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Using ESI-MS to Probe Protein Structure by Site-Specific Noncovalent Attachment of 18-Crown-6

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A new method for probing the equilibrium structures and folding states of proteins utilizing electrospray ionization mass spectrometry is described. Protein structure is explored as a function of side-chain availability as determined by a specific interaction between lysine and 18-crown-6 ether (18C6). Various intramolecular interactions are competitive with the lysine/18C6 interaction and can prevent noncovalent attachment of 18C6. Changes to protein structure modify these inhibiting intramolecular interactions, which leads to a change in the number of 18C6s that attach to the protein. Experiments conducted with cytochrome *c*, ubiquitin, and melittin reveal that the method is sensitive to changes in both tertiary and secondary structure. In addition, the structure of each charge state can be examined independently. Experiments can be performed under conditions where the pH and amount of organic cosolvent are varied. Control experiments conducted with pentalysine, which lacks structural organization, are also presented. (J Am Soc Mass Spectrom 2006, 17, 1209–1215) © 2006 American Society for Mass Spectrometry

Several techniques that rely heavily on electrospray ionization mass spectrometry (ESI-MS) are used to explore the structure and folding states of proteins. Hydrogen/deuterium (H/D) exchange is one method that can be used as an elegant probe of the backbone amide groups [1, 2]. In these experiments, H/D exchange of some amide hydrogens (typically on the interior of the protein) is hindered by intramolecular hydrogen bonding or other factors that are related to macromolecular structure [3]. The degree of exchange for a given set of conditions can be observed directly in a mass spectrum. Under appropriate conditions, specific sub-regions of a protein can be examined individually. This is accomplished by proteolytically digesting the protein and determining the extent of H/D exchange occurring in each individual peptide [4]. Binding sites can be ascertained by recording H/D exchange in the presence and absence of a ligand [5]. In fact, this approach has led recently to the development of MS based techniques for the determination of solution phase binding constants [6, 7]. In addition, the kinetics of protein folding can also be observed by pulsed exchange experiments [8].

Changes in charge state distributions can also be used to monitor protein structure on a coarse level [9, 10]. Numerous experiments have shown that protein charge states tend to increase for unfolded structures when compared with native or folded structures [11, 12]. Under appropriate conditions, both structures for proteins that are two state folders can be observed simultaneously in a bimodal charge state envelope [13]. Nevertheless, charge state distributions yield only limited information and can be misleading. For example, the charge state envelope for cytochrome *c* (cyt *c*) reveals an unfolded state only when the protein is sampled as a cation [14]. Furthermore, it is not possible to distinguish contributions from different structures that give rise to similar charge state distributions. Another drawback is that protein structure cannot be examined on an individual basis for each observed charge state.

Despite these limitations, examining protein structure as a function of charge state remains a desirable methodology because of the elegant simplicity of the experiment itself, i.e., simply electrospraying proteins under various solvent conditions. Although H/D exchange experiments provide detailed information, they are additionally much more complicated to execute and frequently do not directly sample equilibrium states. It is therefore desirable to devise an experiment for examining protein structure, which retains the simplicity of the charge state distribution approach, yet provides additional information.

The present work demonstrates a new approach for examining protein structure and folding based on side-chain availability. In these experiments, 18-crown-6 (18C6) is used to probe the availability of lysine side chains through a specific, three hydrogen bond mediated interaction [15]. The new approach is similar to

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H/D exchange because specific interactions are probed, yet also complimentary because the focus is on side chains and exposed regions rather than the peptide backbone and the protein interior. Simultaneously, the experiment retains the simplicity of a charge state distribution type experiment. Experiments with cyt *c*, ubiquitin, and melittin illustrate that the number of 18C6s that attach to a protein is correlated with the macromolecular structure. In these experiments, the structure for each charge state can be probed independently through observation of the unique pattern of multiple 18C6s that attach to the protein. The relative intensity of each peak in the distribution is determined by lysine side-chain availability, which is a function of the structure of the protein in solution. This approach reveals that changes in the 18C6 distribution can be observed under varying solvent conditions even though no significant change is observed in the overall charge state distribution. The roles that protein secondary and tertiary structure play in lysine availability are explored. Control experiments with pentalysine, which lacks secondary or tertiary structure, are also presented.

