Probing subtle acid-induced conformational changes of ribonuclease A by electrospray mass spectrometry

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Abstract The newly developed technique, electrospray mass spectrometry, was used to probe subtle conformational changes of bovine pancreatic RNase A during acid denaturation. In a dilute acid solution of pH 2.6, RNase A lost nearly all of its activity, whereas its intrinsic fluorescence intensity at 304 nm and its ellipticity at 222 nm were fairly resistant to denaturation by acetic acid. The observed maximum charged state of the enzyme in electrospray mass spectra was increased from 11^+ (at pH 3.3) to 14^+ (at pH 2.6). This could result from exposure of the buried basic amino acid residues R-10, K-41, H-12 and perhaps H-48.

Key words: Ribonuclease A; Electrospray mass spectrometry; Conformational change; Acid denaturation

1. Introduction

Investigations of conformational changes in proteins during the course of unfolding caused by denaturants, heat or pH have been the subject of numerous studies (see [1] and references therein). A variety of techniques have been applied to measure the conformational changes of proteins in solution, including acid-base titration, calorimetry, spectrophotometry, viscometry, circular dichroism (CD), fluorescence and nuclear magnetic resonance [1]. Recent work has demonstrated that conformational changes of proteins can also be detected by electrospray ionization mass spectrometry (ES/MS) [2–8].

Chowdhury et al. [2] described the first use of ES/MS for probing conformational changes in proteins in a manner analogous to spectroscopic techniques. They demonstrated the usefulness of this new technique in an investigation of conformational changes in bovine cytochrome *c*, bovine ubiquitin and yeast ubiquitin induced by pH change and by the addition of organic solvent denaturants. Dramatic differences were observed in the charge state distribution obtained from the native versus denatured proteins [2]. Le Blanc et al. studied the thermal denaturation of eight globular proteins and how the effect was observed using ES/MS. The observed effects ranged from hardly noticeable to a dramatic shift in the mass spectrometric profile and a simultaneous increase in the ion abundance. The change was believed to result from the thermally induced denaturation and transition from a more compact to a less compact conformation of the protein species in solution [7].

These and other published ES/MS experiments deal with protein unfolding transitions with significant conformational change of the proteins. These conformational changes can also be detected by CD, UV and fluorescence spectrometry. Researchers in this laboratory have shown that during unfolding of some enzymes by denaturants, heat or pH, inactivation of the enzymes precedes any noticeable conformational changes of the molecule monitored by CD, UV and fluorescence spectrometry. These results suggested that the enzyme active site is situated in a flexible region of the molecule that is more easily perturbed by denaturants than the molecule as a whole (see [10] and references therein) [9,10]. ES/MS should be investigated to see whether it can probe the conformational changes which cause an enzyme to lose its activity but cause little variation in CD, UV spectrum and fluorescence intensity.

Acid-induced denaturation of bovine pancreatic ribonuclease A (RNase A) is a suitable subject to investigate the ability of ES/MS to probe subtle conformational changes in enzymes. RNase A is probably one of the most extensively studied enzymes, with its sequence and structure being completely known. The course of its unfolding during denaturation by acid, heat, pressure and denaturants has been well recorded in the literature (see [9] and references therein).

This study reports results using ES/MS to probe subtle acid-induced conformational changes of bovine pancreatic RNase A and compares the changes observed using ES/MS with changes of the enzyme activity, CD and fluorescence intensity.

2. Materials and methods

2.1. Materials

Bovine pancreatic RNase A (type XII-A) and cytidine 2,3-(cyclic) monophosphate (C > p) were purchased from Sigma. All other reagents were local products of analytical grade.

2.2. Methods

All mass spectra were obtained in positive-ion mode using an HP 59987 A ES/MS Engine System with an Analytics electrospray source (Analytica of Branford Inc.). The mass spectrometer used for these studies has an effective *m/z* range of 2000. An aqueous solution of RNase A (3.6 μM in distilled water with glacial acetic acid in the range of 0.1–8%, pH 3.3–2.2) was pumped using a syringe pump (Harvard Model 22) at a flow rate of 10 μl/min into the electrospray interface. High purity N_2 was used as the bath/drying gas with a flow rate of 3 l/min and a temperature of 250°C. Electrospray was performed by applying a DC voltage of 4–5 kV to the syringe needle. MS Chemistation software (HP G1034C) was used on an HP computer for data acquisition and processing.

RNase A activity was determined according to the method of Crook et al. [11] at 25°C by following changes in A_{290} with cyclic CMP (C > p) as the substrate in a series of acetic acid concentrations (pH 3.3–2.2) containing 0.06 mg/ml C > p and an enzyme concentration of 0.9 μM.

RNase A concentrations were determined at A_{278}, with a molar

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Abbreviations: RNase A, ribonuclease A; ES/MS, electrospray ionization mass spectrometry; CD, circular dichroism

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absorption coefficient of 9700 M^{-1} cm^{-1} [12]. A molar absorption coefficient of 760 M^{-1} cm^{-1} at 260 nm was used for 2'CMP.

