Matrix-Assisted Laser Desorption Ionization Hydrogen/Deuterium Exchange Studies to Probe Peptide Conformational Changes

Iddys D. Figueroa and David H. Russell
Department of Chemistry, Texas A&M University, College Station, Texas, USA

Hydrogen/deuterium (H/D) exchange chemistry monitored by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry is used to study solution phase conformational changes of bradykinin, α-melanocyte stimulating hormone, and melittin as water is added to methanol-d_4, acetonitrile, and isopropanol-d_8 solutions. The results are interpreted in terms of a preference for the peptides to acquire more compact conformations in organic solvents as compared to the random conformations. Our interpretation is supported by circular dichroism spectra of the peptides in the same solvent systems and by previously published structural data for the peptides. These results demonstrate the utility of MALDI-TOF as a method to monitor the H/D exchange chemistry of peptides and investigations of solution-phase conformations of biomolecules. (J Am Soc Mass Spectrom 1999, 10, 719–731) © 1999 American Society for Mass Spectrometry

Hydrogen/deuterium (H/D) exchange is a commonly used method for studies of conformational changes and folding mechanisms of peptides and proteins in solution [1–7]. The H/D exchange data (extent of exchange and rates of exchange) provide information about the higher order structure of proteins. For example, regions of the molecule that are associated with slow H/D exchange rates are protected, e.g., labile hydrogens that are involved in hydrogen bonding or have low accessibility to the solvent, whereas regions of the molecule that rapidly exchange are highly exposed to the solvent. The H/D exchange process can be monitored using techniques such as nuclear magnetic resonance (NMR) [1, 8–12] and mass spectrometry [5, 13–17]. The information obtained by utilizing each of these techniques is highly complimentary [2, 18, 19]. For example, H/D exchange coupled with NMR can produce site specific information about labile hydrogens in the primary structure, whereas H/D exchange mass spectrometry provides information on the existence of more than one population of conformers. In addition, Smith and co-workers have shown that mass spectrometry combined with limited proteolysis provides a detailed view of higher order structures of proteins too large for NMR analysis [20, 21].

Electrospray ionization (ESI) is the most commonly used method to produce ions from deuterated samples for subsequent mass spectrometric analysis. There are only a few reports of the use of matrix-assisted laser desorption ionization (MALDI) and H/D exchange to study biomolecule conformation [22, 23]. For example, Mandell et al. used H/D exchange and enzymatic digestion with subsequent MALDI analysis of the sample to measure the amide H/D exchange rates on cyclic AMP-dependent protein kinase. They point out specific advantages of MALDI for H/D exchange studies, e.g., elimination of the HPLC separation of proteolytic fragments before mass analysis, ability to analyze smaller sample amounts, better resolution, and faster analysis times.

In previous studies we found a strong dependence of the MALDI ion yields on the water content in sample preparation [23, 24]. These studies lead us to investigate several factors that influence MALDI ion yields, including conformational changes that occur as water is added to the sample solution. Conventional mass spectrometry measurement, e.g., m/z ratios, fragmentation patterns, and ionization efficiencies, are difficult to correlate to secondary and tertiary structure; however, H/D exchange has potential for such investigation. Thus, H/D exchange and circular dichroism (CD) spectroscopy were utilized to monitor changes in conformation of bradykinin, α-melanocyte stimulating hormone, and melittin as the solvent environment is made more hydrophilic. In this paper we expand on our previous H/D exchange experiments using methanol-d_4 to include studies in anhydrous acetonitrile and isopropanol-d_8 in order to compare the results and discuss explanations based on conformational effects caused by

Address reprint requests to Dr. David H. Russell, Laboratory for Biological Mass Spectrometry, Department of Chemistry, Texas A&M University, P.O. Box 30012, College Station, TX 77842-3012.

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organic solvents. The purpose of this study is to investigate the utility of MALDI-TOF to monitor peptide and protein conformational changes. The information obtained is discussed in terms of the effects of solvent environment on the conformation of the model peptides. As shown previously by Desai et al. [25], organic solvents strongly influence the conformation of proteins; however, in many cases key features of secondary and tertiary structure (bioactivity of enzymes) are retained in nonaqueous solvents [26]. We are interested in evaluating MALDI H/D exchange as a method to probe solvent-dependent secondary, tertiary, and quaternary structure of peptides and proteins.

Experimental

MALDI Mass Spectra

The MALDI time-of-flight (TOF) mass spectra were acquired on a PerSeptive Biosystems Voyager Elite XL TOF reflectron mass spectrometer (PerSeptive Biosystems, Inc., Framingham, MA) equipped with a N_2 laser (337 nm). The spectra were all acquired in positive ion mode. The instrument was used in the delayed extraction linear mode for bradykinin and α-melanocyte stimulating hormone. The acceleration voltage for the linear mode was 25 kV with the grid voltage set at 95.5%. The delay times used were 100 and 150 ns for bradykinin and α-msh, respectively [27]. Using these tuning conditions we obtained unit mass resolution for the $[M + H]^+$ ions of both peptides. To obtain unit mass resolution for the $[M + H]^+$ ions of melittin (monoisotopic $m/z = 2845.76$) requires operating the instrument in delayed extraction reflected mode. The instrumental parameters for the reflected mode were 25 kV acceleration voltage, 75% grid voltage, and a delay time of 200 ns [27]. External calibration was performed using the $[M + H]^+$ ion of nondeuterated peptides and the protonated matrix dimer ion. Five spectra for each D_2O content sample solution were acquired.