Experimental

Mass spectra were obtained using a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with a standard electrospray ionization source with no modification. Voltages in the source region were optimized for observation of noncovalent complexes. This was achieved by electrospraying a solution of cyt *c* and 18C6 and optimizing adduct ion intensities by tuning individual voltages. The critical parameters for observing protein-adduct formation are the capillary voltage 9.0 V, capillary temperature 215 °C, and tube lens offset 145–160 V. The tube lens voltage was optimized for maximum total ion count within this range. Peptide mass spectra were taken under instrument settings reoptimized for pentalysine-18C6 adduct ion intensities. Critical parameters yielding peptide-adduct formation are the capillary voltage 49.0 V, capillary temperature 215 °C, and tube lens offset 60 V for maximal ion intensity. The spray voltage was varied from 3.6 to 4.5 kV to achieve best shot-to-shot stability of mass spectra.

Protein and peptide solutions were made in solvents ranging from pure water to mixtures of water/methanol as specified. No acetic acid was added to solutions unless indicated. Analytical reagent-grade methanol (Riedel de Häen, Seelze, Germany) was specifically purchased to limit 18C6 adducts with ammonium, sodium, and potassium cations. The concentrations for all proteins were kept in the ~3 μM to ~5 μM range, and ~15 μM for pentalysine. For all solutions where 18C6 (Alfa Aesar, Pelham, NH) was added, the 18C6 concentration was matched to two times the number of lysine residues to ensure stoichiometric excess. All samples were prepared using NANOpure DIamond (Macalaster Bicknell Co., New Haven, CT) purified water. All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) or EMD (Gibbston, NJ) unless noted otherwise and used without further purification. The biogenic source for ubiquitin was bovine red blood and cyt *c* was obtained from horse heart. Melittin was obtained synthetically from American Peptide Company (Sunnyvale, CA). Solution pHs were measured using a VWR symphony meter. The reported pHs for water/methanol mixtures were measured directly and are uncorrected for methanol content [16].

Results and Discussion

Control Experiments

The experiments described in this work measure the extent to which the attachment of a small molecular probe is sensitive to changes in macromolecular structure. Manipulation of the pH and addition of organic cosolvents are commonly utilized techniques for manipulating protein structure [17]. However, it is also possible that changing these parameters might influence the noncovalent attachment of small molecules in ESI-MS experiments. For example, the addition of organic solvents may alter the solution phase binding constants or the addition of acid may lead to competitive binding with lysine side chains.

To examine the influence of pH and organic content on the complexation of 18C6 to lysine in the absence of structural changes, we conducted several control experiments with pentalysine. Previous work relying on circular dichroism (CD) has demonstrated that pentalysine is typically disordered [18]. In Figure 1, the results for experiments designed to test the influence of organic solvents are shown. The data in Figure 1a reveals that the ratio of naked to complexed peptide does not increase with increasing methanol content. Because the 18C6-lysine interaction is hydrogen bond and ion-dipole mediated, it is anticipated that binding constant will increase as the content of methanol is increased due to reduction of the dielectric constant. If this effect were directly correlated with the observations in ESI-MS, the amount of naked peptide would decrease with increasing methanol. The data in Figure 1a illustrates that this is not the case. Therefore, the introduction of organic solvents does not influence the number of 18C6s that attach to the peptide in a manner consistent with the expected change in binding constant. Although there are some changes in relative intensities in the spectra, all of the major peaks remain present as the methanol percentage is varied from 0 to ~95%. The two extremes are shown in Figure 1b and c. The decline of $[K_5 + 418C6]^{4+}$ appears to be connected with the introduction of sodium (present as a contaminant in the methanol). Smaller changes may be related to structural rearrangement of the peptide or to differences in the electrospray process.

