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## A R T I C L E S

# Water Molecule Adsorption on Short Alanine Peptides: How Short Is the Shortest Gas-Phase Alanine-Based Helix?

Motoya Kohtani and Martin F. Jarrold*

*Contribution from the Chemistry Department, Indiana University, 800 East Kirkwood Avenue, Bloomington, Indiana 47405*

Received February 9, 2004; E-mail: mfj@indiana.edu

**Abstract:** Water adsorption measurements have been performed under equilibrium conditions for unsolvated Ac-A$_n$K+H$^+$ and Ac-KA$_n$+H$^+$ peptides with $n = 4-10$. Previous work on larger alanine peptides has shown that two dominant conformations (helices and globules) are present for these peptides and that water adsorbs much more strongly to the globules than to the helices. All the Ac-KA$_n$+H$^+$ peptides studied here (which are expected to be globular) adsorb water strongly, and so do the Ac-A$_n$K+H$^+$ peptides with $n < 8$. However, for Ac-A$_n$K+H$^+$ with $n = 8-10$ there is a substantial drop in the propensity to adsorb water. This result suggests that Ac-A$_8$K+H$^+$ is the smallest Ac-A$_n$K+H$^+$ peptide to have a significant helical content in the gas phase. Water adsorption measurements for Ac-V$_n$K+H$^+$ and Ac-L$_n$K+H$^+$ with $n = 5-10$ suggest that the helix emerges at $n = 8$ for these peptides as well.

## Introduction

Residues near the center of an extended α-helix form two helical hydrogen bonds (to the backbone CO and NH groups) but at the ends of a helix, half of the hydrogen bonds are missing. The first four N−H groups at the N-terminus and the first four CO groups at the C-terminus of an uncapped peptide do not form helical hydrogen bonds. Consequently, as a helix becomes shorter, it becomes less stable. Short helical sections are common in proteins where their ends are usually capped by interactions with side chains from other residues. But for small isolated peptides, the helical conformation was believed to be unstable until relatively recently.

Helix formation in solution is usually not a two-state problem involving only fully helical and completely nonhelical conformations. Instead, there is an ensemble of partially helical structures that can be characterized in terms of an average fraction or percentage of the residues that are helical. The statistical mechanical models of α-helix formation originally proposed by Zimm and Bragg[1] and Lifson and Roig[2] can account for these observations. These models, which break helix formation into nucleation and propagation steps, also predicted that short peptides should not be helical because it is difficult to overcome the low probability for helix nucleation in a short peptide.[3,4]

The fact that relatively short peptides can assume an α-helical structure is now well established.[5] In particular, peptides such as Ac-A$_4$KA$_4$KA$_4$KA-NH$_2$, with no helix stabilizing side chain

interactions or any other method of inducing a helix, have been shown to be up to 80% α-helical in aqueous solution at 1 °C (primarily because of the high helix propensity of alanine). There is evidence suggesting that for short helical sections in proteins and peptides (<6 residues) a 3$_{10}$-helix (with $i,i+3$ hydrogen bonds) is competitive with an α-helix with ($i,i+4$ hydrogen bonds).[6,7] In proteins, 3$_{10}$-helices often occur at the ends of α-helices.[8] Recent theoretical studies also suggest that the 3$_{10}$-helix is the lowest energy conformation for capped neutral polyalanine peptides in the gas phase.[9,10] In addition to the "natural" peptides mentioned above, a variety of methods have been used to "force" peptides into a helical conformation. Beginning with the first designed peptide,[11] short helices have been made by a variety of methods such as metal ion binding[12−14] (the shortest being a pentapeptide[15]), N-terminal and C-terminal capping templates,[16−20] and side chain tethers or hydrogen bond mimics.[21,22]

