A Quadrupole/Time-of-Flight Mass Spectrometry Study of Trp-Cage's Conformation

Mingxiang Lin, Zeeshan Ahmed, Christopher R. Taormina, and Kasi V. Somayajula*
Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

Trp-cage is a synthetic 20-residue miniprotein that uses tertiary contacts to stabilize its native conformation. NMR, circular dichroism (CD), and UV-resonance Raman spectroscopy were used to probe its energy landscape. In this quadrupole/time-of-flight study, electrospray ionization charge state distribution (CSD) and solution-phase H/D exchange are used to probe Trp-cage's tertiary structure. The CSDs of Trp-cage and its mutant provide spectra showing a pH-dependent conformation change. Solution-phase H/D exchange in 30% deuterated trifluoroethanol solution of the wild type shows increased protection of one labile hydrogen in the native state. Together, CSDs and solution-phase H/D exchange are demonstrated to constitute a simple but effective means to follow conformation changes in a small tertiary protein. (J Am Soc Mass Spectrom 2007, 18, 195–200) © 2007 American Society for Mass Spectrometry

Trp-cage is a synthetic 20-residue miniprotein of sequence N_1LYIQWLKDG$_{10}$GPSSGRPPPS, which resulted from a de novo design effort [1]. The effort began with an examination of a poorly folded 39 residue long saliva protein of a Gila monster. Using an iterative design effort with selective mutations and truncations, Neidigh et al. [1] created different variants and characterized their folded states using NMR and circular dichroism spectroscopy (CD). Trp-cage, originally referred to as Tc5b, exhibited the most structure in terms of the cage motif. It was found to be 95% folded under physiological conditions.

Trp-cage consists entirely of natural amino acids and has been shown to exhibit elements of tertiary structure in the absence of disulfide bridges, metal ion chelation, or stabilization through oligomerization [2]. NMR and CD data of Neidigh et al. [1] suggest a simple two-state unfolding mechanism. Using the intrinsic fluorescence of Trp, Qiu et al. [3] determined the folding time to be 4 μs, which makes Trp-cage the fastest folding polypeptide to exhibit a tertiary structure.

Several groups previously reported theoretical simulations of Trp-cage's folding [4–11]. Simmerling et al. [4], in their all atom molecular dynamics simulation, calculated a native-state topology consistent with the NMR structure reported by Neidigh et al. [1]. Their simulation at 325 K, which modeled the folding of Trp-cage, found that it converges to a native-state topology within 20 ns. Snow et al. [6] carried out stochastic dynamics simulations over a total modeled folding time of 100 μs and found that the unfolded state retains features resembling the native-state topology.

Ahmed et al. [12] recently used UV-resonance Raman spectroscopy (UVRR) to examine the thermal unfolding behavior of Trp-cage. The authors demonstrated that at physiological pH, Trp-cage initially melts to a more compact intermediate state as temperature is increased from 4 to 20 °C, while retaining its native-state secondary structure. Increasing the temperature to 70 °C results in partial unfolding of this maximally compact intermediate, although it still retains some native-like features even at high temperatures, as suggested by the invariance of the trytophan's χ^2 dihedral angle [12].

Recently, Adams et al. [13] applied electron capture dissociation mass spectrometry to examine the conformation of different charge states of Trp-cage. The authors propose that the +2 state retains the solution-phase native conformation, whereas the +3 charge state, dominated by ionic hydrogen bonds, adopts a more open gas-phase conformation. Furthermore, Adams et al. [13] demonstrated that D-Tyr substitution denatures Trp-cage's native structure. The D-isomer prevents stabilization of the tryptophan side chain that destabilizes the hydrogen-bond network and increases the interior solvent accessibility [13].

In this study, we use the conformational dependency of protein charge state distributions (CSDs) in electrospray ionization mass spectrometry (ESI-MS) to examine the pH dependency of Trp-cage's conformation. Well documented for over a decade, protein CSDs in ESI-MS show a shift toward higher charge state values upon protein unfolding [14–17]. Through this marker, we can discriminate between different global conformations of Trp-cage as the solvent pH is varied. Furthermore, we also probe Trp-cage conformation by solution-phase hydrogen/deuterium exchange, which is an established mass spectrometric technique for protein conformation and dynamics studies [18].

