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Stability of Secondary Structural Elements in a Solvent-Free Environment: The α Helix

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ABSTRACT **The stability of the α helix as an element of secondary structure is examined in the absence of solvation, in the gas phase. Mass-analyzed ion kinetic energy (MIKE) spectrometry was applied to measure intercharge repulsion and intercharge distance in multiply protonated melittin, a polypeptide known to possess a stable helical structure in a number of different environments. The experimental results, interpreted in combination with molecular mechanics calculations, suggest that triply charged melittin retains its secondary structure in the gas phase. The stability if the α-helical conformation of the polypeptide in the absence of solvent molecules reflects the fact that a network of intrinsic helical hydrogen bonds is energetically more favorable than unfolded conformations. Proteins 27:165–170**
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Key words: α helix; secondary structure; gas phase; molecular mechanics; mass spectrometry; kinetic energy release; melittin

INTRODUCTION

Noncovalent interactions of biomolecules in solutions are key elements in the biological activities of the vast majority of proteins, peptides, and oligonucleotides. The specificities of such interactions are closely related to the higher order structures of biopolymers, which have been studied by x-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and other techniques. For proteins and peptides in solution, a sharp transition between the native conformation and a random coil usually results from the subtle balance between inter- and intramolecular forces, such as electrostatic interactions, hydrogen bonding, and hydrophobic packing.[1,2] The presence of a solvent with a high dielectric constant reduces the influence of electrostatic repulsions that might otherwise favor unfolded structures with lower charge densities. At the same time, polar solvent molecules can form nonspecific hydrogen bonds with the polar groups of the protein backbone, thus competing with the highly oriented network of hydrogen bonds responsible for the formation and maintenance of the stable elements of secondary structure, α helices and β sheets, in particular. The presence of solvent is also responsible for the hydrophobic interactions that play a very important (but as yet not well understood) role in protein folding.[2,3] This complicated picture makes it difficult to examine the importance of any particular single type of interaction.

One of the approaches to this problem would be complete removal of the solvent molecules, which would enable us to identify and study intrinsic intramolecular forces. Such simplification of the problem may serve as a very important first step in our understanding of the behavior of this complex system. Such a solvent-free environment is offered by the gas phase under vacuum.[4,5] Until recently, however, this approach was limited to studies using theoretical methods, molecular mechanics in particular, due to apparent difficulties associated with the evaporation of large biomolecules and their storage in the gas phase.

The introduction of electrospray ionization[6,7] and matrix-assisted laser desorption ionization[8] mass spectrometry have enabled researchers to desorb macromolecules in the mass range up to and above 200,000 Da and to study their behavior in the gas phase. Stable conformations of polypeptides and proteins, once thought to be impossible outside the aqueous environment, have become subjects of experimental studies in the gas phase during the last several years. The existence and relative stabilities of the gas phase conformations of peptides and proteins are suggested by target capture[9] and by gas-phase H/D exchange[10] experiments, as well as by

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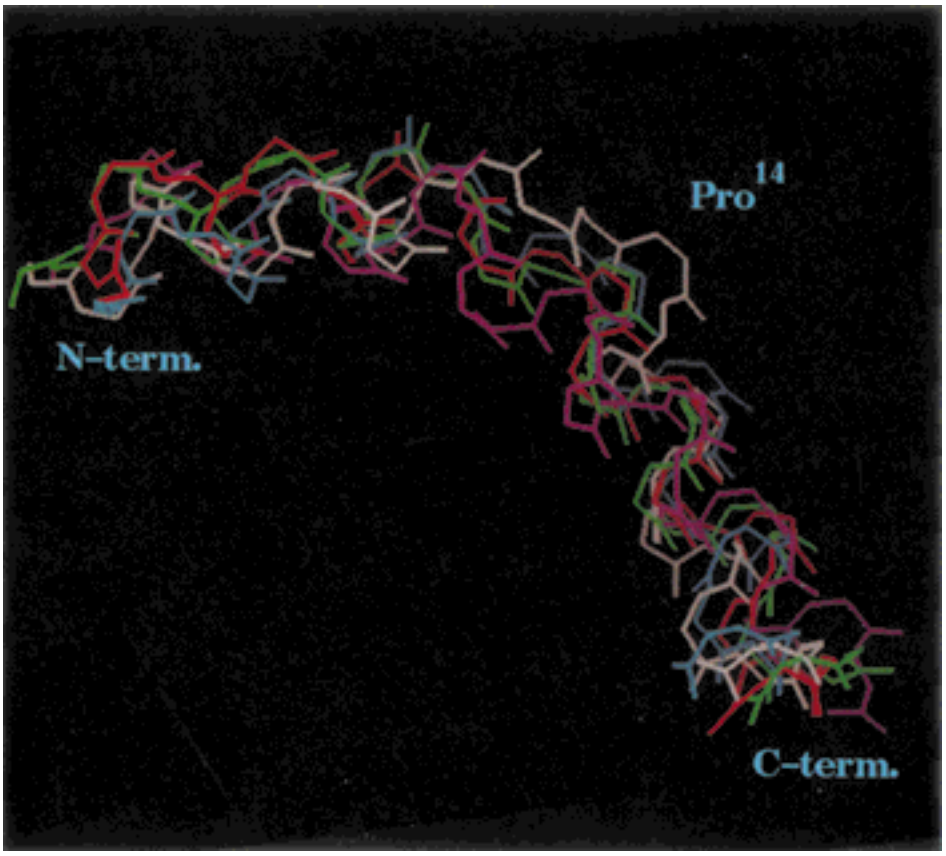