Similarly, experiments with varying amounts of acid were performed to test the extent to which acidic

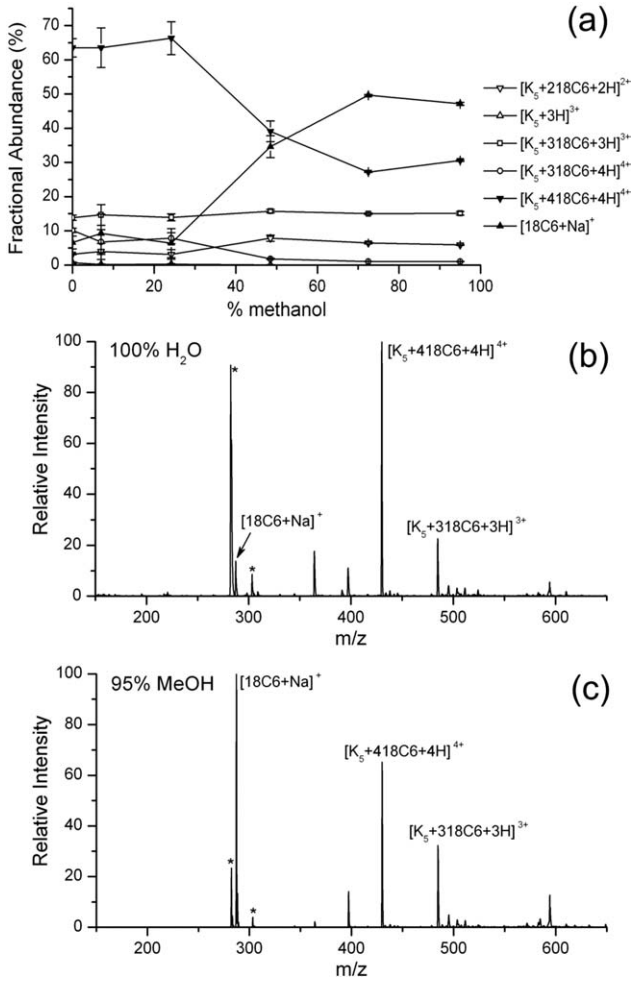

Figure 1. (**a**) The fractional abundance of several species sampled from a solution of pentalysine and 18C6 is plotted as a function of the percentage methanol in solution (with the remainder being water). As the organic content increases, the number of 18C6 adducts does not change significantly, suggesting that small changes in binding constant do not strongly influence the observed attachment of 18C6. The spectra representing the two extreme cases are shown in (**b**) and (**c**). Asterisks denote chemical noise from NH_4^+, Na^+, and K^+ present in the solvents that attach to 18C6.

molecules might competitively interfere with complexation of pentalysine by 18C6. The addition of acid leads to several changes in the resulting spectra. The total ion intensity increases significantly, the intensity of naked peptide peaks increases, and the relative intensity of some 18C6 adduct peaks is reduced. It is unclear whether the increased abundance of naked peptides is a result of reduced 18C6 attachment or simply represents an enhancement of the intensity of previously unobserved ions in the presence of acid. In either case, the net results suggest that some interference due to the addition of acid may occur and reduce the amount of 18C6 complexes that are formed. However, the number of 18C6 adducts does not change (only the relative intensity of some contributing peaks). Furthermore, an experiment resulting in an increase in the number of 18C6 adducts after the addition of acid would unambiguously indicate a structural rearrangement for an experiment involving a macromolecule.

Protein Structure in Methanol

Many studies have shown that organic solvents can influence the equilibrium structures that proteins adopt [19]. It is also well known that the addition of methanol or trifluoro-ethanol can be used to stabilize and even increase the helical content of a protein [20]. Cyt *c* is an important heme containing electron-transfer protein with a predominantly helical structure [21] and no disulfide bonds. The data in Figure 2 show that the addition of 50% methanol does not lead to a significant shift in charge state distribution for cyt *c*, in agreement with previous results [22]. However, from spectroscopic