Experimental

Mass spectrometry measurements were performed on a hybrid quadrupole time-of-flight mass spectrometer (Q-ToF MS, API-US, Micromass UK Ltd., Manchester, UK), equipped with a Z-spray electrospray source. The typical instrument operation parameters at the source region for this study were: capillary voltage 3.5 kV, cone voltage 40 V, source temperature 80 °C, desolvation temperature 150 °C, cone gas flow 60 L/h, and desolvation gas flow 600 L/h. Sample solutions were injected from the embedded syringe pump at a flow rate of 10 μL/min. Argon was used as the collision gas with a pressure of 5×10^{-5} bar at the analyzer pressure. The collision energy was kept at 10 V in the ToF MS mode. The instrument was calibrated externally by a cesium iodide solution up to 2500 amu. Spectra were accumulated at 1 s per scan at reflectron V mode with a full-width half-maximum resolution of 10,000. Data acquisition and processing were performed by use of MassLynx V4.0 software (Waters Corp., Milford, MA).

Trp-cage peptide was obtained at >95% purity from the Pittsburgh peptide synthesis facility and was used at 40 μM concentrations. D-Tyr substituted Trp-cage peptide was provided by Dr. Zubarev at Uppsala University and was used at 18 μM concentrations. The pH in all of the experiments was adjusted using glacial acetic acid (Aldrich, St. Louis, MO) and NH_4OH (Aldrich).

Charge state distribution analysis was carried out by measuring the intensity differences of the ToF spectrum of Trp-cage aqueous solutions at pH 3 and pH 7. A complete deuterium-exchanged Trp-cage spectrum was obtained from a 100% D_2O Trp-cage solution buffered at pH 7 using deuterated acetic acid and ammonium hydroxide (Sigma Chemical, St. Louis, MO). By dissolving Trp-cage directly in 70% D_2O and 30% deuterated trifluoroethanol, CF_3CH_2OD (d-TFE, Sigma Chemical), we examined the amide hydrogen protection against hydrogen–deuterium (H/D) exchange in a 30% TFE (Aldrich) solution by continuously monitoring the temporal dependency of the spectrum up to 30 min.

Results and Discussion

Trp-cage charge state distributions were obtained at pH 3 and pH 7. As shown in Figure 1a, the ESI mass spectrum of the wild-type Trp-cage at pH 7 shows three peaks corresponding to the +1, +2, and +3 charge state, with +2 being the dominant state. The intensity ratio of charge state +2/charge state +3 is 2.0. The intensity ratios obtained at different solvent conditions and pH values are summarized in Table 1. As the solution pH is decreased from pH 7 to pH 3, the CSD shifts from the +2 to the +3 state and the intensity ratio of +2 to +3 charge states decreases from 2.0 to 0.3. The weak +1 state observed at pH 7 is no longer observed at pH 3. Our results indicate that at low pH Trp-cage denatures, adopting a more open conformation results in a CSD shift to the +3 charge state. Contrary to the behavior of wild-type Trp-cage, the D-Tyr substituted mutant shows a dominant +3 state at either pH with the intensity ratio of +2 to +3 charge states being 0.2 at pH 3 and 0.6 at pH 7. The spectrum obtained at pH 7 is shown in Figure 2b. The difference in intensity ratio at the two pH values likely arises from charge repulsion. The D-Tyr substituted mutant adopts a more open conformation at either pH value compared to that of the wild-type Trp-cage at pH 7. Our results offer further support of the proposal introduced by Adams et al. [13] that the substitution with the D-Tyr isomer within Trp-cage denatures the native Trp-cage conformation.