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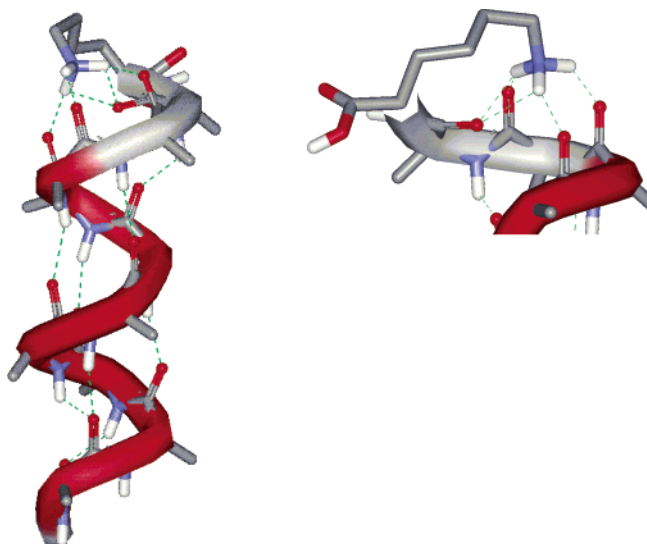
**Figure 1.** A representative Ac-A$_{10}$K+H$^+$ $\alpha$-helix from molecular dynamics simulations with the CHARMM force field. The C-terminus end is magnified to show the hydrogen bonds (green dashed lines) between the lysine $-NH_3^+$ group and the dangling carbonyl groups at the C-terminus.

In our laboratory, we have studied helix formation in unsolvated peptides. The motivation for these studies is that the intrinsic structural properties of the peptide can be resolved from those imposed by interactions with the solvent. A variety of different environments are important in biological systems, ranging from aqueous solution to the hydrophobic interior of membranes. Studies of unsolvated peptides provide a starting point for investigating how the geometry depends on the environment. We have found that two conformations are important for unsolvated peptide ions based on nonpolar residues (alanine, valine, and leucine): the helix and the globule (a compact, random-looking, three-dimensional structure). The conformation of peptide ions in vacuo is strongly influenced by the location of the charge. Placing a protonated lysine side chain[23] or an alkali metal cation[24] at the C-terminus stabilizes an $\alpha$-helix through favorable interactions of the charge with the helix macrodipole. Helices with a C-terminus lysine (as considered here) are also stabilized by helix capping interactions where the protonated amine from the lysine side chain forms hydrogen bonds to the dangling CO groups at the C-terminus of the helix, as illustrated in Figure 1 (which shows representative Ac-A$_{10}$K+H$^+$ $\alpha$-helix from molecular dynamics simulations with the CHARMM force field). On the other hand, if the basic lysine residue is located at the N-terminus, the interaction of the charge with the helix macrodipole destabilizes the helix and the peptide adopts a globular conformation. Thus Ac-A$_n$K+H$^+$ peptides (Ac = acetyl, A = alanine, and K = lysine) with $n$ = 15$-$20 are completely helical in the gas phase while the Ac-KA$_n$+H$^+$ analogues are globular.[26$-$28]

The structures described above were assigned using ion mobility measurements (the main mass spectrometry based method for probing conformation). The mobility of an ion, how rapidly it travels through an inert buffer gas under the influence of a weak electric field, depends on its average collision cross section, which in turn depends on the ion's geometry. For peptides with more than 10 residues, it is easy to distinguish between helical and globular conformations. However, for smaller peptides it becomes increasingly difficult to make this distinction because the difference between the cross sections of the helices and globules becomes small. Thus it is difficult to determine the size of the shortest stable helix from ion mobility measurements. Since the larger Ac-A$_n$K+H$^+$ peptides are completely helical in the gas phase, the size of the shortest stable helix provides important information about the intrinsic stability of the helical conformation. Spectroscopic approaches to studying the conformations of unsolvated peptides (such as IR ion dip spectroscopy) are currently limited to individual amino acids and peptides with only few amino acids.[26$-$28] Thus both ion mobility measurements and spectroscopic methods are currently unable to address the question of the shortest unsolvated helix.

