′,5′-bisphosphate (pdGp) ternary complex. Not only does the dG residue differ from the dT residue in the obvious properties of size and chemical composition, but the dG residue has significantly greater ring current shifts than does dT. This implies that protons on amino acids near the dG in the ternary complex might experience different chemical shifts in the pdGp complex than in the pdTp complex even if the structures are rather similar.

Materials and Methods

The recombinant plasmid pNJS was used to prepare N-terminal modified SNase in which the heptapeptide Met-Asp-Pro-Thr-Val-Tyr-Ser is appended to the N-terminal alanine of the 149 amino acid nuclease A using the methods previously described (Hibler et al., 1987; Wilde et al., 1988; Pourmotabbed et al., 1990). The protein samples were prepared in the laboratory of John A. Gerlt at the University of Maryland. The protein samples were prepared for the NMR studies by heating the proteins to 45 °C in ^2H_2O for 15 min to exchange the amide protons. The proteins were subsequently lyophilized and dialyzed against the NMR buffer which consisted of 0.1 M NaCl, 50 mM borate buffer, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). After dialysis, the samples were lyophilized and dissolved in ^2H_2O. The pH values of the NMR samples were adjusted with ^2HCl and NaO^2H, and the pH of the sample was determined in the NMR tube. The NMR samples were at 2 mM concentration in protein. The samples of pdTp and pdGp were obtained from Pharmacia, and the NpdTp was from Ash Stevens.

The rate of hydrolysis of NpdTp was monitored under the NMR conditions by ^{31}P NMR. The hydrolysis of the NpdTp changes the 5′-phosphate from a diester to a monoester, and the rate of hydrolysis was determined from the rate of disappearance of the diester resonance. Each sample of NpdTp–Ca^{2+}–SNase was examined by ^{31}P NMR after each two-dimensional NMR experiment to determine the extent of hydrolysis which occurred during the NMR experiment. All NMR spectra were obtained using a Varian XL-400 spectrometer. The NOESY and ROESY spectra were obtained as described previously (Hibler et al., 1987; Withka et al., 1991). The NOESY experiments were performed with a 150-ms mixing time, and the ROESY experiments were performed with a 100-ms mixing time.

Results and Discussion

Experimental Conditions for a Stable NpdTp–Ca^{2+}–SNase Ternary Complex. To be able to obtain two-dimensional NMR data on the ternary complex containing NpdTp, it was necessary to define experimental conditions under which the NpdTp formed the ternary complex without being hydrolyzed. Studies by Anfinsen and co-workers have shown that NpdTp is a substrate for SNase with a pH optimum at around 10 (Anfinsen et al., 1971; Cuatrecasas et al., 1967a–c). They also showed that the rate of hydrolysis of NpdTp by SNase is dependent on the pH but that the binding constant is not a strong function of pH. Extrapolation of the published results suggested that near pH 7 the NpdTp would be hydrolyzed slowly and that the ternary complex would be formed which is what is needed for NMR studies to be carried out on the complex. Since the rate of hydrolysis is so strongly pH dependent, this suggests that some functionality of the NpdTp–Ca^{2+}–SNase complex is being protonated as the pH is lowered, which has a profound effect on the catalytic power.