We further probed the Trp-cage by examining its conformation in an aqueous solution of 30% TFE. Helix-stabilizing effects of aqueous TFE are well documented [19]. Extensive work on helix-stabilizing effects of TFE indicate that 30% vol/vol is the optimum concentration of TFE needed for helical stabilization [1, 19]. Neidigh et al. [1] showed that Trp-cage exhibits a pronounced resistance to thermal denaturation in 30% vol/vol aqueous TFE solution. They reported an increased amide protection of Leu HN and Trp Hϵ1 hydrogens in TFE solution, which indicates a more stable native state. Based on this evidence Neidigh et al. argued that the addition of TFE led to increased "nativeness" of the protein.

Figure 2 shows ESI-ToF spectra of 40 μM Trp-cage in 30% TFE and 70% H_2O solution at pH 3 and pH 7. The intensity ratio of +2 and +3 charge states at pH 7 is 2.5 and the value is comparable to the value of 2.0 observed in aqueous solution. The stabilizing effect of TFE can be seen from the intensity ratio of +2 and +3 charge states at pH 3. The ratio is 0.3 in pure water, whereas the ratio changes to 1.3 in 30% TFE aqueous solution. This signifies an increase in "nativeness" or compactness of Trp-cage in aqueous TFE solutions. Our conclusions agree with the observations of Neidigh et al. [1].

We further probed the Trp-cage conformation by examining the H/D exchange protection of labile hydrogens. The spectrum of Trp-Cage in 100% H_2O is shown in Figure 3a and in 100% D_2O, obtained within 1 min after dissolution, is shown in Figure 3b. The
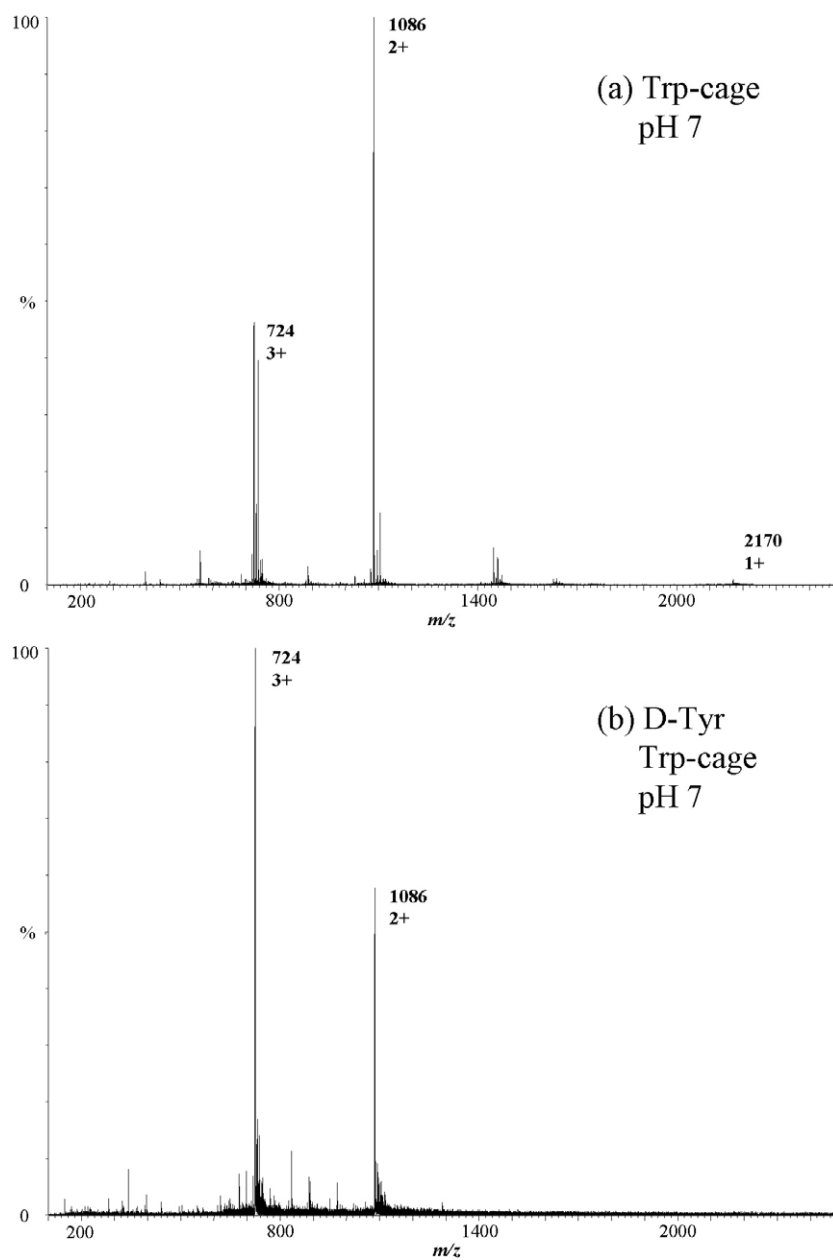
Figure 1. ESI-ToF spectrum of (**a**) 40 μM Trp-cage in H_2O at pH 7; (**b**) 18 μM D-Tyr substituted Trp-cage in H_2O at pH 7.