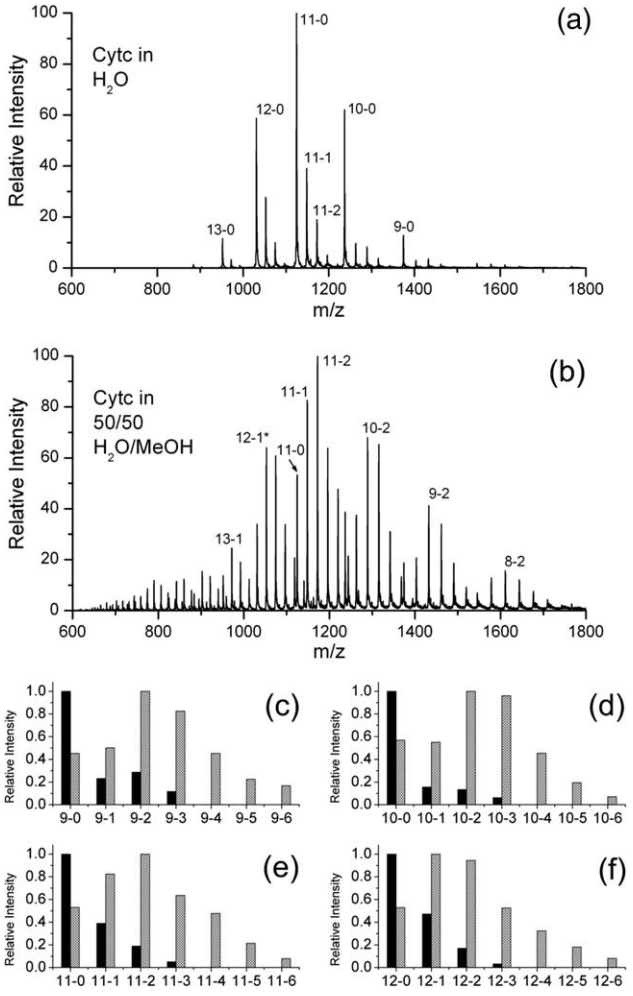

Figure 2. ESI-MS spectra of cyt *c* and 18C6 with no acid in (**a**) water and (**b**) 50/50 water/methanol. Several peaks are identified by two numbers (charge state-#18C6). The resulting spectra are noticeably different. In (**c**)–(**f**), the data from (**a**) and (**b**) are shown for individual charge states for easy comparison. The black bars are data extracted from (**a**) while the gray bars represent data from (**b**). Intensities are shown relative to the base peak for each distribution. Asterisk denotes that the peak overlaps with 13–5.

data, clearly cyt *c* undergoes a significant structural rearrangement in 50:50 water/methanol [22]. The spectra for cyt *c* with 18C6 in water and 50:50 water/methanol are shown in Figure 2a and b. It is very clear that the number and distribution of crown adducts is very different, suggesting that at least two different structures are being sampled from solution. In Figure 2c–f, the 18C6 adducts associated with a particular charge state from Figure 1a and b are isolated. The intensities are shown relative to the base peak for easy comparison between the two distributions. The data in Figure 1c–f demonstrate that in 50% methanol more 18C6s attach to cyt *c* both in number and in total relative intensity, suggesting a significant structural shift.

The number of 18C6 adducts that attach to cyt *c* in Figure 2a is qualitatively consistent with the number of lysines that are not in close proximity to a strong hydrogen bond partner in the known crystal structure [21]. The contrast with the results shown in Figure 2b are particularly intriguing in light of the fact that the charge state distribution does not shift substantially, which typically indicates that the protein is still in a compact form. However, from the dramatic increase in the number of 18C6s that attach in Figure 2b, clearly the observed structure is not the native structure. This idea is further supported by previous work showing a change in heme coordination in 50% methanol [22]. A substantial portion of the protein in the crystal structure of cyt *c* is in the random coil configuration. The addition of methanol may induce organization of this region into more helical arrangements.

It is clear from the results in Figure 2 that structural rearrangement not involving the complete unfolding of a protein can still lead to substantial modification of the availability of lysine residues for binding by 18C6. There are several potential factors that may influence lysine availability. For example, intramolecular side-chain–side-chain interactions, such as salt bridges, could potentially interfere. However, there are 19 lysines in cyt *c* but only a few of these are involved in salt bridges in the crystal form. Other intramolecular hydrogen bonding interactions between side chains or with the peptide backbone may play a significant role in determining lysine availability for cyt *c*. If these interfering interactions are optimized in the native state, then lysine availability should typically increase if the native structure is perturbed. In the case of cyt *c*, non-native helical regions induced by the addition of methanol may disrupt intramolecular interactions that otherwise inhibit attachment of 18C6. All of the above mentioned interfering interactions are individually weak in solvated environments; however, 18C6 is also a weak binder. This allows it to be an efficient, noninterfering probe. In fact, 18C6 does not interfere with and can be used advantageously in conjunction with enzymatic catalysis [23].