The rate of hydrolysis of the NpdTp ternary complex as a function of pH was performed on samples under the NMR conditions. The rate of hydrolysis was monitored by ^{31}P NMR with the rate of disappearance of the diester phosphate signal being taken to equal the rate of hydrolysis. It was found that at pH 7 the rate of hydrolysis at 30 °C is on the time scale of many days, and hence the NpdTp–calcium–SNase complex could be studied by NMR at pH 7. On the basis of our investigations of the rate of hydrolysis as a function of pH, it was found that pH 7 was the highest pH at which the rate of hydrolysis was sufficiently slow to allow the NMR experiments to be performed. The rate of hydrolysis was not found to be a strong function of temperature, and the NMR experiments were performed at 30 °C, which is the temperature used in our previous NMR studies of SNase. For comparison purposes, all of the NMR spectra reported here were obtained on samples prepared at pH 7.0 whereas our previous studies have all been carried out on samples at pH 7.8 (Hibler et al., 1987; Wilde et al., 1988; Pourmotabbed et al., 1990). The NMR results on the pdTp–Ca^{2+}–SNase ternary complex at pH 7.0 and pH 7.8 are quite similar with a number of small changes in chemical shifts and NOESY cross-peak intensities. Attempts to find a cryosolvent system to allow the NpdTp–Ca^{2+}–SNase complex to be studied at higher pHs were unsuccessful primarily due to the low solubility of the ternary complex in the cryosolvents examined at subzero temperatures.

Identification of Chemical Exchange and Magnetization Transfer Cross-Peaks. NOESY spectra can contain cross-peaks which arise from magnetization exchange, from chemical exchange, or from zero quantum coherence. Since in this investigation we are interested in the possible conformational differences between the different ternary complexes, as mon-

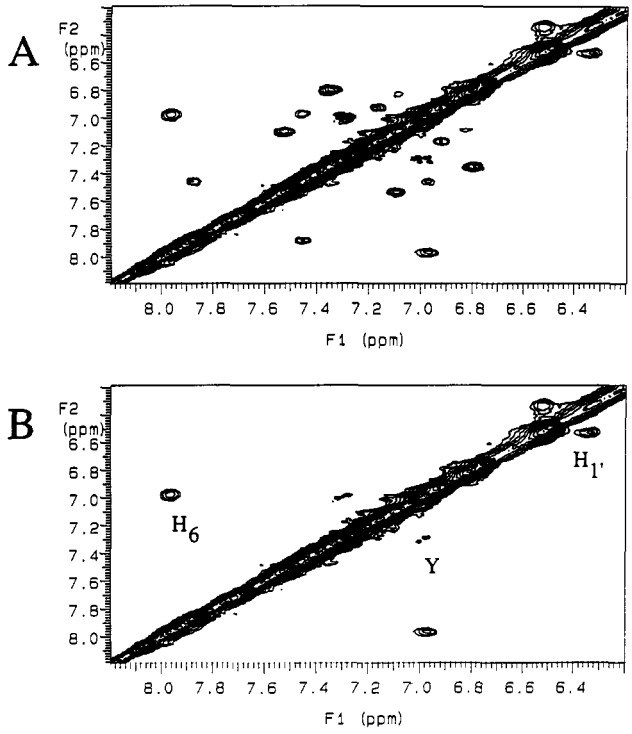

FIGURE 1: Aromatic–aromatic region of the two-dimensional ROESY spectrum of the pdTp–SNase–Ca^{2+} ternary complex. In panel A, all of the contours are plotted, and in panel B, only the positive contours are plotted. The chemical exchange cross-peaks of H6 and H1′ of the pdTp are indicated in panel B as are the zero quantum signals of the tyrosine (Y) in the heptapeptide tail of the SNase.

itored by differences in NOESY spectra, it is important to distinguish between cross-peaks on the basis of the physical process by which they arise. The results of ROESY experiments can be used to distinguish between magnetization transfer and chemical exchange cross-peaks since for a complex of the size of the SNase ternary complex the two types of cross-peaks have different algebraic signs (Ernst et al., 1987; Wüthrich, 1986). The magnetization transfer cross-peaks, analogous to NOESY cross-peaks, are of negative algebraic sign whereas the chemical exchange cross-peaks are positive and zero quantum cross-peaks contain both positive and negative cross-peaks.