In a recent publication, we reported that globular Ac-KA$_{15}$+H$^+$ and Ac-KA$_{20}$+H$^+$ peptides adsorbed a single water molecule much more strongly than helical Ac-A$_{15}$K+H$^+$ and Ac-A$_{20}$K+H$^+$.[29] In fact, we were not able to observe the Ac-A$_{15}$K + H$^+$+H$_2$O and Ac-A$_{20}$K+H$^+$+H$_2$O complexes under equilibrium conditions for any combination of temperature and water vapor pressure we employed. This observation suggests the possibility of using water adsorption as a probe of helix formation for the smaller peptides where ion mobility measurements are not able to effectively distinguish helical and globular conformations. The idea of using ion equilibrium measurements as a structural probe is not new. Mautner[30$-$32] and Kebarle[33,34] have studied the hydration properties of simpler molecular ions extensively and used the results to make inferences about structure on many occasions. Thus, the method itself is well established.

Here we report the results of water adsorption measurements (performed under equilibrium conditions) for Ac-KA$_n$+H$^+$ and Ac-A$_n$K+H$^+$ ($n$ = 4$-$10) to probe helix formation in the smaller Ac-A$_n$K+H$^+$ peptides (the Ac-KA$_n$+H$^+$ peptides are expected to be globular for all values of $n$). A substantial difference between the water adsorption properties of the Ac-KA$_n$+H$^+$ and Ac-A$_n$K+H$^+$ peptides first emerges at $n$ = 8. This observation is interpreted as indicating that Ac-A$_8$K+H$^+$ is the smallest Ac-A$_n$K+H$^+$ peptide to show a significant helical content. Water adsorption measurements were also performed for AcV$_n$K+H$^+$ ($n$ = 5$-$10) and AcL$_n$K+H$^+$ ($n$ = 5$-$10). The results suggest the helix first emerges at $n$ = 8 for these peptides as well.

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## Experimental Section

All experimental data were obtained using a temperature-variable injected-ion drift tube apparatus that has been described in detail elsewhere.[35] Briefly, desolvated ions are produced by an electrospray source with a heated capillary interface and pass through a differentially pumped region before being injected into a 30.5 cm long drift tube. As the ions enter the drift tube, they are collisionally heated and then rapidly cooled to the drift tube temperature by collisions with the buffer gas. Because of this transient heating cycle, the peptide ions are not expected to retain a memory of their solution phase conformations. The drift tube consists of four sections that can be cooled with liquid nitrogen. The temperature of each section is regulated to better than $\pm 0.5$ K with microprocessor-based temperature controllers coupled to solenoid valves that regulate the liquid nitrogen flow. The drift tube contains a series of guard rings that establish a uniform electric field along its length. A helium buffer gas pressure of around 4 Torr was employed. After traveling down the drift tube under the influence of a weak electric field, some of the ions exit through a small aperture. These ions are focused into a quadrupole mass spectrometer, and after being mass analyzed, they are detected by an off-axis collision dynode and dual microchannel plates. Drift time distributions were obtained by admitting a short pulse of peptide ions into the drift tube and recording their arrival time distribution at the detector. The arrival time distribution is then converted into a drift time distribution by accounting for the flight time outside of the drift tube. The drift times for the main features are converted into collision cross sections using standard methods.[36]

Water adsorption measurements were performed by admitting a known partial pressure of water vapor into the drift tube and obtaining the intensity of the reactant and product ions from the mass spectrum. The equilibrium constant for water adsorption is calculated from the following expression:

