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International Journal of Mass Spectrometry 222 (2003) 397–412

International Journal of Mass Spectrometry

www.elsevier.com/locate/ijms

A mass spectrometric study of noncovalent gas phase interaction of staphylococcal nucleases with 5′-nucleoside phosphate inhibitors

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Received 10 May 2002; accepted 28 August 2002

Abstract

A series of electrospray ionization (ESI) mass spectral studies of noncovalent gas phase complexes of staphylococcal nucleases (SNases) and tailored SNase mutants with 5′-nucleoside phosphate inhibitors are reported. Both quadrupole ion trap (IT) and external source Fourier transform mass spectrometers (FTMS) were used to obtain low and high resolution results, respectively. It is concluded that competitive binding comparisons are possible with FTMS, but not quadrupole IT mass analysis, due to the need for the high resolving power provided by the FTMS mass analyzer. It is found that SNase–inhibitor complexes can be observed. In contrast to previous solution studies and X-ray crystallography results, the gas phase complexes do not contain Ca ions. (Int J Mass Spectrom 222 (2003) 397–412)

Keywords: Proteins; Noncovalent interactions; ESI; FTMS

1. Introduction

Proteins play key roles in a variety of biological processes. Some carry out the transport and storage of small molecules; others make up a large part of the structural framework of cells and tissues. They also form specific noncovalent complexes with DNA, small ligands, and other proteins in a number of biological processes [1]. The driving forces for noncovalent interactions include hydrogen bonds, van der Waals, hydrophobic interactions, and electrostatic interactions. An important step in understanding these biological processes is to define the stoichiometry of protein quaternary structure and to evaluate the strength of the protein complexes involved.

In recent years, mass spectrometry (MS) with soft ionization methods—electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) has been increasingly employed for investigation of protein–protein noncovalent interactions. It provides complementary information to other commonly used techniques, such as X-ray crystallography and nuclear magnetic resonance (NMR) and offers the often-noted advantages of specificity, sensitivity, speed, and stoichiometry information [2,3]. Specific or nonspecific interactions can be differentiated by control experiments [4], such as whether corresponding changes are observed in mass spectra upon changes in solution temperature, pH, absence or presence of buffer components, the addition of organic solvents, etc. It also is possible to find binding sites by systematic modification of amino acids at possible

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1387-3806/02/$ – see front matter
PII S1387-3806(02)00995-8

active sites. Speed and sensitivity are the most obvious advantages of MS for the study of noncovalent complexes. Detection limits as low as the picomole to femtomole range can be achieved. Additionally, MS provides accurate stoichiometry of complexes, especially for those comprised of heterogeneous subunits. On the other hand, MS has some drawbacks. It does not provide direct structural data as with NMR and X-ray crystallography. Furthermore, weakly bound complexes may not always be observed by mass spectrometer and changes in instrument parameters can change the spectra obtained.

In this paper, a series of systematic mass spectral studies of gas phase noncovalent complexes of tailored staphylococcal nucleases (SNases) are reported. The original objective was to use observations of the noncovalent complexes of nucleases modified at both active and nonactive sites to provide a measure of relative inhibitor–protein binding affinities. By comparing mass spectra of the modified nucleases, the influence of specific protein structure on the affinities can be probed.

SNase is a phosphodiesterase enzyme originally isolated from the *Staphylococcus aureus* bacterium which catalyzes Ca^{2+}-dependent DNA and RNA hydrolysis with a rate enhancement of 10^{16} [5,6]. This model protein has been extensively studied in attempts to understand protein structure and stability. The crystal structure has been refined by modern methods to a resolution of 1.65 Å [7]. The solution structure and structural dynamics also have been intensively examined by NMR [8]. It is a relatively small protein, 149 amino acids, and has a single domain containing both an α-helix and a β-sheet. It can be purified in a single

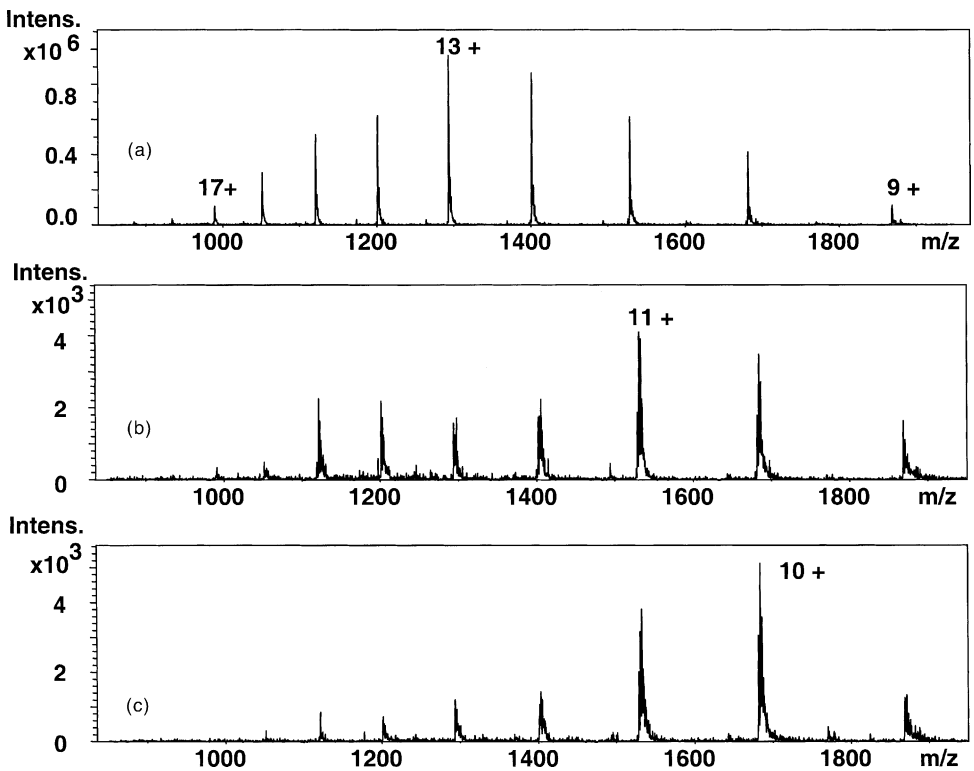

Fig. 1. Positive ESI-IT mass spectra of WT SNase under a variety of experimental conditions. (a) WT SNase in methanol/water (1:2 v/v) with 0.1% formic acid (pH = 4.8) at drying gas temperature of 300 °C. (b) and (c) WT SNase in aqueous solution (pH = 6.3) at drying gas temperature of (b) 300 °C and (c) 150 °C.

chromatographic step, folds and unfolds reversibly, and contains no disulfides, indeed no cysteine at all. It has fairly strong affinity to 5′-nucleoside phosphate inhibitors. The objective of the present study is to use both quadrupole ion trap (IT) and Fourier transform ESI (ESI-FT) mass spectrometric techniques to investigate the noncovalent complexes of wild type (WT) SNase and SNase mutants with such inhibitors. The analysis also addresses the possibility of performing a series of competition experiments among several SNases to determine whether there is preferential binding in the gas phase. If noncovalent interactions of SNase in the gas phase actually reflect their solution behavior, and if MS is efficacious in studying these interactions, understanding of the relationship between protein structure and function could be improved.

Here, ESI studies using either quadrupole IT or 7 T FTMS are reported. The protein complex is efficiently transferred from solution to the gas phase for subsequent mass analysis by ESI, where the noncovalent complexes can be detected.