ROESY data has been obtained on the pdTp, NpdTp, and pdGp ternary complexes with SNase. Typical ROESY data obtained with a 100-ms mixing time on the ternary complex of SNase–Ca^{2+}–pdTp are shown in Figure 1. The spectrum in panel A contains all of the cross-peaks, and the spectrum in panel B contains only the positive cross-peaks. The results show that there is a cross-peak at 6.9, 7.85 ppm which arises from the exchange of the H6 of the thymidine in the free and bound states, which is labeled H6 in panel B of Figure 1. There is also a cross-peak associated with the exchange of the H1′ of the thymidine between the free and bound states, which is labeled H1′ in panel B of Figure 1. The other positive signals which are observed at 6.7, 7.2 ppm are part of the zero quantum signals from the tyrosine in the heptapeptide tail, Y-2, with the cross-peak labeled Y in panel B of Figure 1. The chemical exchange between free and bound pdTp has been previously noted (Torchia et al., 1989; Wang et al., 1990).

Since the nucleotides in the NpdTp and pdGp ternary complexes are in fast exchange, these samples do not have signals corresponding to those observed for the H6 and H1′ arising from the chemical exchange of pdTp between the free and bound states. Thus, the chemical exchange cross-peak of the thymidine H6 will not be included in the comparison of the results on the complexes. The zero quantum cross-peaks for Y-2 were also observed for the NpdTp and pdGp complexes (data not shown).

Comparison of Aromatic–Aromatic NOEs of the Ternary Complexes. The NOESY cross-peaks in the aromatic region of the pdTp–Ca^{2+}–SNase ternary complex have been assigned and are primarily due to intraresidue NOEs and the chemical exchange cross-peaks noted above. Therefore, the comparison of the NOESY cross-peaks in this region will primarily report on the changes in the chemical shifts of the protons giving rise to the NOEs rather than on the conformation of the complex. It is noted that the NOESY cross-peaks in the aromatic–aromatic region were not sensitive probes of the conformational changes induced by amino acid substitutions at the active site.

The NOESY spectra containing the aromatic–aromatic region of the pdTp, NpdTp, and pdGp ternary complexes are shown in panels A, B, and C of Figure 2 respectively. A comparison of the results for the pdTp and NpdTp ternary complexes shows that the only significant differences are the presence of the H6 chemical exchange peak only in the pdTp ternary complex spectrum and in the diagonal signals of the two spectra. The differences in the diagonal signals correspond to the chemical shifts of the pdTp and NpdTp aromatic resonances. The NOESY cross-peaks which arise from the SNase are nearly identical in the two ternary complexes. This result suggests that there is not a profound structural difference between the pdTp and NpdTp ternary complexes.

The comparison of the NOESY spectra containing the aromatic–aromatic region of the pdTp and pdGp ternary complexes indicates a similar result. That is, most of the cross-peaks associated with the SNase have the same chemical shifts and intensities in both ternary complexes with the exception of the cross-peaks associated with Y85, Y113, and Y115. The assignments for the pdTp ternary complex (Torchia et al., 1989; Wang et al., 1990) were used to make these assignments. The chemical shifts, in parts per million, of the [Hϵ, Hδ] cross-peaks in the pdTp ternary complex are [6.71, 7.27] for Y115, [6.84, 7.07] for Y113, and [7.07, 7.11] for Y85, and in the pdGp ternary complex they are [6.80, 7.26] for Y115, [6.84, 7.14] for Y113 with the shifts in parts per million, with the chemical shifts for the Hϵ and Hδ sites of Y85 being nearly degenerate at 7.09 ppm. These relatively small chemical shift changes, the largest is for the Hϵ site of Y115 which is just under 0.1 ppm, are consistent with the expected ring current shift differences for these tyrosine residues in the pdGp and pdTp ternary complexes. These three tyrosines are thought to make up part of the nucleotide binding pocket on the basis of the crystal structure of SNase and hence are in close enough physical proximity for the observed chemical shift changes between the pdGp and pdTp ternary complexes to be attributed to the ring current changes rather than a structural change.