$$K_{eq} = \frac{I_{p+w}}{I_p \cdot P_w}$$

where $I_p$ and $I_{p+w}$ are the integrated intensities of the peptide and peptide−water complex peaks in the mass spectrum, and $P_w$ is the partial pressure of water vapor in the drift tube in atmospheres. A leak valve was used to regulate the water vapor pressure, and the measured pressure was corrected for the buffer gas flow. The corrected water vapor pressures were around 1−12 mTorr with 4 Torr of He buffer gas. We have considered possible sources of error in these measurements in detail in previous publications.[29,37] The present study was conducted with an $E/p$ (drift field/total pressure) of ∼2.3 V/cm Torr which meets the low-field criteria[36] in similar studies.[38,39] The effective temperature increase for the ions in the drift tube due to acceleration from the electric field is estimated to be well below 1 K.[40] Lowering the drift voltage from 280 to 180 V gave identical results, which confirms that we are measuring equilibrium constants (rather than reaction kinetics) and that the measured values are independent of conditions. The measurements reported here were performed with a drift tube temperature of 223 K. This low temperature is necessary to promote water adsorption. The conditions employed here are similar to those used in our previous study of water adsorption on helical and globular alanine-based peptides.[29]
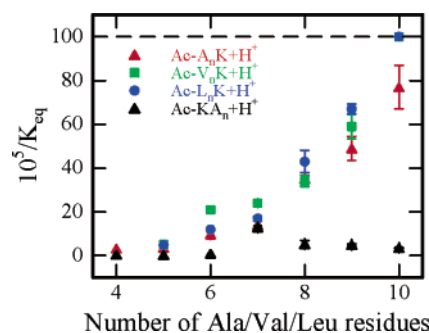
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**Figure 2.** Plot of $10^5/K_{eq}$ (where $K_{eq}$ is the measured equilibrium constant for adsorption of the first water molecule onto the peptide in question) against the number of nonpolar residues for Ac-KA$_n$+H$^+$, Ac-A$_n$K+H$^+$, Ac-V$_n$K+H$^+$, and Ac-L$_n$K+H$^+$. The error bars are the standard deviation of the mean.

All peptides were synthesized on an Applied Biosystems model 433A peptide synthesizer using *Fastmoc* chemistry. After cleavage with a cocktail of 95% trifluoroacetic acid (TFA) and 5% water, the peptides were precipitated, centrifuged, and then lyophilized. The peptides were used without further purification. The electrospray solutions consisted of 2 mg of peptide in 1.0 mL of TFA and 0.1 mL of water.

## Experimental Results

Figure 2 shows a plot of $10^5/K_{eq}$ (where $K_{eq}$ is the measured equilibrium constant for adsorption of the first water molecule) against the number of nonpolar amino acid residues (Ala, Val, or Leu) in the peptides. Peptides that adsorb water weakly have large values of $10^5/K_{eq}$, while peptides that adsorb water strongly have small values. The dashed line at $10^5/K_{eq} = 100$ represents a (somewhat conservative) limit beyond which it is difficult to quantify the peptide water complex. The value of $10^5/K_{eq} = 100$ corresponds to a peptide−water complex abundance of around 1% of the unsolvated peptide intensity. As described in the Introduction, peptides with globular conformations are expected to adsorb a water molecule (and have small values of $10^5/K_{eq}$), while peptides with helical conformations are not expected to adsorb water under the conditions employed (and so they are expected to have a large value for $10^5/K_{eq}$). Thus the $10^5/K_{eq}$ values provide a measure of helix content. For the Ac-KA$_n$+H$^+$ peptides (black triangles in Figure 2), the $10^5/K_{eq}$ values remain small from $n = 4$ up to $n = 10$ but show a small maximum for $n = 7$. For Ac-A$_n$K+H$^+$, the $10^5/K_{eq}$ values start small and closely track the values for the Ac-KA$_n$+H$^+$ peptides up to $n = 7$. Beyond $n = 7$, the $10^5/K_{eq}$ values increase sharply for Ac-A$_n$K+H$^+$ and approach the $10^5/K_{eq} = 100$ limit for $n = 10$. The $10^5/K_{eq}$ values for Ac-V$_n$K+H$^+$ and Ac-L$_n$K+H$^+$ closely track the results for the Ac-A$_n$K+H$^+$ peptide.

Collision cross sections were measured at the temperature used for the hydration studies (223 K). The 223 K cross sections are slightly but systematically larger (by around 6 Å$^2$) than the values measured at room temperature. This uniform increase is not indicative of a structural change but is due to the long-range interactions between the peptide ion and the helium buffer gas becoming more important as the temperature is lowered. Hence, there is no indication that the peptide structures probed in the hydration experiments at 223 K are different from those present at room temperature.