2. Experimental

2.1. Instrumentation

2.1.1. ESI-ITMS

All the ESI-IT mass spectra were obtained in positive ion mode with a Bruker-HP Esquire LC Quadrupole Ion Trap mass spectrometer. Samples were injected into a fused silica capillary by an automated syringe pump (Cole-Parmer 74900 Series) at a rate of

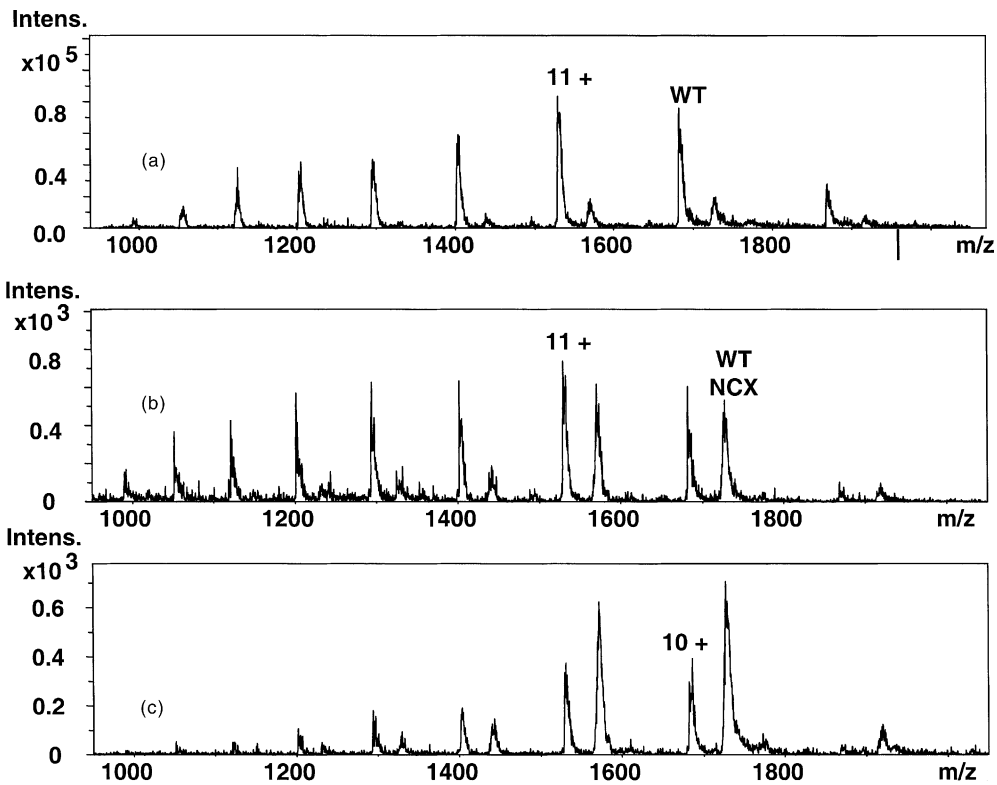

Fig. 2. Positive ESI-IT mass spectra of WT SNase/PdTp complex in the presence of CaAc under a variety of experimental conditions. (a) WT SNase/pdTp complex in methanol/water (1:2 v/v) at drying gas temperature of 300 °C. (b) and (c) WT SNase in aqueous solution (pH = 6.7) at drying gas temperature of (b) 300 °C and (c) 150 °C. WT NCX stands for noncovalent complex of WT SNase with pdTp.

90 μL/h with a nebulizer gas pressure of 15 psi. Drying gas temperature was set at 300 °C in most experiments except those intended to test temperature effects, where the drying gas temperature was set at 150 °C. The voltage on the capillary end cap was set at 4000 V, and the drying gas flow rate was adjusted to 6 L/min. Usually 100 scans were collected for each spectrum.

2.1.2. ESI-FTMS

FTMS spectra were obtained in positive ion mode using a 7 T Fourier transform Ultima mass spectrometer (IonSpec, Irvine, CA) equipped with both MALDI and ESI sources. Samples were continuously injected into a fused silica capillary by an automated syringe pump (Harvard Apparatus 55-2222 Series) at a rate of 120 mL/h. ESI conditions were 5000 V capillary voltage, 200 °C drying gas temperature, and 10 L/min drying gas flow rate. Capillary current was maximized for each sample by adjusting the needle gas pressure. Seven in-cell accumulations (cool in hexapole and transfer to FTMS cell seven times, excite, and acquire spectrum once) were used for each spectrum.

2.2. Sample preparation

SNase samples were obtained from the research group of Dr. Wesley Stites at the University of Arkansas. The WT SNase and mutants D21K, D21N, and Gail (T41I, P117G, H124L, and S128A) were prepared according to a method described by Kunkel et al. [9,10]. In order to remove the salts that have been shown to have detrimental effects on the droplet formation and ionization efficiency in ESI interface, the samples were dialyzed against Milli-Q water overnight at 4 °C. Dialysis tubing (Spectrum Laboratories) with 16 mm diameter and molecular cut-off of

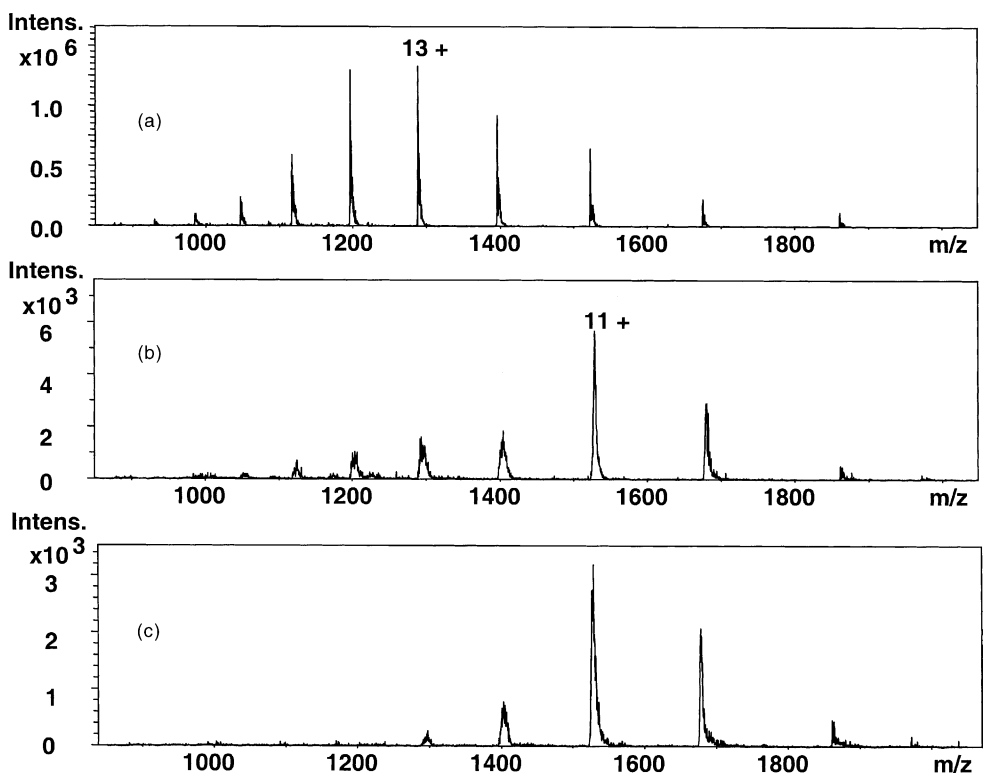

Fig. 3. Positive ESI-IT mass spectra of SNase mutant Gail under a variety of experimental conditions. Experimental conditions: (a) SNase Gail in methanol/water (1:2 v/v) with 0.1% formic acid (pH = 4.9) at drying gas temperature of 300 °C. (b) and (c) SNase Gail in aqueous solution (pH = 6.5) at drying gas temperature of 300 °C (b) and 150 °C (c).

12,000–14,000 Da was obtained dry, which was used after previously treating as follows: soak in Milli-Q water for 30 min; place in a large volume of 2% sodium carbonate, and 1 mM EDTA at 60 °C for 30 min; then rinse thoroughly with Milli-Q water. After treatment, the dialysis tubing should be stored in an aqueous preservative solution (0.1% sodium azide, 1% sodium benzoate, 1% hydrogen peroxide, and 1% formaldehyde) at 4 °C before use. The concentrations of SNase were measured by UV spectroscopy, based on the A_{280} of a 1 mg/mL solution of WT SNase as 0.93. Concentrations of WT, Gail, D21K, and D21N SNase were 7.1, 11.7, 1.4, and 17.1 mg/mL, respectively.