Comparison of Aromatic–Upfield Shifted Methyl NOEs of the Ternary Complexes. In our previous examinations of the effects of amino acid substitutions on the structure of SNase it was found that the NOESY cross-peaks between aromatic protons and those of the upfield shifted methyls could be a sensitive probe of structural changes (Hibler et al., 1987; Pourmotabbed et al., 1990; Wilde et al., 1988). The intensities of some of the cross-peaks in this region were found to have significantly different intensities for wild type and mutant proteins in ternary complexes with pdTp and Ca^{2+}. The changes in the intensities were taken to indicate structural changes, and the assignments of the cross-peaks indicated that the structural changes occur at a considerable distance from

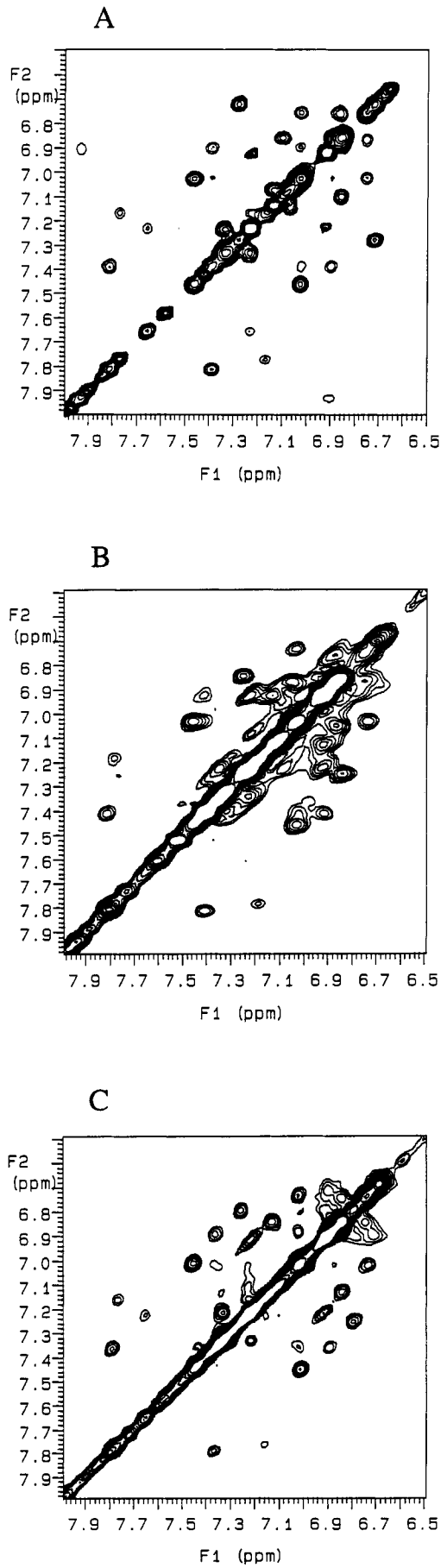

FIGURE 2: Aromatic–aromatic region of the two-dimensional NOESY spectrum of the pdTp–SNase–Ca^{2+} ternary complex (panel A), the spectrum of the NpdTp–SNase–Ca^{2+} ternary complex (panel B), and the spectrum of the pdGp–SNase–Ca^{2+} ternary complex (panel C).

the site of amino acid substitution. The aromatic–upfield shifted methyl regions of the NOESY spectra of the pdTp, NpdTp, and pdGp ternary complexes were examined to determine if structural changes analogous to those induced by amino acid substitution were also induced by changes in the nucleotide bound at the active site of SNase.

The spectral region of interest contains the NOE cross-peaks of many aromatic protons to many upfield shifted methyls and other protons as the spectra in Figure 3 indicate. The comparison of the spectra in this region for pdTp, in panel A of Figure 3, and NpdTp, in panel B of Figure 3, shows that the results obtained on the two ternary complexes are nearly identical. The changes observed between the results for these two ternary complexes are much less than the changes observed in comparing the results of the pdTp–Ca^{2+}–wild-type SNase ternary complex with the corresponding ternary complexes of E43D, E43S, R87K, or R35K. This implies that the structures of the pdTp and NpdTp ternary complexes are more similar, as monitored by either the aromatic–aromatic NOEs or the aromatic–upfield shifted methyl NOEs, than the wild-type pdTp ternary complex is to the ternary complexes of the four mutants listed above.