Table 1. Intensity ratio of charge state +2/charge state +3 of Trp-cage and D-Tyr substituted Trp-cage under different solvent conditions

Experimental conditions	Intensity of charge state +2 / Intensity of charge state +3
Trp-cage in H_2O, pH 3	0.3
Trp-cage in H_2O, pH 7	2.0
D-Tyr substituted Trp-cage in H_2O, pH 3	0.2
D-Tyr substituted Trp-cage in H_2O, pH 7	0.6
Trp-cage in 30% TFE and 70% H_2O, pH 3	1.3
Trp-cage in 30% TFE and 70% H_2O, pH 7	2.5
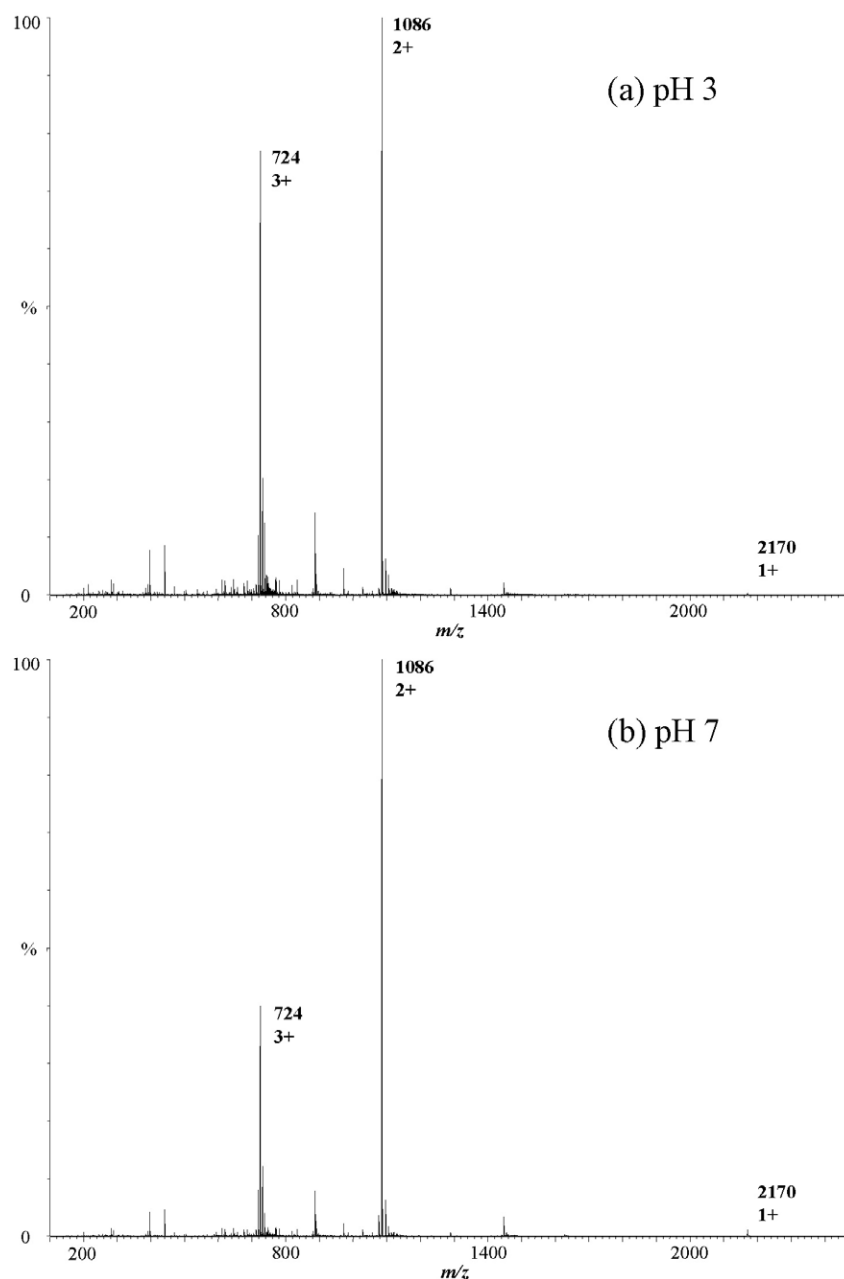
Figure 2. ESI-ToF spectrum of 40 μM Trp-cage in 30% TFE and 70% H_2O solution at (**a**) pH 3 and (**b**) pH 7. Some minor fragments arising from in-source fragmentation are observed.