Methanol and formic acid were purchased from Aldrich and EM Science, respectively. Calcium acetate (CaAc, Fluka), 3′,5′-thymidine-diphosphate tetralithium salt (pdTp, Calbiotech), 2′-deoxyadenosine-5′-monophosphate (dAMP, Sigma–Aldrich), and thymidine-5′-monophosphate (dTMP, Sigma-Aldrich) were dissolved in Milli-Q water to make stock solutions without further purification. For ESI-IT experiments, SNase spectra were obtained by running a solution of ∼30 μM SNase in a mixture of Milli-Q water and methanol (2:1 v/v) with 0.1% formic acid, unless mentioned otherwise. For ESI-FTMS experiments, all spectra were obtained by running a solution of ∼7 μM SNase in 2.5 mM aqueous ammonium acetate/methanol (3:1 v/v), unless noted otherwise. To observe the noncovalent complexes, the appropriate SNase and inhibitor 3′,5′-thymidine-diphosphate (pdTp) were mixed in the specified solvent systems in a molar ratio of 1–1.2 in the presence of CaAc (molar ratio of SNase to CaAc = 2) or absence of CaAc. Solution pH was measured using a portable pH meter

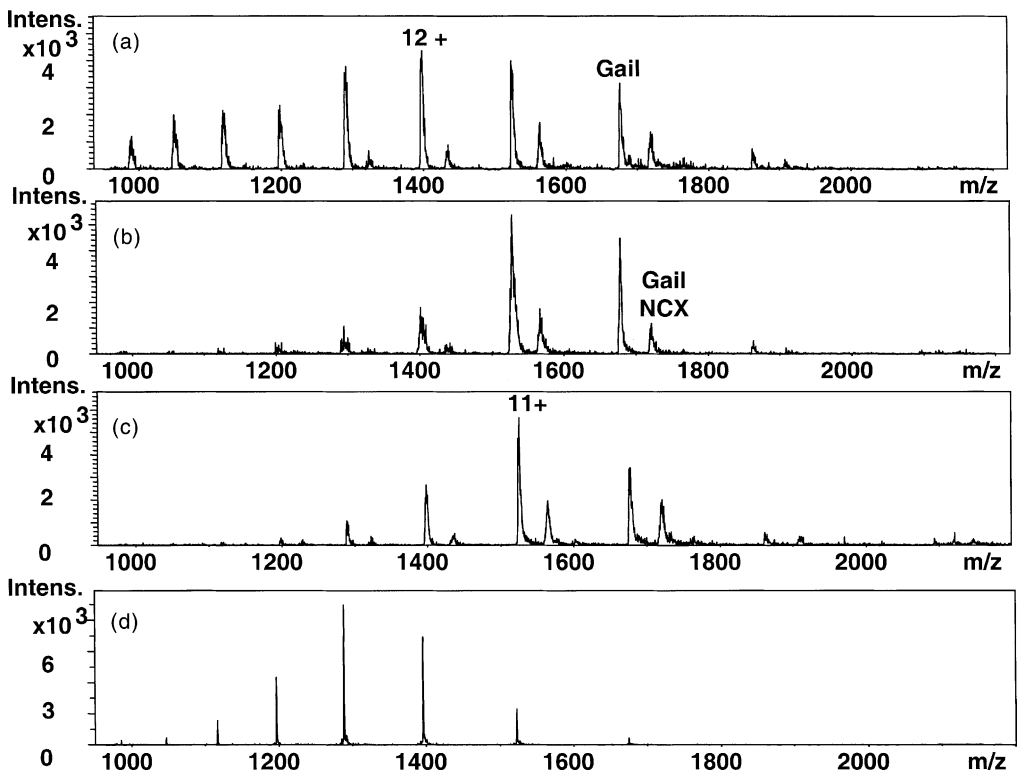

Fig. 4. Positive ESI-IT mass spectra of SNase mutant Gail/pdTp complex in the presence of CaAc under a variety of experimental conditions. (a) SNase Gail/pdTp complex in methanol/water (1:2 v/v) (pH = 6.8) at drying gas temperature of 300 °C. (b) and (c) SNase Gail in aqueous solution (pH = 6.8) at drying gas temperature of 300 °C (b) and 150 °C (c). (d) Aqueous solution pH was adjusted to 3.3 at drying gas temperature of 150 °C. Gail NCX stands for noncovalent complex of SNase Gail with pdTp.

(Thermo Orion, Model 230A) with a microelectrode (Thermo Orion, Model 98-03).

3. Results and discussion

Various means can be employed to infer the degree of folding of proteins and their noncovalent complexes observed by ESI-MS. For example, one can vary the electrospray solvent, solution pH, and the drying gas temperature. If such changes cause logical changes in the resulting ESI spectra, they can be interpreted in terms of the underlying protein behavior.

3.1. Observation of SNase by ESI-quadrupole ITMS

The mass spectra of WT SNase obtained under a variety of ESI conditions are shown in Fig. 1. The three spectra are a low pH spectrum (Fig. 1a), and near-neutral pH spectra at higher (Fig. 1b, 300 °C) and lower temperature (Fig. 1c, 150 °C). Proteins electrosprayed from native conditions tend to have a narrower charge state distribution, while proteins electrosprayed from denatured conditions tend to have a wider distribution [11,12]. Thus, Fig. 1a is the mass spectrum of WT SNase electrosprayed from a 2:1 (v/v) water/methanol solution in the presence of 0.1% formic acid (pH = 4.8). Under these denaturing conditions, the charge states observed range from +9 to +17. In contrast, when the enzyme is electrosprayed from aqueous solution (pH = 6.3) the maximum abundance peak shifts from the +13 to the +11 or +10 charge states, with the observed range being reduced to +9 to +15 (Fig. 1b and c). Furthermore, the relative abundances of higher charge states (≥+12) decrease dramatically when the temperature of the ESI drying gas is reduced to 150 °C. This observation is consistent with a temperature-dependent conformation change, an observation similar to a previous report [13]. The deconvoluted molecular weight of the WT SNase is

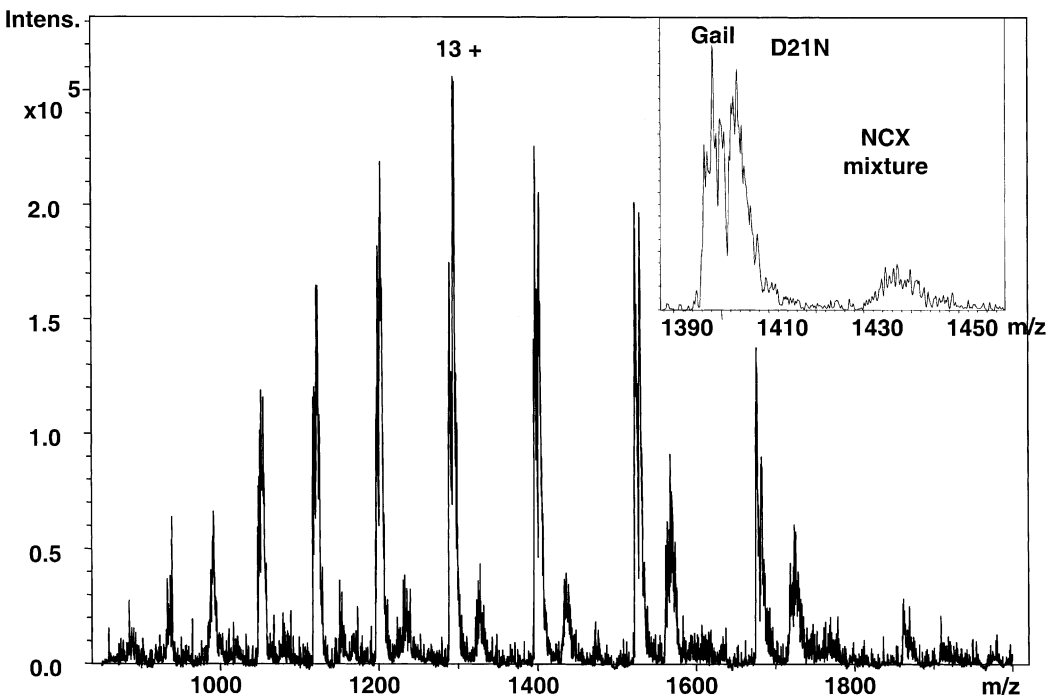

Fig. 5. Positive ESI-IT mass spectra of noncovalent complex of SNase mutants D21N and Gail with pdTp in the presence of CaAc. The concentrations of both D21N and Gail are 32.2 μM.