## Discussion

Ion mobility measurements for Ac-X$_n$K+H$^+$ and Ac-KX$_n$+H$^+$ peptides with X = Ala, Val, and Leu and $n > 10$

show that the Ac-X$_n$K+H$^+$ peptides have systematically larger collision cross sections than their Ac-KX$_n$+H$^+$ analogues.[23,25,41,42] The lowest energy conformations found in molecular dynamics (MD) simulations for Ac-X$_n$K+H$^+$ peptides with $n$ >10 are helices, while, for Ac-KX$_n$+H$^+$ peptides, the lowest energy conformations are globules. Average cross sections calculated for the low energy conformations derived from the MD simulations match the measured cross sections to within the expected uncertainty. In the simulations, it is assumed that the lysine carries that charge and, hence, the structural difference between the Ac-X$_n$K+H$^+$ and Ac-KX$_n$+H$^+$ peptides results because in the former the charge interacts favorably with the helix macrodipole (stabilizing the helix), while, in the latter, where the charge is at the N-terminus, the interaction with the helix macrodipole is helix destabilizing. The helical conformation is also stabilized by hydrogen bonds between the protonated amine group of the lysine side chain and the dangling carbonyl groups at the C-terminus of the helix (see Figure 1). This helix capping interaction is stabilizing for short and long peptides, while the interaction between the charge and the helix macrodipole is expected to show a dependence on the length of the helix. Comparison of the measured cross section for Ac-A$_{10}$K+H$^+$ (234 Å$^2$) with cross sections calculated for ideal $\pi$-helix (224 Å$^2$), $\alpha$-helix (234 Å$^2$), 3$_{10}$-helix (251 Å$^2$), and a polyproline II helix (325 Å$^2$) show that the ideal $\alpha$-helix provides the best match to the experimental value and that the 3$_{10}$-helix (suggested by some calculations to be the lowest energy conformations for capped neutral polyalanine peptides[9,10]) can be ruled out.

For $n$ < 10, the difference between the calculated cross sections for the helical and globular conformations becomes small enough that it is no longer possible to make structural assignments with confidence. While the Ac-KX$_n$+H$^+$ peptides are expected to retain globular conformations for $n$ < 10, the Ac-X$_n$K+H$^+$ peptides are expected to undergo a transition from helical to globular as $n$ decreases because the helix becomes less stable as the peptide gets shorter. The measured cross sections for the smaller Ac-A$_n$K+H$^+$ peptides remain slightly but significantly larger than those for the Ac-KA$_n$+H$^+$ analogues all the way down to $n$ = 4. Thus they do not provide much of a clue as to where the transition to the globular conformation occurs.

As mentioned above, previous work has shown that unsolvated Ac-A$_{15}$K+H$^+$ and Ac-A$_{20}$K+H$^+$ (which are helical) do not adsorb water under equilibrium conditions, while Ac-KA$_{15}$+H$^+$ and Ac-KA$_{20}$+H$^+$ (which are globular) readily adsorb a water molecule.[29] Molecular dynamics simulations were performed in an effort to understand this difference, and it was attributed to the globular conformation being able to bind the water molecule through a network of hydrogen bonds. The most favorable water binding sites found for the globular peptides are pockets or clefts where the water molecule is embedded and surrounded by multiple hydrogen bonding partners. The water molecule does not appear to be bound to the protonation site. In the simulations of the globular AcKA$_{15}$+H$^+$ and AcKA$_{20}$+H$^+$ peptides, the protonation site is already involved in multiple hydrogen bonds, and in order to introduce a water molecule into the self-solvation shell around the protonation site, it is necessary to displace one of the existing hydrogen bonding partners. For smaller peptides, the formation

of strongly binding clefts and pockets is more difficult. At the same time, the self-solvation shell around the protonation site is expected to become less crowded. So at some point it should become favorable to bind the water molecule to the protonation site.[43,44] It is evident from Figure 2 that the Ac-KA$_n$+H$^+$ peptides (which are expected to be globular) remain strong adsorbers of water (low values of 10$^5$/$K_{eq}$) from $n$ = 10 to $n$ = 4. There is a small maximum in the 10$^5$/$K_{eq}$ values at $n$ = 7 which may be related to a change in the nature of the interaction between the peptide and the water molecule with peptide size.