number of labile hydrogens in Trp-cage is 34, including two N-terminal hydrogens, 15 backbone amide hydrogens, one C-terminal hydrogen, and 16 side-chain hydrogens. The number of labile hydrogens on each side chain is indicated below:

$$
\begin{array}{cccccccccccccccccccc}
N & L & Y & I & Q & W & L & K & D & G & G & P & S & S & G & R & P & P & P & S \\
2 &   & 1 &   & 2 & 1 &   & 2 & 1 &   &   &   & 1 & 1 &   & 4 &   &   &   & 1
\end{array} \quad (1)
$$

A total of 34 labile hydrogens are observed from comparing isotopic distribution of singly charged Trp-cage peak at 100% H_2O and 100% D_2O (Figure 3a and b).

Note that a 35-mass-unit shift is observed as a result of the addition of a deuterium atom, instead of a proton, in the singly charged Trp-cage ion electrosprayed from 100% D_2O. Likewise, an 18-mass-unit shift is observed in the doubly charged Trp-cage ion obtained from 100% D_2O as a result of the addition of two deuterium atoms.

The isotopic distribution of singly charged Trp-cage ions in 70% D_2O and 30% d-TFE is shown at 1- and 20-min intervals after dissolution in Figure 3c and d. After 20 min, the isotopic distribution showed an increase of m/z value by 1 amu and is identical to the spectrum obtained from Trp-cage in 100% D_2O (Figure
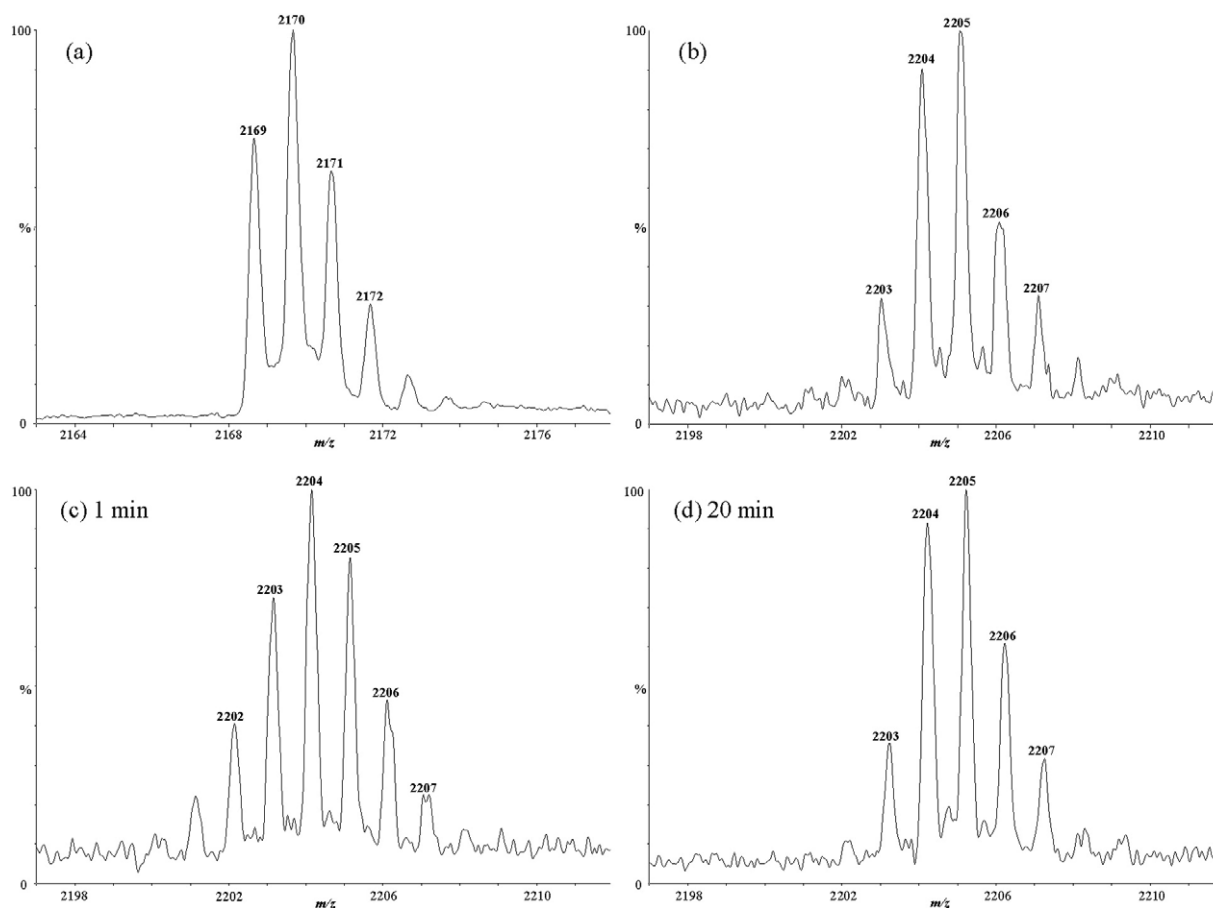
Figure 3. Isotopic distribution of singly charged Trp-cage in (**a**) 100% H_2O, pH 7; (**b**) 100% D_2O, pH 7; (**c**) in 30% d-TFE D_2O solution pH 7 after 1-min incubation; (**d**) in 30% d-TFE D_2O solution pH 7 after 20-min incubation.