16811.2 ± 1.1 Da (n = 6) in close agreement with theoretical value 16,810.8361 Da calculated according to amino acid sequence. Thus, the spectra from these different conditions are clearly distinct, indicating that the WT SNase retains a history of its solution environment in gas phase.

3.2. Observation of noncovalent SNase complexes by ESI-quadrupole ITMS

When inhibitor pdTp and CaAc were added to the WT SNase solution, mass spectral behavior was similar to that in the absence of calcium salt and inhibitor. The primary difference, apparent in Fig. 2, is that now noncovalent complex peaks are seen. Also, all primary peaks are now accompanied by one or more sodium attachment peaks in all spectra. The peak abundance of the SNase/pdTp complex relative to uncomplexed SNase under standard ESI conditions is the smallest (Fig. 2a), it increases in the absence of methanol at 300 °C (Fig. 2b), and becomes most abundant when the drying gas temperature is decreased to 150 °C (Fig. 2c). Unfortunately, neither the resolving power nor the mass accuracy was sufficient to determine whether or not calcium was present in these complexes.

3.3. Observation of Gail SNase and its noncovalent complexes by ESI-quadrupole ITMS

When similar mass spectral measurements were performed on SNase mutant Gail (T41I, P117G, H124L, and S128A), *pH/solvent* effects parallel to those obtained with WT SNase were obtained. Significantly, there is not a major change in charge state distribution when the ESI drying gas temperature is changed from 300 °C (Fig. 3b) to 150 °C (Fig. 3c).

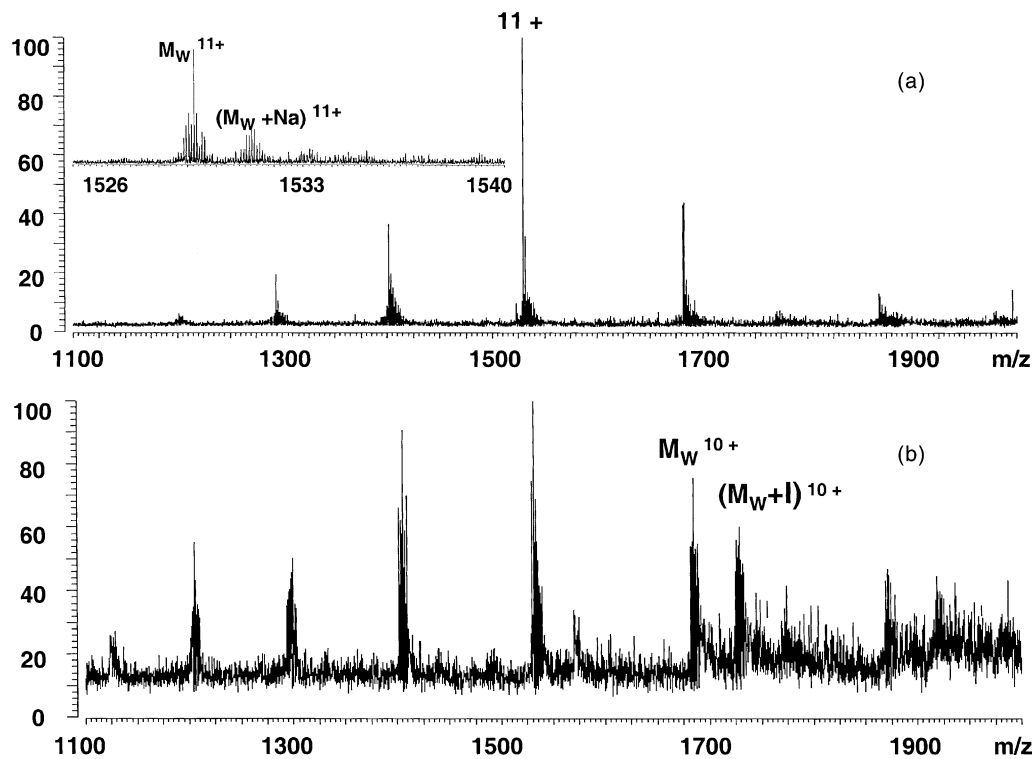

Fig. 6. Comparison of positive ESI-FT mass spectra of WT SNase and its complex with pdTp in methanol/water (1:2 v/v). (a) WT SNase. (b) WT SNase and inhibitor pdTp in the presence of CaAc. M_W stands for WT and I stands for inhibitor pdTp. The inset is the expanded scale spectrum of WT SNase at charge state +11.

This gas phase behavior is consistent with solution phase results. This observation could be a consequence of the fact that Gail was purposely engineered to have the maximum thermal stability, resulting in a solution stability constant of 10 kcal/mol [14].

Noncovalent complexes of Gail with pdTp were also observed under different conditions. No big difference was observed regarding the ratio of complex to Gail under different experimental conditions (shown in Fig. 4a–c). Thus, the noncovalent complex formed between Gail and pdTp appears to have a greater tolerance for higher temperature and the presence of organic solvent than WT SNase. To test the effect of pH on the Gail noncovalent complex, formic acid was added to its aqueous solution to adjust the pH from 6.8 to 3.3. When the resulting solution was subjected to ESI-MS noncovalent complex was not observed (Fig. 4d). Thus, it is concluded that at pH 3.3 Gail SNase is unfolded and, therefore, does not retain binding affinity to the inhibitor pdTp.

Therefore, for these SNases, observation of inhibitor noncovalent complexes is dependent upon solution pH, ESI drying gas temperature, and the presence or absence of organic solvent. Furthermore, the effect of amino acid substitution in the SNase sequence is reflected in the relative affinity for inhibitor. The observations are consistent with the noncovalent complexes being the result of specific, as opposed to nonspecific associations between SNases and inhibitors.

3.4. Effect of amino acid mutations on noncovalent binding affinity of SNase to inhibitor pdTp by ESI-quadrupole ITMS

An important aim of the present project is to perform competitive studies of the binding affinity

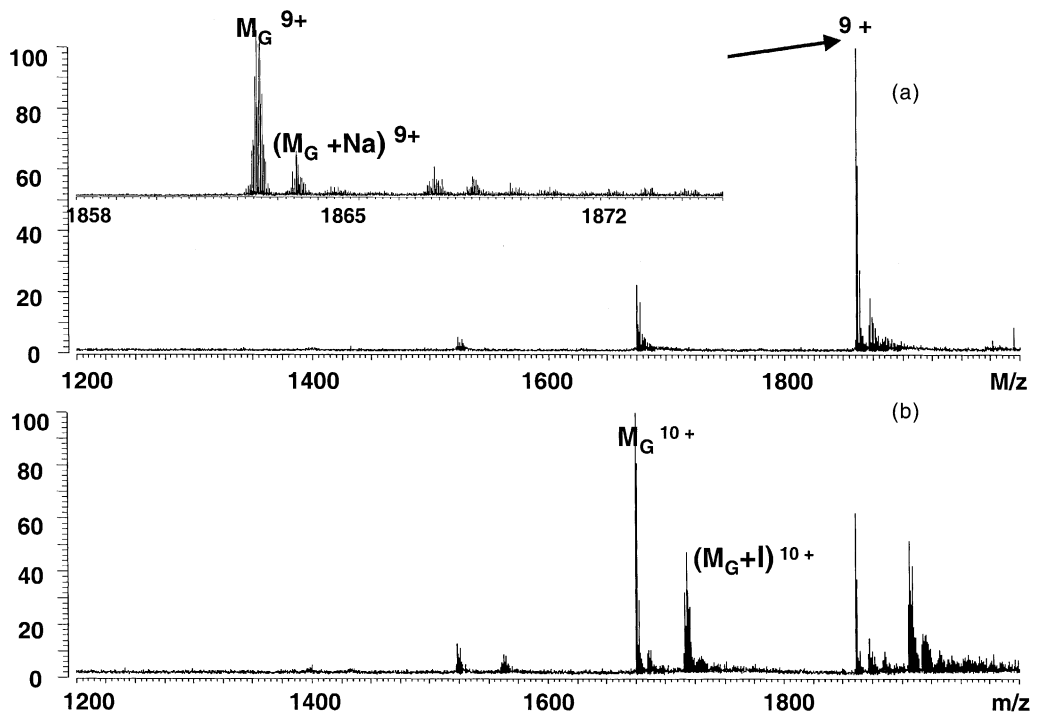

Fig. 7. Comparison of positive ESI-FT mass spectra of SNase mutant Gail and its complex with pdTp in methanol/water (1:2 v/v). (a) Gail SNase. (b) SNase Gail and inhibitor pdTp in the presence of CaAc. M_G stands for SNase Gail and I stands for inhibitor pdTp. The inset is the expanded scale spectrum of SNase Gail at charge state +9.