Under identical experimental conditions, ubiquitin yields very different results as shown in Figure 3. Ubiquitin is a 76 residue protein with mixed helical and

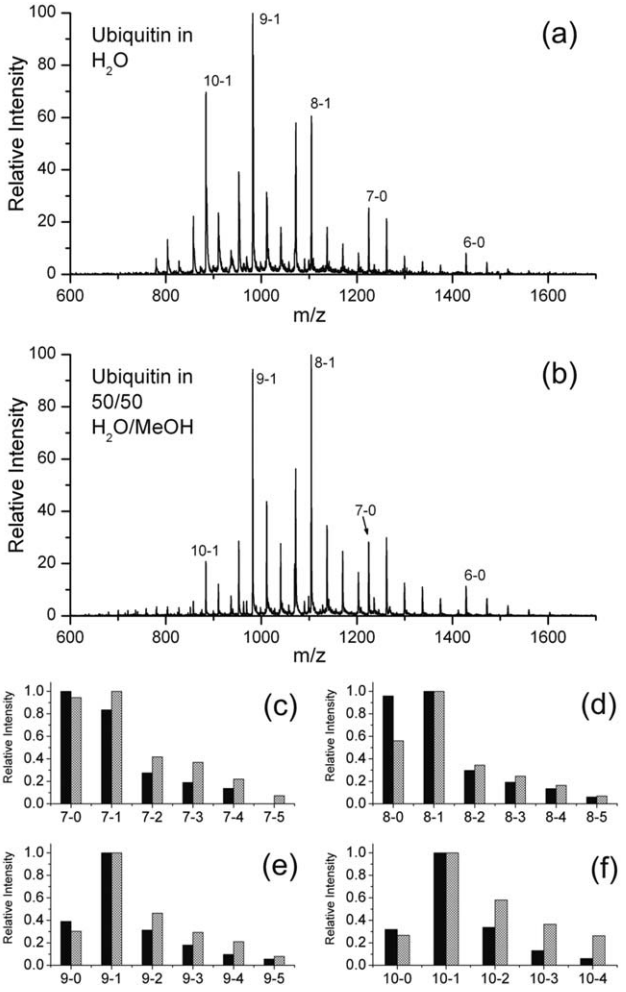

Figure 3. ESI-MS spectra of ubiquitin and 18C6 with no acid in (**a**) water and (**b**) 50/50 water/methanol. Several peaks are identified by two numbers (charge state-#18C6). The resulting spectra are fairly similar, indicating similar structures are sampled in both experiments. In (**c**)–(**f**), the data from (**a**) and (**b**) are shown for individual charge states for easy comparison. The black bars are data extracted from (**a**) while the gray bars represent data from (**b**). Intensities are shown relative to the base peak for each distribution.

β sheet structure and no disulfide bonds [24]. Comparison of the spectra obtained in water (Figure 3a) and 50/50 water/methanol (Figure 3b) reveals very similar looking results, suggesting that no significant structural reorganization takes place in the case of ubiquitin. The data shown in Figure 3c–f confirm that the 18C6 adduct distributions associated with each charge state are similar in both shape and relative intensities. The similarity of the results in both solvent systems may be related to the overall stability of the structure of ubiquitin [25].

However, closer inspection suggests the presence of two structures in both systems. In water, the first structure is present in the +7 and +8 charge states, as noted by the similar relative intensities of the 7-0, 7-1 and 8-0, 8-1 peaks. In 50/50 water/methanol, the first structure is present only in the +7 charge state. Nearly identical 18C6 distributions are observed in both sol-