Fluorescence measurements were made with a Perkin-Elmer MPF-66 Fluorescence spectrophotometer at 25°C with an excitation wavelength of 280 nm. The enzyme was incubated with the required concentration of acetic acid at a concentration of 0.2 mg/ml at 25°C for 24 h.

CD spectra were recorded on a Jasco J-720 spectropolarimeter. The same samples as those used in the fluorescence measurements were scanned four times from 250 to 190 nm at 20°C using a quartz cuvette with a 1 mm pathlength.

3. Results

Fig. 1 line (a) shows the enzyme activities assayed at different pH. The enzyme activity decreases sharply with increasing acetic acid concentration. At pH 3.3 RNase A retains 70% of its native activity indicating that most of the enzyme molecules are in their native structure. At pH 2.6 the enzyme loses most of its activity. 200-Fold dilution of the acetic acid (pH 2.6) inactivated enzyme into a pH 4 buffer resulted in 66% reactivation, assayed immediately after diluting, and in 90% reactivation when the assay was carried out 10 min after diluting. The time dependence of the reactivation suggests that the inactivation of the enzyme arises not only from protonation but also from reversible conformational changes, because the protonation reaction occurs in a very short time scale. In contrast, the intensities of the maximum emission intrinsic fluorescence at 304 nm and the value of ellipticity at 222 nm for RNase A are fairly insensitive to denaturation by acetic acid (Fig. 1 lines (b) and (c)). In the transition during which the enzyme loses its activity, intrinsic fluorescence intensity remains constant and CD intensity increases slightly. The above results are in general agreement with those reported in the literature [9].

The mass spectra of RNase A shown in Fig. 2 were obtained by electrospraying aqueous solutions with different acetic acid concentrations at pH values of 3.3, 2.9, 2.6, 2.5 and 2.2. The mass spectrum obtained using the pH 3.3 solution (Fig. 2a) exhibited five peaks, each corresponding to different charged states of RNase A. These charged states ranged from 11^+ to 7^+ with 8^+ being the most intense. As the pH of the sprayed solution decreases the charge states show a wider distribution and a much higher average, with the range of the charge states shifting upward to between 9 and 14 (pH 2.9, Fig. 2b). Upon further decrease of the pH to 2.2 (Fig. 2c–e), the distribution exhibits charge states that are not significantly different from that measured at pH 2.9.

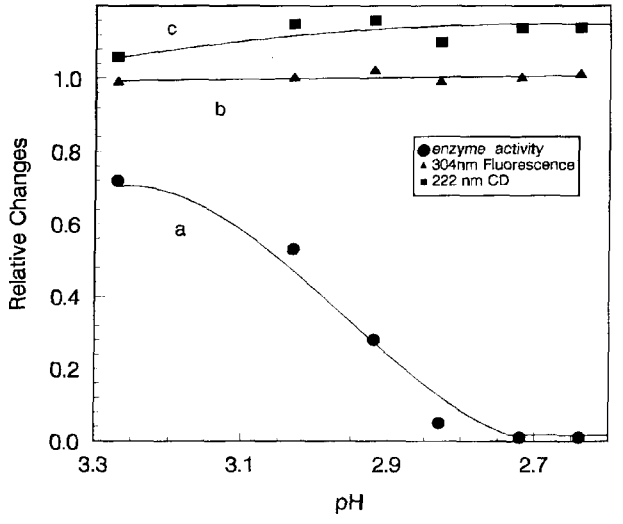

Fig. 1. Activity and conformation change of RNase A during denaturation with acetic acid. a: Enzyme activity; b: fluorescence emission intensity at 304 nm; c: ellipticity at 222 nm.

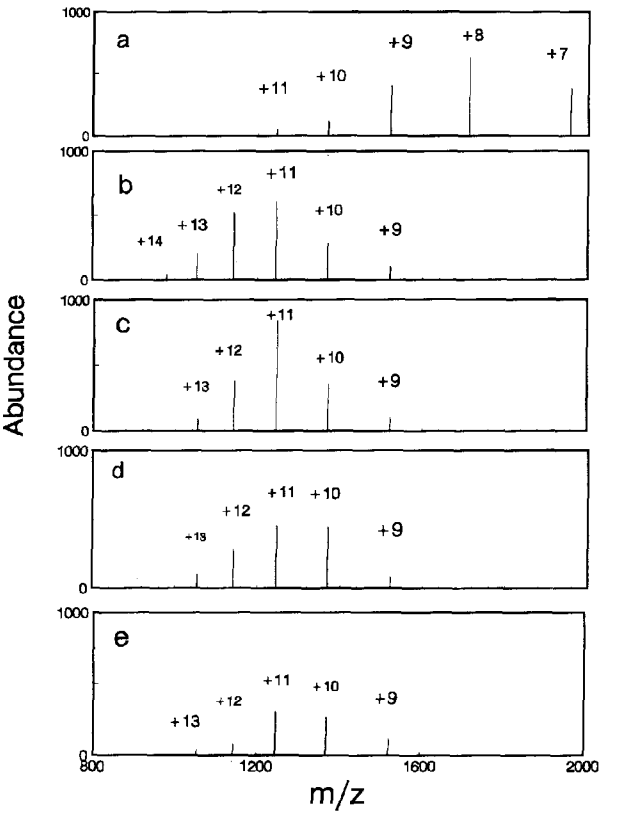

Fig. 2. ES/MS spectrum of a 3.6 μM solution of RNase A in aqueous solutions with different acetic acid concentrations. a: 0.1% acetic acid, pH 3.3. b: 0.5% acetic acid, pH 2.9. c: 2% acetic acid, pH 2.6. d: 4% acetic acid, pH 2.5. e: 8% acetic acid, pH 2.2. For details see Section 2.2.