Fig. 1. Backbone traces of several low-energy conformations of melittin collected throughout the 30 ps dynamics simulation of the triply charged polypeptide at 500 K in the absence of solvent. The purple structure represents melittin conformation in crystals.[18]

the results of collisional cross section measurements for multiply protonated biomolecules.[11–13] Although the results of many experiments do suggest that peptides and proteins in many instances maintain rather compact conformations in the absence of solvent, no strong evidence has been presented that these compact three-dimensional structures bear any resemblance to the native conformations in solution. The preservation of higher order structures of peptides and proteins upon their transition from solution to the vacuum has been questioned in the literature.[14] In this report, we present experimental evidence that the elements of the secondary structures of polypeptides may be preserved upon their desorption from solution by means of electrospray ionization.

We and others have recently applied the classical technique of mass-analyzed ion kinetic energy (MIKE) spectrometry to measure intercharge distances in peptides in the gas phase.[15,16] In this work, we use MIKE spectrometry in combination with molecular mechanics methods to evaluate the higher order structure of the multiply protonated polypeptide melittin unaffected by solvent molecules (i.e., in the gas phase).

Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2) is a 26-residue amphipathic polypeptide found in bee venom.[17] Structural studies of this polypeptide by x-ray crystallography,[18] NMR,[17,19–21] CD, fluorescence and FTIR spectroscopy,[20] as well as titration studies[22] have revealed that it exists in an α-helical form (two helices connected by a hinge; see purple structure on Figure 1) in a number of different environments. These include crystals[18] and lipophilic environments,[19] as well as melittin solutions in methanol, isopropanol, water/methanol/acetic acid, and so on.[17,19,20] The stability of the α-helical conformation of melittin in solution has also been studied by H/D exchange using electrospray ionization mass spectrometer as a detector.[23]

Because of its amphipathic nature, melittin forms multimers very readily in water-based environments (solutions and crystals).[17,18] Multimer formation helps to stabilize the helical structure, although monomers may also exist as α helices.[17,19] The gas phase provides an ideal environment, in which the intrinsic stabilities of the elements of the secondary structure are unaffected by the solvent or other intermolecular interactions. In experiments carried out using mass spectrometry, intramolecular hydrogen bonding,

which is responsible for formation of α helices, will compete only with electrostatic repulsion between the charges (protons) required for the analysis.

MATERIALS AND METHODS

Melittin was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Multiply charged ions of melittin were desorbed from a 0.2 mM solution of 1:1:0.1 (v:v:v) water: methanol:acetic acid in the electrospray ion source of a tandem four-sector mass spectrometer (JEOL HX110/HX110, Tokyo, Japan). The desorbed ions were separated by their kinetic energies and masses in the first two sectors of the mass spectrometer (electrostatic analyzer E_1 and magnetic sector B_1), and then guided into the reaction cell, where a fraction of these ions undergo spontaneous (metastable) unimolecular dissociations. Fragment ions formed in the charge separation reaction

$$m_1^{x+} \rightarrow m_2^{y+} + m_3^{(x-y)+} \quad (1)$$

possess a significant amount of kinetic energy in the center-of-mass frame due to electrostatic repulsion between the products bearing positive charges. Therefore, the kinetic energy release in the dissociation process (1) can be used as a measure of the electrostatic repulsion in the transition state for decomposition of the m_1^{x+} ion, assuming that most of the electrostatic repulsion energy is partitioned directly into kinetic energy of the product ions m_2^{y+} and $m_3^{(x-y)+}$.[15,16,24–26] In these experiments kinetic energies of the fragment ions were determined in the second electrostatic analyzer E_2.