3b). Beyond the 20-min mark the spectrum remains unchanged. The isotopic distribution of doubly charged Trp-cage ion obtained at 1 and 20 min yields the same result. Thus the time-dependent spectra of Trp-cage show increased H/D exchange protection of only one labile hydrogen atom. This increased protection against H/D exchange supports the TFE's stabilizing effect on Trp-cage's native state. The fact that native Trp-cage does not show any significant protection of the labile hydrogens in a water solution indicates that the native and nonnative unfolded conformations are separated by a small energy barrier. This allows for rapid interconversion between the folded and unfolded conformations. In 30% TFE solution, the rate of interconversion is sufficiently slowed down to afford some protection of the protein's labile hydrogen.

In this work we successfully applied ESI-MS CSD methodology to Trp-cage, which is one of the smallest known peptides to show conformationally dependent CSD shifts [15–17, 20]. Our results indicate that Trp-cage conformation is pH sensitive and has maximum stability at around physiological pH. The lowering of the pH beyond the pH 6–7 region disrupts ionic interaction in the native conformation leading to denatur-ation of Trp-cage. TFE solutions are shown to stabilize the native conformation and provide resistance against acid denaturation. This study demonstrates the utility of ESI-MS CSD in determining the compactness of tertiary proteins/polypeptides in a rapid and efficient manner.

Acknowledgments

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