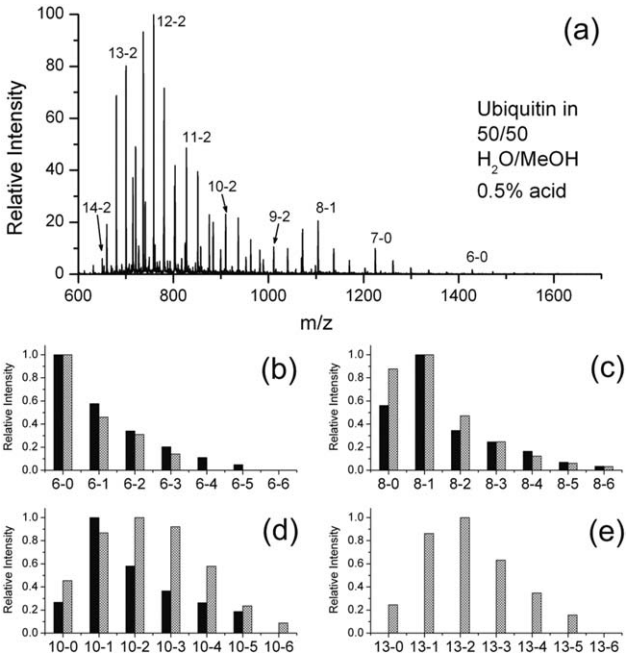

Figure 4. ESI-MS spectra of ubiquitin and 18C6 with 0.5% acetic acid (pH 3.6) in 50/50 water/methanol. Several peaks are identified (charge state-#18C6). The spectrum is compared with data from Figure 3b in (**b**)–(**e**). The black bars are data extracted from Figure 3b while the gray bars represent data from (**a**). Intensities are shown relative to the base peak for each distribution. Acid induces a noticeable structural shift, increasing the number of attached crowns.

vent conditions for the +9 and +10 charge states. These results suggest an equilibrium between the native structure and a very slightly more accessible structure under both solvent conditions, with the more accessible structure being more favored in methanol. It has been shown previously by the addition of a non-native disulfide bond that ubiquitin must be flexible to retain functionality [26]. This observation is in excellent agreement with our observation of two "native" structures. The shift in the 18C6 distribution to include one additional 18C6 suggests that the more accessible form is the result of a structural rearrangement exposing one additional lysine. Previous results suggest that the c-terminal domain (Gly^{53}-Gly^{76} is less stable than the *n*-terminal portion of the structure [27]. There is a single lysine residue (Lys^{63}) in this region near the c-terminus, which may be the lysine that 18C6 attaches to in the more accessible structure.

Acid Induced Unfolding

The addition of acid is frequently used to denature proteins [28]. As discussed above, this type of denaturation is commonly accompanied by a shift to higher charges states. The spectrum for ubiquitin and 18C6 in 50/50 water/methanol with 0.5% acetic acid added is shown in Figure 4a. Comparison with Figure 3b reveals that the charge state distribution has shifted significantly. The data shown in Figure 4b–e compares the 18C6 distributions for several charge states in the absence and presence of acid. The +6 charge state is still present in very small abundance with the addition of acid, yet the structure appears to be unchanged when compared with the +6 charge state in the absence of acid as shown in Figure 4b. This observation is in accord with previous studies where it was found that the native structure was still present under acidic conditions [29]. The +8 charge state shown in Figure 4c also appears to have a similar distribution with or without acid, although the structure is potentially different from that observed in the +6 charge state. The +10 and +13 charge states clearly reveal the presence of a more open structure which attaches 18C6 in higher abundance. This is most likely the a-state of ubiquitin, which has been studied previously [25, 27]. All of the charges states over +10 yield very similar distributions to that shown in Figure 4d. It can also be clearly observed that the structure yielding the 10+ charge state is different in the presence and absence of acid, a distinction that cannot be made without the addition of 18C6.

The shift in charge state implies a more open structure, which is confirmed by the addition of more 18C6 adducts. The distribution of 18C6 also shifts at the point that would be predicted by the bimodal charge state distribution, in agreement with previous predictions. Furthermore, three different structures are predicted by comparison of the 18C6 distributions in Figure 4b, c, and e. The presence of three structures is in agreement with previous results obtained by combined H/D exchange and charge state distribution analysis [8]. In this study, the three structures were detected by comparing results from both techniques [8]. With the current approach, all three structures are observed with a single method.

Melittin and Secondary Structure

The results obtained with cyt *c* and ubiquitin suggest that the attachment of 18C6 is sensitive to changes in both the overall tertiary structure (i.e., compact or extended) and to more subtle changes in the secondary structure. To probe this possibility more thoroughly, we conducted experiments with melittin, which is known to undergo a transition from a random coil to an α helix under appropriate conditions. Melittin is a 26 residue peptide with an amidated c-terminus that comprises the principle component of honey bee venom. Melittin has a random coil structure in water, which becomes primarily α helical in ~25% TFE or >50% methanol [30, 31]. The spectra for melittin and 18C6 in water and 20:80 water/methanol are shown in Figure 5. Acid (0.5% acetic acid) was added to both samples to enhance the signal, but melittin has no acidic groups and is not sensitive to structural changes under pH 7 [32]. Examination of the 18C6 distributions shown in Figure 5c–f reveals that lysine availability changes significantly with the transition to an α helical structure.