Because the backbone carbonyl and amide groups are involved in helix hydrogen bonds in the helical Ac-A$_{15}$K+H$^+$ and Ac-A$_{20}$K+H$^+$ peptides, they are not available to form hydrogen bonds to a water molecule. Thus, in the simulations, the most favorable site to bind a water molecule is at the ends of the helices, where hydrogen bonding sites are available. However, it is not possible to generate a network of hydrogen bonds, and the water is only weakly bound to the ends of the helices. This behavior is not expected to depend strongly on the length of the helix, so water adsorption should be unfavorable for all helices, regardless of their length.

It is evident from the results shown in Figure 2 that the Ac-A$_n$K+H$^+$ peptides with $n$ < 8 bind water strongly and the 10$^5$/$K_{eq}$ values are similar to those for the globular Ac-KA$_n$+H$^+$ peptides. Thus the Ac-A$_n$K+H$^+$ peptides with $n$ < 8 are assigned to globular conformations. For Ac-A$_8$K+H$^+$, there is a substantial difference in the 10$^5$/$K_{eq}$ values for the Ac-KA$_n$+H$^+$ and Ac-A$_n$K+H$^+$ peptides. For $n$ > 8, the 10$^5$/$K_{eq}$ values for the Ac-A$_n$K+H$^+$ peptides increase sharply and approach the limiting value of 10$^5$/$K_{eq}$ = 100 at $n$ = 10. Thus the transition between the helix and globule appears to occur between $n$ = 8 and $n$ = 10, with $n$ = 8 being the smallest peptide with a significant helical content.

If the helix and globule coexist in the transition region but do not interconvert, the measured equilibrium constants may depend on the water vapor pressure, while the absence of a pressure dependence suggests that the helical and globular conformations interconvert on the experimental time scale (around 10 ms). The measured equilibrium constants do not display a significant pressure dependence; however, this result cannot really be used to distinguish between the two possibilities mentioned above because only a small fraction of the peptides adsorb a water molecule in the transition region.

Water adsorption measurements were also performed for the valine and leucine analogues of the Ac-A$_n$K+H$^+$ peptides. 10$^5$/$K_{eq}$ values for Ac-V$_n$K+H$^+$ and Ac-L$_n$K+H$^+$ show the same behavior as that found for the alanine peptides (see Figure 2). The 10$^5$/$K_{eq}$ values increase sharply in the $n$ = 8 to $n$ = 10 size range and approach the 10$^5$/$K_{eq}$ = 100 limit at $n$ = 10. Thus it appears that helices first appear for Ac-V$_n$K+H$^+$ and Ac-L$_n$K+H$^+$ at around the same size as that for the alanine peptides.

## Conclusions

In this work we have explored the possibility of using water adsorption measurements to distinguish between helical and

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globular conformations of unsolvated Ac-X$_n$K+H$^+$ peptides with X = Ala, Val, and Leu. For $n < 10$, ion mobility measurements are not effective in distinguishing between these two forms because they have similar cross sections. We find that Ac-KA$_n$+H$^+$ with $n = 4-10$ adsorb water strongly, which is consistent with these peptides retaining globular conformations, as expected. The Ac-A$_n$K+H$^+$ peptides, on the other hand, are expected to undergo a transition from helical to globular conformations for $n < 10$ because the helix becomes intrinsically less stable as the length decreases. We find that Ac-A$_n$K+H$^+$ with $n < 8$ adsorb water strongly and have $10^5/K_{eq}$ values that are very similar to those for the globular Ac-KA$_n$+H$^+$ peptides.

For $n = 8-10$. the $10^5/K_{eq}$ values increase sharply suggesting that a transition to helical conformations occurs in this size range. Results for Ac-V$_{10}$K+H$^+$ and Ac-KL$_n$+H$^+$ suggest that the transition to a helical conformation occurs in a similar size range to the alanine peptides.