RESULTS AND DISCUSSION

Figure 2 shows a MIKE spectrum of the products of the unimolecular dissociation of metastable melittin molecules carrying three protons. The spectrum shows a cluster of rather abundant fragment ions, labeled here y_{18}^{2+} through y_{21}^{2+}, formed by cleavages of the amide bonds 18–21 from the carboxyl terminus.[27] These bond cleavages are centered around Lys-7. The shapes of these peaks are very different from that of the "slim," near-gaussian peak of the precursor ion MH_3^{3+}. The wide, clearly "dishtopped" peak shapes for y_n^{2+} ions suggest that these fragmentations are accompanied by substantial kinetic energy release (KER), and that the KER distributions are rather narrow and have maxima at nonzero values.[28–30] Under these circumstances the average KER value can be estimated from the widths of the fragment ion peaks, using the following formula[28–31]:

$$T = \frac{y^2 m_1^2 eV}{16 x m_2 m_3} \cdot \frac{(\Delta E)^2}{E^2} \quad (2)$$

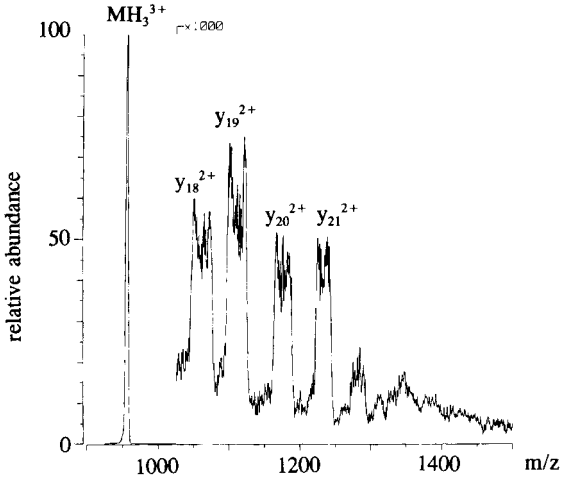

Fig. 2. A MIKE spectrum of the products of the unimolecular dissociation of metastable triply charged melittin ion. Fragment ion nomenclature is taken from ref. 27.

where V is the acceleration voltage, E is the position of the center of the peak of the precursor ion m_1^{x+} in the MIKE spectrum, and ΔE is the peak width of either of the fragment ions m_2^{y+} or $m_3^{(x-y)+}$. Estimation of the average KER, based on the widths of these peaks, gives the following numbers: $T(y_{18}^{2+}) = 1.27 \pm 0.12$ eV and $T(y_{19}^{2+}) = 1.22 \pm 0.12$ eV. These numbers represent electrostatic repulsion between the pair of protons on the y_n^{2+} fragment and the proton on the complementary b_{26-n}^{+} fragment.

It must be noted that only a small fraction of the triply charged melittin ions undergo metastable dissociation (the magnification factor for y_n^{2+} ions on Figure 2 is 1000). This is because cleavage of a covalent amide bond in a multiply charged peptide requires substantial activation energy,[32] and only a limited number of ions may accumulate enough internal energy in a particular bond for it to be broken. The activation energy may be lowered (and fragmentation yields increased) if the cleavage site is a relatively weak hydrogen bond, rather than covalent one. This is the case when the ion undergoing unimolecular dissociation is a proton-bound dimer, formed by melittin (M) and another molecule (B). Since the only significant intermolecular interaction between M and B in a transition state of the complex $[B{\cdot\cdot}H^+{\cdots}MH_2^{2+}]^{\ddagger}$ is through the "shared" proton, the melittin conformation should not be affected much by its partner in the dimer. Therefore, kinetic energy release in the unimolecular dissociation process

$$[B{\cdot\cdot}H^+ \cdots MH_2^{2+}]^{\ddagger} \rightarrow BH^+ + MH_2^{2+} \quad (3)$$

can also be used as a measure of the electrostatic repulsion in triply protonated melittin. The average value of the KER in the charge separation process (3) in melittin was measured using several small pep-