of different mutants of SNase to inhibitor pdTp or WT SNase to different inhibitors. Accordingly, it is necessary that the mass analyzer be able to accurately differentiate mass differences less than 100 Da for analytes with molecular weights in excess of 16,000 Da. In a preliminary experiment, two mutants Gail and D21N, with a mass difference of 67 Da, were mixed together with pdTp and CaAc (molar ratio SNase/pdTp of 1:1.2) in aqueous solution. A typical spectrum obtained with a drying gas temperature of 150 °C is shown in Fig. 5. Both Gail and D21N and their noncovalent complexes are observed. As is clear from the inset, the SNase/pdTp complex peaks of D21N and Gail could not be resolved and the two mutant proteins barely can be differentiated. Thus, ESI-quadrupole ITMS is inadequate to accomplish this aspect of the research. Fortunately, FTMS possesses the necessary high resolving power to distinguish species of high molecular weight. Therefore, an external source 7 T ESI-FTMS instrument was used for the remainder of the study.

3.5. Effect of amino acid mutations on noncovalent binding affinity of SNase to inhibitor pdTp by ESI-FTMS

3.5.1. Competitive binding of WT vs. Gail SNase with pdTp

Initially, it was planned to determine the competitive binding of pdTp by WT and Gail SNase using a 1:2 methanol/water solution. As Figs. 6 and 7 show, it is possible to obtain ESI-FT mass spectra of the pure enzymes (Figs. 6a and 7a) and their noncovalent complexes (Figs. 6b and 7b). However, when the two enzymes were mixed in this solvent system, only ions from WT SNase are observed in the ESI spectra. Therefore, different solvent systems were tested in order to find one that permits observation of both WT and Gail enzymes in a mixture with similar abundances. A 2.5 mM aqueous ammonium acetate/methanol (3:1 v/v) solution of a 1.6:1 molar ratio of WT/Gail satisfies this requirement (Fig. 8). When a competitive binding affinity experiment (WT

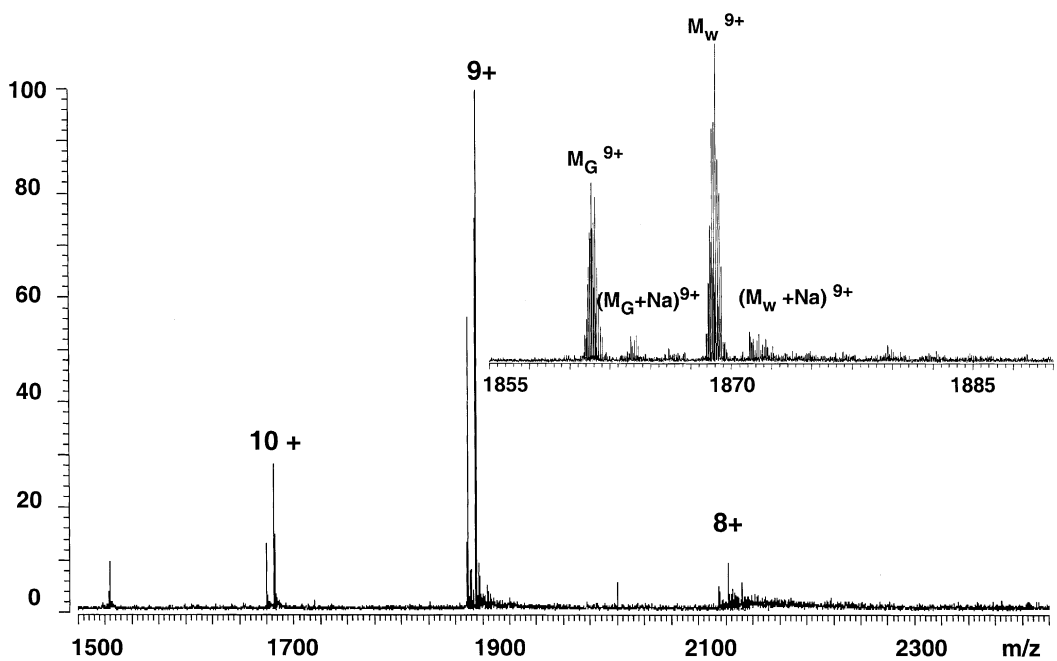

Fig. 8. Positive ESI-FT mass spectrum of WT SNase and Gail mixture (molar ratio 1.6:1) in 2.5 mM ammonium acetate/methanol (3:1 v/v). M_W stands for WT SNase and M_G stands for SNase mutant Gail. The inset is the expanded scale spectrum of SNase mixture at charge state +9.

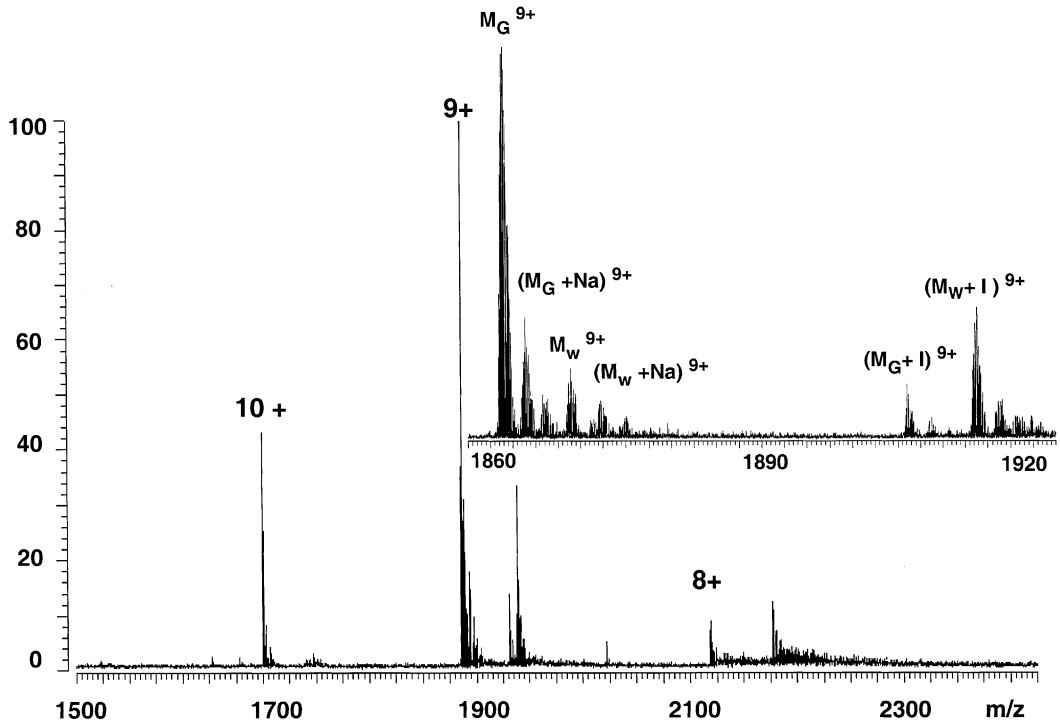

Fig. 9. Positive ESI-FT mass spectrum of competitive binding affinity study of WT and Gail SNase in the absence of CaAc in 2.5 mM ammonium acetate/methanol (3:1 v/v). The molar ratio of WT/Gail is 1.6:1. M_W stands for WT SNase, M_G stands for SNase mutant Gail, and I stands for inhibitor pdTp. The inset is the expanded scale spectrum of charge state +9.

vs. Gail SNase) is performed the results shown in Fig. 9 are obtained. Based upon the instrument software peak list, the ratio of the highest +9 charge state peak SNase/pdTp complex to SNase was 2.0 ± 0.3 ($n = 5$) for WT; and it was 0.14 ± 0.02 ($n = 5$) for SNase mutant Gail. Surprisingly, there is no evidence that the inhibitor complexes contain calcium.