The comparison of the results for the pdTp and pdGp ternary complexes offers a quite similar conclusion on the basis of the comparison of the results in Figure 3. The aromatic–upfield shifted methyl NOESY cross-peaks are nearly identical in the two cases. There are some small chemical shift differences between the two results which are due to the changes in the chemical shifts of the tyrosine protons discussed above.

The intensities of the NOE cross-peaks of the upfield shifted L25, V74, and other methyls (Wilde et al., 1988; Pourmotabbed et al. 1990) were also compared in detail. The three ternary complexes investigated here exhibit NOESY cross-peaks of essentially the same intensity for all of the cross-peaks examined (data not shown), which is consistent with the contour plot representation of the results presented in Figure 3. Thus, the differences in NOE cross-peak intensities between the pdTp ternary complex and the pdGp and NpdTp ternary complexes are smaller than the differences between the pdTp ternary complexes of the wild-type SNase and those of E43D, E43S, R35K, or R87K.

Conclusions. The crystal structure of the pdTp–Ca^{2+}–SNase ternary complex indicates that the 5′-phosphate group of pdTp interacts with the solvent *inaccessible* cations Arg 35, Arg 87, and Ca^{2+} and that the 3′-phosphate group of pdTp interacts with the solvent *accessible* ε-ammonium group of Lys 84 and the phenolic hydroxyl group of Tyr 85. Previous investigations have examined the effects of amino acid substitutions for Arg 35, Arg 87 and Tyr 85. Grissom and Markley have used both kinetic and ^{13}C NMR methods to study the hydrogen bonding interaction between the phenolic hydroxyl group of Tyr 85 and substrates and/or inhibitors with 3′-phosphate groups (Grissom & Markley, 1989), and we have previously examined the effects of amino acid substitutions for Arg 35 and Arg 87.

The studies on the differences between wild-type SNase and the mutant in which Tyr 85 was replaced with Phe (Y85F) have indicated that with a mononucleotide substrate containing a dianionic 3′-phosphate group the apparent pK_a for wild-type SNase of 9.67 for k_{cat}/K_m is increased slightly to 10.1 for Y85F. With a mononucleotide substrate containing a monoanionic 3′-methylphosphonate group, an apparent pK_a of 10.1 for both wild-type SNase and Y85F is observed. On the basis of this data, it has been concluded that the interaction of a terminal 3′-phosphate group (for exonucleolytic cleavage)