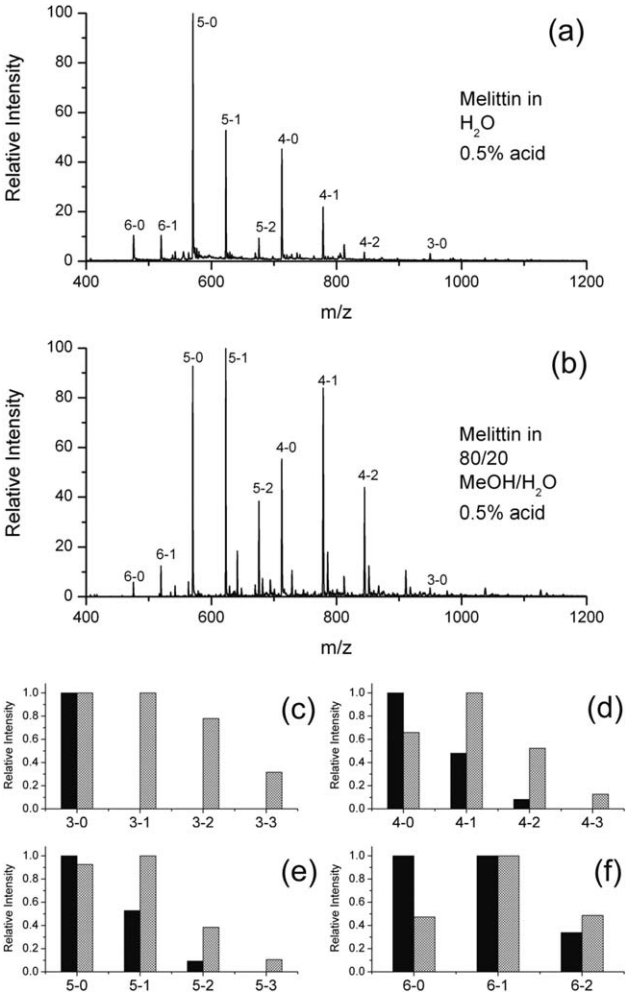

Figure 5. ESI-MS spectra of melittin and 18C6 with 0.5% acetic acid in (**a**) water (pH 3.05) and (**b**) 20/80 water/methanol (pH 2.97). Several peaks are identified (charge state-#18C6). The 18C6 distributions shift significantly as shown in (**c**)–(**f**), where the data from (**a**) and (**b**) are extracted for individual charge states. The black bars are data extracted from (**a**) while the gray bars represent data from (**b**). Intensities are shown relative to the base peak for each distribution.

This observation can be rationalized in terms of hydrogen bonding with the peptide backbone. In the absence of acidic groups, the backbone carbonyls offer the best potential intramolecular sites for hydrogen bonding. The backbone carbonyls are available to interact with the side chains if the peptide is in a random coil conformation. On the other hand, the carbonyls become largely unavailable if the peptide adopts an α helical structure, which should lead to enhanced availability of the side chains. Indeed, it is observed in Figure 5 that lysine availability increases substantially with the shift to the helical structure. Both the number and relative abundance of 18C6 adducts increases for the helical structure as seen in Figure 5c–f. Our data show the formation of three 18C6 adducts suggesting that all three lysines are available, in agreement with the predicted availability based on the X-ray structure [33]. It is important to note that the charge state distribution does not change significantly, which implies that evidence for the structural transition cannot be obtained through charge state analysis alone. However, with the addition of 18C6, the transition is easily detected, confirming that the present technique is sensitive to changes in secondary structure.

Conclusions

A new method for probing protein structure has been described. The technique relies on ESI-MS for the detection of noncovalent reporter molecules that reveal the folding state of the protein to which they attach. In the present work, 18C6 is used to recognize the side chain of lysine, but nothing prevents the technique from being applied with other small molecules that also interact specifically with amino acid side chains. It is possible to probe changes in both secondary and tertiary structure, while retaining experimental simplicity. The facile application of this technique to examine the folding state of proteins will provide a complimentary method for addressing the complex problems of protein folding and structure determination.

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