The results do show that the inhibitor pdTp binds preferentially with WT SNase. This observation can be understood by considering the modifications to WT SNase that the Gail mutant represents. First, threonine 41 (known to be at the active site of inhibitor binding) is replaced with isoleucine in Gail. Furthermore, the positively charged amino acid histidine 124, and the uncharged polar amino acids threonine 41 and serine 128 in WT SNase are replaced in Gail by nonpolar amino acids leucine, isoleucine, and alanine, respectively. Removal of the solvent during the ESI process should destabilize hydrophobic interactions between nonpolar groups, but should stabilize H-bonding interactions. Thus, it might be expected that WT SNase would have greater binding affinity to pdTp than does Gail.

As mentioned earlier, the surprising observation in this competitive binding study is that calcium appears to be absent from the gas phase complex. As presently understood, to maintain SNase activity, calcium is essential for binding both the attacked phosphoryl group and the attacking water. Other metals also bind to the enzyme, but none can activate it fully, and most support no activity at all [15,16]. In contrast to evidence from NMR and X-ray crystallography studies, ESI-FT mass spectra of the complex reveal that it contains no Ca^{2+}. Accordingly, control experiments

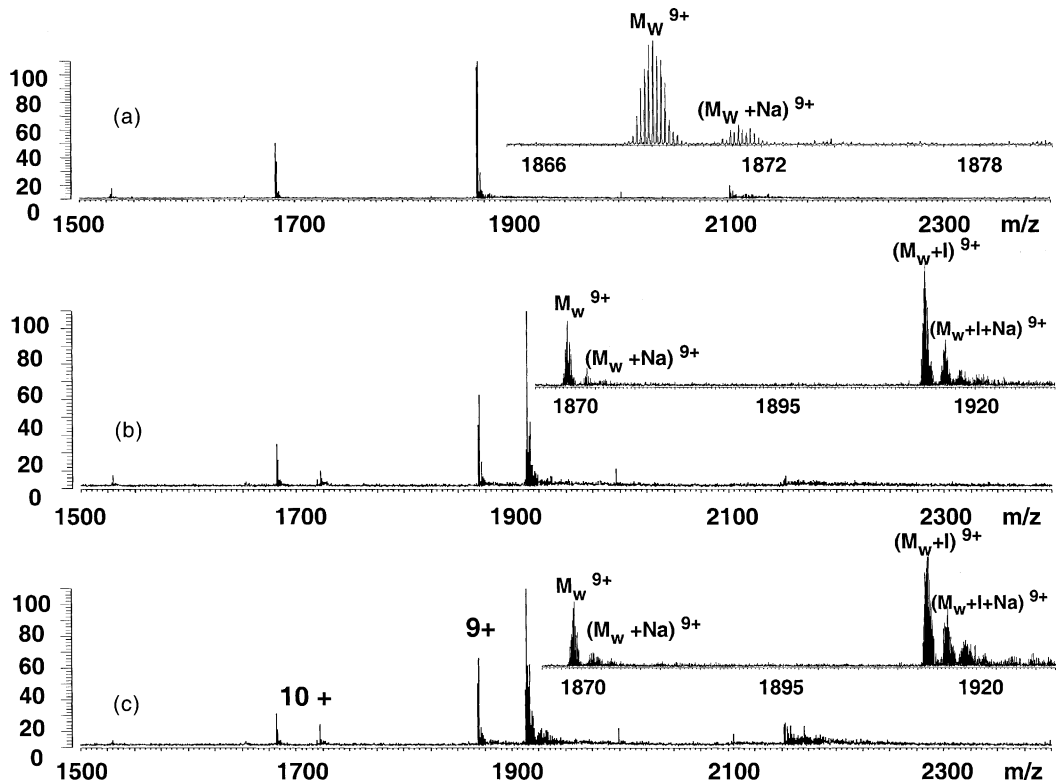

Fig. 10. Comparison of positive ESI-FT mass spectra in 2.5 mM ammonium acetate/methanol (3:1 v/v). (a) WT SNase. (b) WT/pdTp complex in the presence of calcium acetate. (c) WT/pdTp complex in the absence of CaAc. M_W stands for WT SNase and I stands for inhibitor pdTp. The insets are the expanded scale spectra of charge state +9.

in the absence of CaAc were carried out. The spectra shown in Figs. 10 and 11 show that noncovalent complexes of both WT and Gail are still observed and, in each case the spectra in the presence and in the absence of calcium acetate are indistinguishable. These results verify that the presence of Ca^{2+} is not necessary for SNase to bind to its inhibitor pdTp in gas phase. The gas phase stoichiometry of the complex, therefore, includes one SNase and one inhibitor molecule. Sodium adduct ions are also seen, although in limited abundance for the complexes.

3.5.2. Competitive binding of WT vs. D21K SNase with pdTp

Another active site mutant D21K, 13 Da bigger than WT SNase, also was studied. Both D21K and D21K/pdTp complex signals are observed, primarily with +8, +9, and +10 charge states (Fig. 12). When the molar ratio of D21K to pdTp is 2:1 in the absence of calcium, the signal ratio of D21K/pdTp complexes to D21K for the +9 charge state is greater than 32, as compared with the expected theoretical ratio 1:1 if the uncomplexed and noncovalent complex signal abundances correctly reflect their relative amounts. To test that premise, another ESI mass spectrum was obtained employing a different ratio of D21K to pdTp (4:1) and the signal ratio changed to about 2. Although these data do not quantify the difference in proton affinity between D21K and the D21K/pdTp complex, it is clear that D21K/pdTp gives a stronger signal than D21K at the same concentration.

In order to examine the competitive binding of WT and D21K to the inhibitor pdTp, it is necessary to know whether the signals from WT and D21K

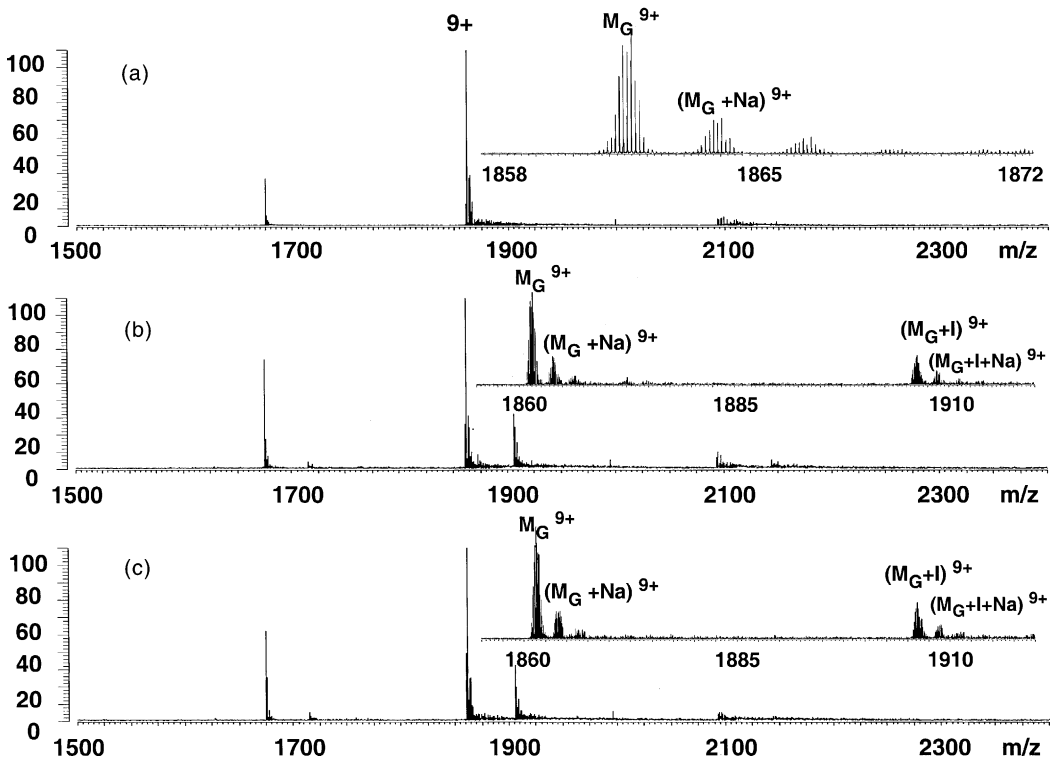

Fig. 11. Comparison of positive ESI-FT mass spectra in 2.5 mM ammonium acetate/methanol (3:1 v/v). (a) SNase Gail. (b) Gail/pdTp complex in the presence of CaAc. (c) Gail/pdTp complex in the absence of CaAc. M_G stands for SNase mutant Gail and I stands for inhibitor pdTp. The insets are the expanded scale spectra of charge state +9.