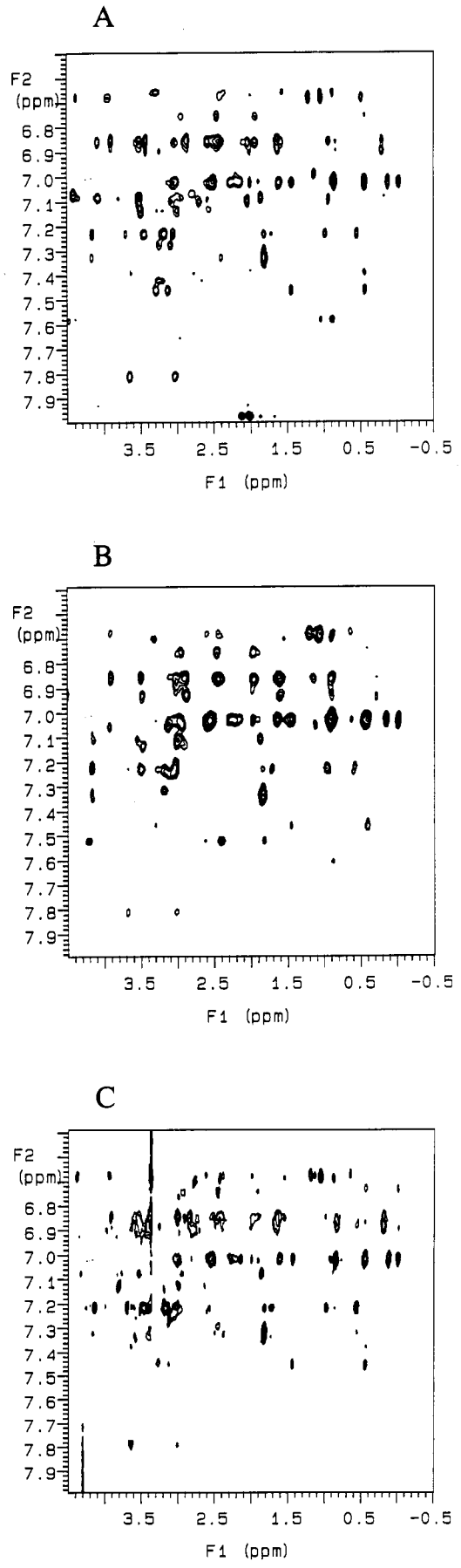

FIGURE 3: Aromatic–upfield shifted methyl region of the two-dimensional NOESY spectrum of the pdTp–SNase–Ca^{2+} ternary complex (panel A), the spectrum of the NpdTp–SNase–Ca^{2+} ternary complex (panel B), and the spectrum of the pdGp–SNase–Ca^{2+} ternary complex (panel C).

differed slightly from the interaction with an internal phosphodiester linkage (for endonucleolytic cleavage). The apparent pK_a of 9.67 could be uniquely assigned to the ionization of Tyr 85 but the residue associated with the apparent pK_a of 10.1 was not specified. The comparison of the NMR properties of wild type and Y85F ternary complexes with pdTp have indicated that the structures are very similar. Therefore, it appears that both amino acid substitution of the Tyr 85 involved in interacting with the 3′-phosphate and alteration in the 3′-phosphate from a monoester to a diester has relatively minor effects on the activity and structure of SNase.

The substitution of lysine for either of the arginines thought to be interacting with the 5′-phosphate leads to significant lowering of the catalytic power, by more than 4 orders of magnitude, as well as to structural disruption. R87K has NMR properties more different from those of wild-type SNase than any of the other mutants we have reported on, and the binding of pdTp to R87K is very weak. The results reported here indicate that alterations in the nucleotide portion of the ternary complex of wild-type SNase do not lead to structural changes of the magnitude induced by substitutions of Arg 35 or Arg 87. This suggests that the same general conformation of wild-type SNase can bind a range of inhibitors and substrates and hence that the structural information obtained on the ternary complex containing the inhibitor pdTp may indeed be a useful guide to the properties of a productive complex.

While this study was in progress, a report appeared on the NMR-based investigation of the conformation of dTpdA in ternary complexes with SNase using various metal ions (Weber et al., 1991). The results indicated that the dinucleotide binds to SNase in an extended conformation and did not directly examine the conformation of the protein portion of the ternary complexes. One conclusion of the study is that the SNase-induced conformational changes at the phosphorus and at the 3′ leaving group are important in catalysis. The studies reported here on the conformation of SNase in the presence of the three different nucleotides is consistent with this notion that the conformational features of the substrate in the ternary complex are important determinants of the catalytic reaction whereas the conformation of the wild-type enzyme in the Ca^{2+} ternary complexes is generally the same for the different complexes.

The ability to characterize the details of the ternary complexes in solution at the resolution needed to explain catalysis, a resolution on the order of 0.1 nm or less, will require a comprehensive analysis of the dynamical and distance information present in proton and heteronuclear NMR data. We are now beginning to develop the methodology required for this level of analysis on small proteins, following the general approach we have recently demonstrated for duplex DNA (Withka et al., 1992), with the expectation of applying the methods to wild-type and mutant forms of SNase in the future.

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