TABLE I. Energy of Coulombic Repulsion δ Between the Proton on the Lys-7 Side Chain and the Pair of Protons on the Arg-22 and Arg-24 Side Chains

Calculated	δ, eV	Determined by Kinetic Energy Release	δ, eV
Charges-on-a-string (fully extended) conformation	0.52	Unimolecular dissociation $MH_3^{3+} \rightarrow y_{18}^{2+}$ (y_{19}^{2+})	1.25 ± 0.12
Crystal structure (α-helical conformation polar side chains extended)	1.15	Unimolecular dissociation $B\cdots H^+\cdots MH_2^{2+} \rightarrow BH^+ + MH_2^{2+}$	1.34 ± 0.12
Crystal structure, molecular dynamics (α-helical conformation, polar side chains solvated by the backbone)	1.26 ± 0.06		

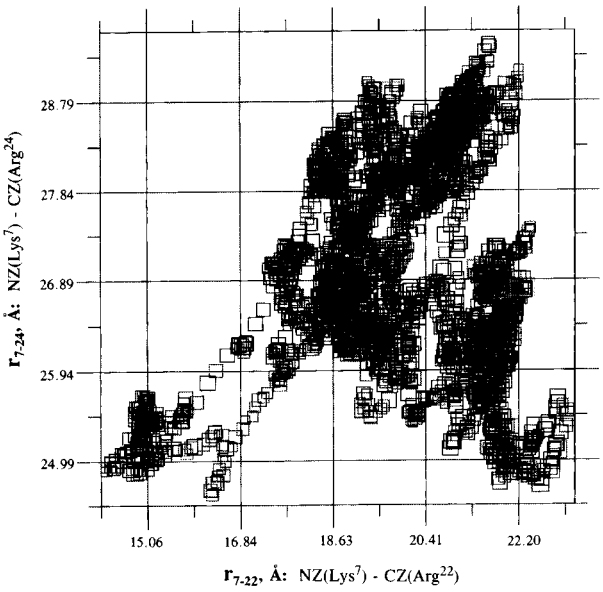

Fig. 3. A pairwise plot of intercharge distances (Lys-7—Arg-22 and Lys-7—Arg-24) for melittin conformations generated throughout the dynamics simulation of the triply charged polypeptide at 500 K in the absence of solvent. Each data point represents a single conformation retrieved from the dynamics trajectories file. NZ is the nitrogen atom of the Lys-7 side chain amino group, CZ is the central carbon atom of the Arg guanidinium group. The electrostatic repulsion between the proton on the Lys-7 side chain and the pair of protons on the Arg-22 and Arg-24 side chains for each conformation is calculated as

$$\delta = e^2/r_{7-22} + e^2/r_{7-24}.$$

tides YGGFL, FGGFL, and *N*-methyl-FGGFL as partners. Again, the "dishtopped" shapes of the peaks representing MH_2^{2+} product ions suggest that the kinetic energy release distributions in charge separation processes (3) are rather narrow and have maxima at nonzero values. Calculation of the average KER value using the widths of these peaks gives 1.34 ± 0.12 eV.

In order to find a correlation between the electrostatic repulsion measured experimentally, and a structure or structures of melittin, we have to assign the positions of charges in the polypeptide. Melittin has six basic amino acid residues (including N-terminal Gly), and four of them occur within a four residue chain near the C terminus of the polypeptide chain. Protonation of all four of these basic sites may be restricted by the abnormally high electrostatic repulsion.[33] It has been shown earlier that Lys-21 and Lys-23 in melittin have unusually low p*K* values, suggesting that electrostatic repulsion effectively drives protons away from these residues even in the presence of polar solvent molecules.[22] Apparently, electrostatic interactions play an even more important role in vacuo, where they are not mitigated due to charge screening by the polar solvent. We therefore assume that Lys-21 and Lys-23 are unlikely to carry charges in the gas phase. Arginine is the most basic amino acid and its proton affinity is higher than that of the next highest (lysine) by at least 20 kcal/mol.[34,35] Furthermore, the lengths of the Arg side chains provide sufficient spatial separation for two protons on the guanidinium groups of Arg-22 and Arg-24 (almost 14 Å).[15,16] Therefore, the Arg-22 and Arg-24 side chains are assigned as sites of protonation. There are two "candidate" sites on which the third proton can be located: the N-terminal amino group and the side chain of the Lys-7 residue. The latter one seems to be a more favorable site for protonation, since the flexibility of the lysine side chain allows charge stabilization by hydrogen bonding with polar groups on the polypeptide backbone. The formation of y_n^{2+} fragment ions by cleavages centered around Lys-7 in the spectrum of metastable triply charged melittin also suggests that the side chain of Lys-7 carries a proton.