accurately reflect their relative concentrations. Several different molar ratios of D21K to WT (1:2.5, 4:1, and 2:1) were employed to investigate this issue. Using the +9 charge states, the signal ratios of D21K to WT were estimated. When the D21K to WT solution ratio is 1:2.5, D21K peaks partially overlap with WT sodium adduct peaks, complicating ESI mass spectral estimation of the WT to D21K signal ratio (Fig. 13). When the molar ratios of D21K to WT are 4:1 and 2:1, the peak intensity ratios of D21K to WT using the +9 charge state is 4.1 ± 0.9 ($n = 5$) and 2.2 ± 0.2 ($n = 8$). This establishes that WT and D21K can be clearly resolved and the concentration ratio of the uncomplexed forms can be accurately estimated by ESI-FTMS although the mass difference is only 13 Da out of 17 kDa (Fig. 14 is a typical spectrum for the 2:1 D21K/WT ratio).

The situation is more complicated for the complexed forms of the two nucleases, in view of the significantly higher proton affinity of D21K/pdTp complex. For the competitive binding experiments two sets of molar ratios were used. When the molar ratio of WT to D21K to the inhibitor pdTp is 2:4:1, signals from WT SNase, D21K Snase, and D21K/pdTp complexes *but no WT/pdTp complexes* are observed. This result is reasonable if pdTp preferentially binds with D21K instead of WT SNase. Because D21K consumes all the inhibitor pdTp, no pdTp is available to bind with WT SNase. When the molar ratio of WT to D21K to pdTp is changed to 2:1:1.5, signals from WT, WT/pdTp complex, and D21K/pdTp complex *but no uncomplexed D21K are observed.* This is as expected if D21K has bigger binding affinity than WT SNase. With the concentration ratios used, there was still

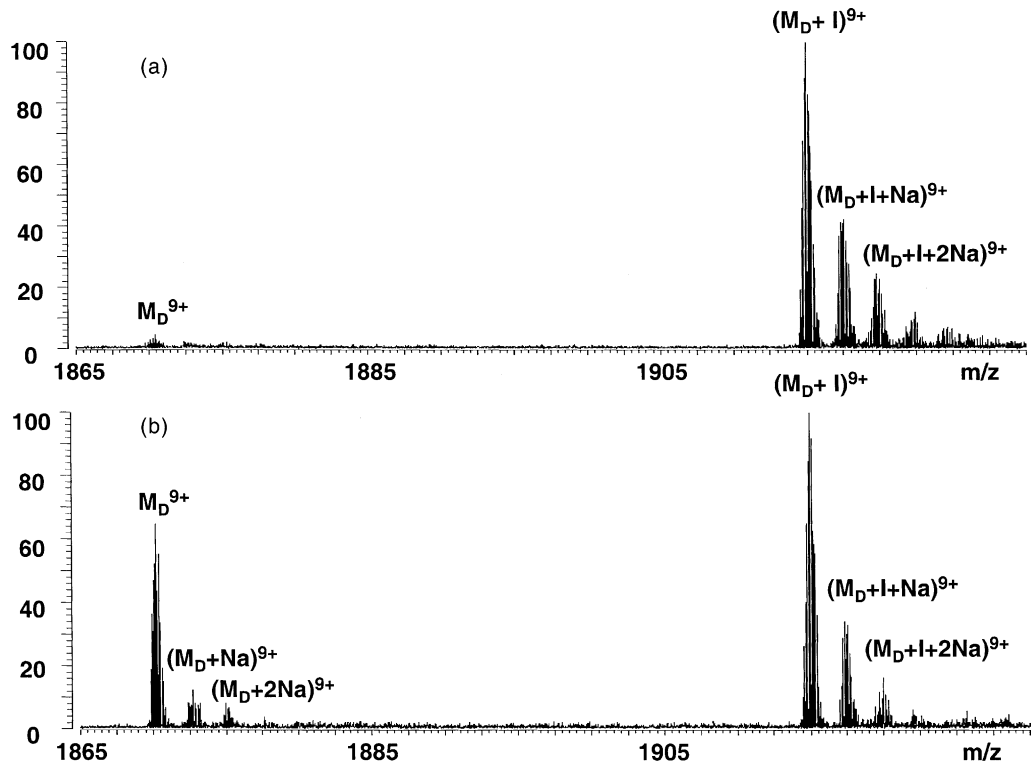

Fig. 12. Expanded scale positive ESI-FT mass spectra of D21K and pdTp in the absence of CaAc in 2.5 mM ammonium acetate/methanol (3:1 v/v). (a) The molar ratio of D21K to pdTp is 2:1. (b) The molar ratio of D21K to pdTp is 4:1. M_D stands for D21K SNase and I stands for inhibitor pdTp.

pdTp left to bind with WT SNase after all D21K was converted to the noncovalent complex. The preferential binding affinity between D21K and pdTp is probably caused by the stronger electrostatic interaction of positively charged lysine (K) at the D21K active site with the phosphoryl group of pdTp. This is not an unreasonable suggestion based upon previously reported crystal structures of SNase–inhibitor complexes [7,17].

3.6. Competitive study of noncovalent complexes of WT SNase with various inhibitors by ESI-FTMS

Cheng et al. [18] reported that a competition study by ESI-FTMS could identify inhibitors tightly bound to carbonic anhydrase II, and demonstrated this approach by applying it to two small libraries. Freitas et al. [19] used the same approach to examine binding of two peptides with the oligopeptide binding protein, OppA. Similarly, here the competitive binding of SNase was examined with two additional inhibitors. The best-known inhibitor of SNase is pdTp ($K_i = 10^{-7}$ M), other 5′-phosphate nucleotides, such as dAMP and dTMP, are also good inhibitors ($K_i = 10^{-5}$ M), although having binding constants about a factor of 100 smaller [17].

The first step was to observe the WT/dAMP complex by ESI-FTMS because this complex is two orders of magnitude weaker than WT/pdTp. When the molar ratio of SNase to dAMP was 1:1.2, the same as for pdTp, no SNase/dAMP complex was observed in different solvent systems, such as 2.5, 5, and 10 mM aqueous ammonium acetate/methanol in 4:1 or 3:1 ratios (v/v), where a strong signal of the WT/pdTp

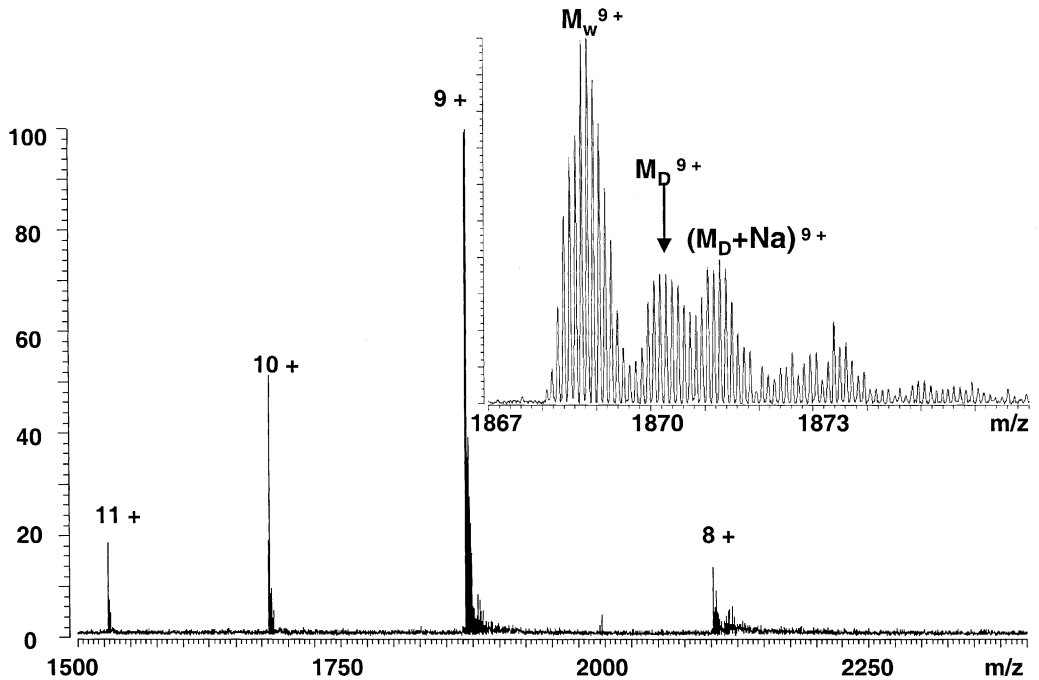

Fig. 13. Positive ESI-FT mass spectrum of WT SNase and D21K mixture in 2.5 mM ammonium acetate/methanol (3:1 v/v). The molar ratio of D21K/WT is 1:2.5. M_W stands for WT SNase and M_D stands for D21K SNase. The inset is the expanded scale spectrum of charge state +9.