4. Discussion

Fluorescence changes at 304 nm and CD changes in the ultraviolet indicate environmental changes of Tyr residues and second structural changes of the enzyme respectively. The inactivation of the enzyme that occurs between pH 4 and 2.6 without significantly affecting either the fluorescence or the CD properties of the enzyme shows that the active site of the enzyme is more fragile and is consequently more easily perturbed by the denaturant than the molecule as a whole. Based on susceptibility to proteolysis of RNase A in dilute guanidium chloride, Yang and Tsou [13] have reported that although gross unfolding of the RNase A molecule as a whole cannot be detected by conventional methods, a subtle change in conformation has indeed occurred at the active site during inactivation, which causes the exposure of the active site residues to proteolysis. Our ES/MS experimental results have shown that these subtle conformational changes do increase the positive charges. This increase can only be observed in the transition region of enzyme inactivation and the charge state of the inactivated enzyme is not changed by further increasing

the concentration of acetic acid. So the difference in charge distribution at different pH values could not be caused by a general increase in positive charges due to a lower pH or depend on a difference in ion production in the electrospray inlet due to differences in solvent composition.

In the electrospray ionization process, small multiply charged droplets form initially and then rapidly evaporate forming gas-phase ions. The mechanisms of ion production in electrospray mass spectrometry are still unknown, because the evolution of the effective pH of the rapidly evaporating charge droplets is not known. Nevertheless, ES/MS experimental results indicate that in a dilute acid solution (pH 2–4) the maximum charged state of simple polypeptides consisting of 10–20 amino acid residues correlates with the number of basic amino acid residues (arginine, lysine and histidine; pK_a of side chains = 12.5, 10.5 and 6.0, respectively) plus one for the α-amino group [4].

RNase A consists of 124 amino acid residues with 10 lysines, 4 arginines and 4 histidines. Although all these basic residues on RNase A are protonated in acidified solution (pH 2–4) [14], the observed ES/MS charge distribution of the proteins may also depend on its conformation. It has been demonstrated that only after reduction of disulfide bonds can all basic amino acid residues of RNase A be charged in the ES/MS process, otherwise there are still four left without a positive charge even in 5% acetic acid solution (pH about 2.3) [8]. Because the structure of the protein is partly kept by disulfide bonds, some basic amino acid residues are relatively buried in the interior without contact with the solvent. This suggests that the charge distribution in the ES/MS process is also correlated with the accessibility of the basic amino acid residues to the solvent molecules. In its native structure the calculated accessibility of solvent molecules to the side chain groups of basic amino acid residues decreases in the order [15]: K-1, K-91, K-66, R-39, K-98, K-31, K-37, K-7, K-61, H-119, K-104, R-10, R-33, H-105, K-85, K-41, H-48 and H-12. In a pH 3.3 solution the enzyme retains 70% of its native activity and the observed ES/MS maximum charged state of RNase A is 11^+. This means that 10 basic amino acid residues of the RNase A molecule which are more exposed to solvent in the protein native structure, i.e. K-1, K-91, K-66, R-39, K-98, K-31, K-37, K-7, K-61 and H-119, might be charged, while the eight other basic amino acid residues that are less exposed and buried in the interior might not be charged.

Among the eight basic amino acid residues that are hidden in the protein native structure, K-104, H-105 and K-85 are in the antiparallel β-strands and R-33 is in the α-helix structure held by four disulfide bonds [16]. The observed CD and fluorescence intensities in solutions in the pH range of 2.2–3.3 suggest that the secondary structure of the enzyme molecule is not changed. Because the structure is held by four disulfide bonds, the accessibility of solvent molecules with side chain atoms of these residues may also be unlikely to be changed. Residues K-41 and H-48 are in random region and residues R-10 and H-12 are in a relatively flexible α-helix near the N-terminus. Therefore, the most reasonable explanation is that the increased positive charge is due to the exposure of residues R-10, K-41, H-12 and H-48. Notably, the residues R-10, K-41 and H-12 are located in the active site of the enzyme while H-48 is not. It is believed that the increase of the observed ES/MS maximum charged states of the acid denatured enzyme is caused by the conformational changes of the active site residues R-10, K-41, H-12 and perhaps H-48.

Our studies demonstrate the ability of ES/MS to probe the subtle conformational changes of proteins in solution which are hardly observable by UV, CD and fluorescence measurement. ES/MS measurements provide a basis for a better understanding of the role of the active site conformation in the biological activity of proteins.

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