Based on this charge site assignment (Arg-22, Arg-24, and Lys-7), we can estimate the amount of the coulombic repulsion energy released upon the cleavage of the peptide or dimer. To perform such an estimation, we energy-minimized the x-ray structure of melittin[18] in the CHARMm22 semiempirical

force field.[36] The resulting intercharge distances of 28.6 Å (Lys-7—Arg-24) and 22.5 Å (Lys-7—Arg-22) correspond to an overall electrostatic energy of 1.15 eV for a proton on the Lys-7 residue. However, this value can be used only as a very rough (zero-order) approximation of coulombic energy in the gas phase, since it is entirely based on the crystal structure of melittin. The presence of polar solvent molecules in the crystal structure[18] allows the polar side chains (charge-bearing Arg-22, Arg-24, and Lys-7 in particular) to stretch out to increase the solvation of the charges, and shielding of the electrostatic repulsion. It is very likely that in the absence of such effective shielding in the gas phase, the charges will be stabilized by the hydrogen bonding.[37,38] To test this hypothesis, we performed a cycle of molecular dynamics calculations using the crystal structure of melittin as a starting conformation. This structure was heated from 0 K to 500 K in 1 ps (10^{-3} ps time step), equilibrated at that temperature for 1 ps, followed by dynamics simulation for 30 ps, with every tenth conformation being saved in a trajectory file. The selection of 500 K as the temperature for the dynamics simulation was based on the typical values reported in ref. 39 for the effective temperatures of metastable proton-bound dimers. The choice of the rather short simulation time was based on the need to optimize the conformations of the side chain no longer extended by solvation without significantly alterating the global (backbone) conformation of the peptide. Indeed, the 30 ps dynamics simulation in the absence of solvent resulted in only limited distortion of the polypeptide backbone: the majority of the hydrogen bonds responsible for the helix formation have been preserved (see also Fig. 1). However, the orientations of polar side chains have changed significantly: the proton-carrying side chains are now involved in intramolecular hydrogen bonding that apparently helps to stabilize the charges. Analysis of the dynamics trajectories shows that the most probable electrostatic repulsion between the proton on the side chain of the Lys-7 residue and the two protons on the Arg-22 and Arg-24 side chains is 1.26 ± 0.06 eV. Figure 3 represents a pairwise plot of the intercharge distances retrieved from the trajectory file.

Denatured polypeptides carrying several charges in the gas phase have been assumed in one model[40] to be fully extended, since such conformations would allow minimization of the electrostatic repulsion. If we consider this "charge-on-the-string" model as a representation of triply charged melittin in the gas phase, the intercharge distances will be 59.3 Å (Lys-7—Arg-24) and 52.7 Å (Lys-7—Arg-22), giving us an overall energy of the coulombic repulsion experienced by the proton on the Lys-7 side chain of only 0.52 eV.

The results of the molecular modeling and experimental measurements of electrostatic repulsion are summarized in Table I. The good agreement between the experimental data and the calculations based on the helical structure of melittin suggests that the secondary structure of this polypeptide is maintained in the absence of solvent.

The stability of the helical conformation of melittin in the absence of solvent probably reflects the fact that this element of secondary structure is an intrinsic property of the polypeptide rather than a result of interaction with the solvent molecules. Indeed, the helical structure is maintained by a highly oriented network of hydrogen bonds along the polypeptide backbone. The presence of the polar solvent molecules may actually destabilize the helix due to the entropy-driven process of formation of "random" hydrogen bonds between polar solvent molecules and polar groups of the backbone. The helical conformation of melittin is more stable in less polar solutions (alcohols) than in aqueous solutions.[17] In that respect, vacuum can be viewed as an ultimate apolar environment causing minimal distortions to intrinsic networks of hydrogen bonds.

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