complex could be observed. However, when the molar ratio of SNase/dAMP was changed to 1:2.4, the noncovalent complex of WT SNase/dAMP was observed in 10 mM aqueous ammonium acetate/methanol (4:1 v/v) at low intensity (Fig. 15). Competitive experiments were performed with the molar ratio of WT/pdTp/dAMP = 2:1:4 using a drying gas temperature of 150 °C. The WT/dAMP complex was barely observed compared to the strong signal from WT/pdTp complex, although dAMP was present at four times the concentration of pdTp in the mixture. Accurate quantitative comparison is not possible because the signal from WT/dAMP barely can be distinguished from the baseline. The ratio of WT/pdTp to WT/dAMP (40) was approximated, based on the highest peak intensities from each group. Thus, preferential binding of WT SNase to pdTp over dAMP is clearly demonstrated.

Similar experiments were also performed for dTMP. WT SNase/dTMP complexes began to be observed at low abundance when the molar ratio of WT to dTMP was increased to 1:2.4 in 10 mM aqueous ammonium acetate/methanol (4:1 v/v) (1024 K data points were collected). Competitive binding affinity was studied using a molar ratio of WT/pdTp/dTMP = 2:1:5 and drying gas temperature of 150 °C. The relative abundance ratio of the noncovalent complexes WT/pdTp to WT/dTMP was 2.8 ± 0.7 ($n = 8$), although dTMP was present in fivefold excess over pdTp. Thus, preferential binding of WT SNase to pdTp over dTMP was also demonstrated.

Interestingly, although previous results showed that D21K has greater binding affinity to pdTp than WT SNase, when noncovalent interaction between D21K and dAMP was examined, no complex was observed in any of the solvent systems investigated, including 2.5, 5, and 10 mM aqueous ammonium acetate/methanol (4:1 or 3:1 v/v). At present, this result is not understood.

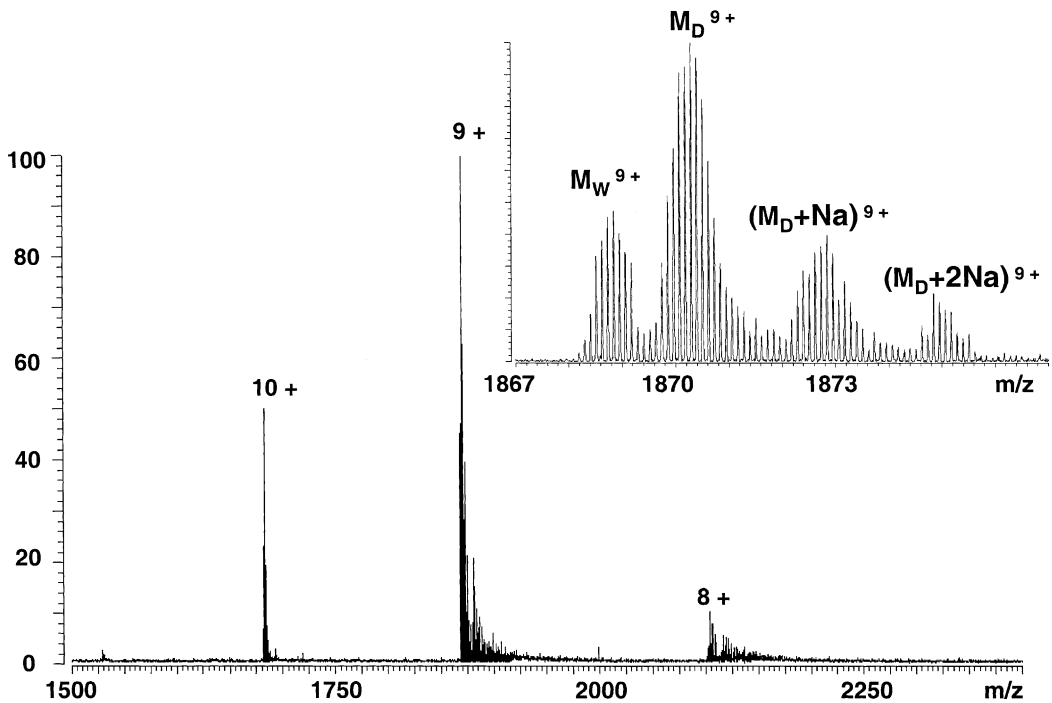

Fig. 14. Positive ESI-FT mass spectrum of WT SNase and D21K mixture in 2.5 mM ammonium acetate/methanol (3:1 v/v). The molar ratio of D21K/WT is 2:1. M_W stands for WT SNase and M_D stands for D21K SNase. The inset is the expanded scale spectrum of charge state +9.

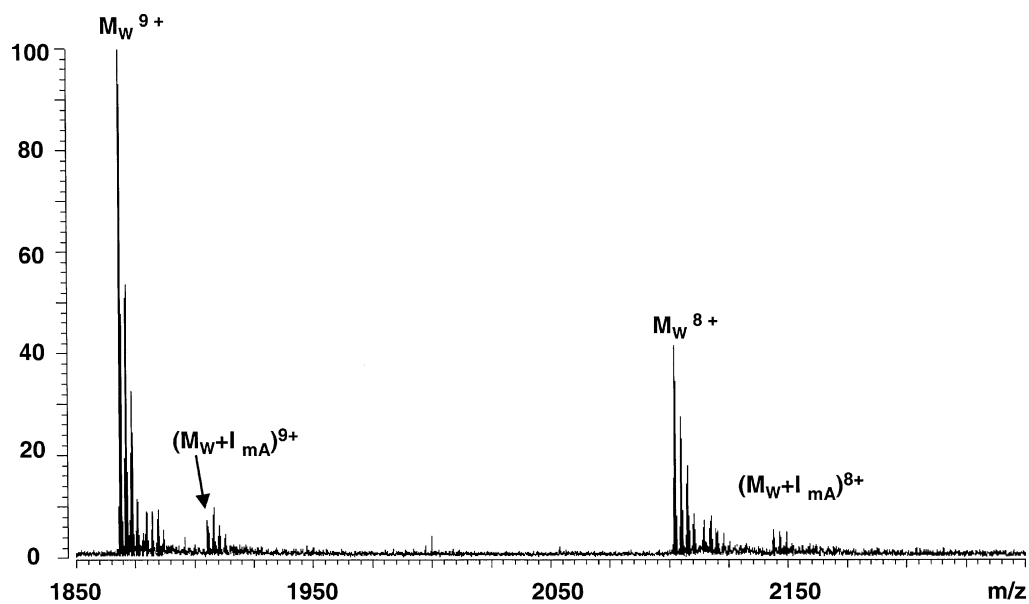

Fig. 15. Expanded scale positive ESI-FT mass spectrum of WT and dAMP in the absence of CaAc in 10 mM ammonium acetate/methanol (4:1 v/v). The molar ratio of WT to dAMP is 1:2.4. M_W stands for WT SNase and I_{mA} stands for inhibitor dAMP.

Acknowledgements

This research was supported by the National Science Foundation (Grant Nos. CHE-00-91868 and CHE-00-96780).

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