Sample Preparation for the MALDI H/D Exchange Experiments

The samples were prepared and handled inside a nitrogen-filled drybox where the relative humidity was maintained at less than 7%. Stock solutions of the peptides, bradykinin (RPPGFSPFR, 1.5 mol of H_2O per mol of peptide), α-melanocyte stimulating hormone (α-msh) (N-acetyl-SYSMEHFRWGKPV-NH_2), and melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2) (all purchased from Sigma, St. Louis, MO) were prepared by dissolving the peptides in D_2O (99.8%, Cambridge Isotopes Laboratories, Inc., Andover, MA).

The matrix compound used for these experiments was α-cyano-4-hydroxycinnamic acid (HCCA, Aldrich, Milwaukee, WI) dissolved in three different solvents: methanol-d_4 (99.8%, Cambridge Isotope Laboratories, Inc., Andover, MA), isopropanol-d_8 (99.9%, Cambridge Isotope Laboratories, Inc., Andover MA), and anhydrous acetonitrile (99.8%, Aldrich, Milwaukee, WI). Aliquots for each of the peptide solutions (1×10^{-4} M) were mixed with an aliquot of the matrix solution to obtain a matrix to analyte molar ratio of 3000:1. The stock solutions were diluted with specific proportions of the deuterated or anhydrous organic solvent and D_2O such that a microliter of the final sample solution (1×10^{-6} M) contained 1 pmol of peptide and the desired amount (%*v*/*v*) of D_2O in solution. These solutions were incubated for a period of 24 h before mass spectrometric analysis.

We utilized the humidity information for bradykinin provided by the manufacturer to calculate the moles of hydrogen in the final sample solution that originate from the peptide stock solution. The results from these calculations show that 2.8×10^{-10} mol of hydrogen stem from the peptide stock solution. Hence, the moles of hydrogen originating from the peptide are negligible in comparison to the moles of hydrogen originating from the deuterated solvents (4×10^{-6} mol). The manufacturer reports that melittin is 100% peptide content of 70% purity (the remaining 30% is phospholipase A_2, common impurity) and that the amount of water in the peptide is insignificant. The humidity information for α-msh is not available but the peptide content is 78% and a large part of the remaining 22% is salts. The hydrophobicity of α-msh is higher than for bradykinin; therefore, we expect the humidity of α-msh to be similar to or lower than that of bradykinin. To avoid undesired water condensation on the peptides, precautions were taken to weigh the peptides at room temperature and to keep the peptide vial capped.

The dried droplet sample preparation method was used to prepare the MALDI sample spots. One microliter of the sample solution was laid on top of the stainless steel sample stage and allowed to dry inside the drybox to protect it from back-exchange. The sample was moved immediately after drying from the drybox to the instrument by placing the sample stage inside a nitrogen-filled Ziploc bag (DowBrands L.P., Indianapolis, IN). The Ziploc bag containing the sample was placed inside an Atmosbag (Aldrich, Milwaukee, WI), taped around the instrument inlet and continuously flushed with dry nitrogen. We did control experiments to evaluate the possibility of H/D exchange in the mass spectrometer. A sample deposited on the sample probe was stored in the instrument at a background vacuum of 1×10^{-6} Torr maintained by bleeding D_2O through a variable leak valve. Following a 48-h incubation period the MALDI spectra were recorded and no detectable H/D exchange was observed.

Calculation of the Centroid of the Isotopic Peak Distribution

To quantify the amount of H/D exchange the peptides underwent in the different D_2O content solutions, we

first calculated the centroid of the isotopic peak envelope for the $[M_{exch} + D]^+$ ions. The areas of the peaks that comprise the isotopic envelope in the spectrum were integrated and exported to a Microsoft® Excel 97 spreadsheet. The areas for the individual signals were then summed to calculate the total area of the isotopic distribution. A weighed area for each peak was calculated by dividing the area of each peak by the total area. Finally, the centroid of the isotopic distribution was calculated by adding the products of multiplying the weighed areas and the mass-to-charge ratio (m/z) of all the peaks in the isotopic peak distribution. The calculated centroid was assumed to correspond to the m/z of the deuterated H/D exchanged species ($[M_{exch} + D]^+$ ion) rather than to protonated H/D exchanged species ($[M_{exch} + H]^+$ ion). This assumption is reasonable because all the solutions were prepared in deuterated solvents (with the exception of acetonitrile, which is an aprotic solvent and anhydrous; therefore, there are no sources of H^+ ions). The exchange percentages are calculated using the following equation:

$$\% \text{ exchange} = \frac{(m/z)_{[M_{exch}+D]^+} - (m/z)_{[M+H]^+}}{(n+1) \times 1.006} \times 100 \quad (1)$$

where $(m/z)_{[M_{exch} + D]^+}$ is the calculated mass to charge ratio of the centroid of the isotopic peak envelope, $(m/z)_{[M+H]^+}$ is the calculated average mass of the nondeuterated protonated peptide ion, and n is the number of exchangeable hydrogens on the neutral peptide. Average masses of the protonated molecules (1061.23, 1665.91, and 2847.50 Da) and a number of exchangeable hydrogens for the neutral molecules of 17, 26, and 47 for bradykinin, α-msh, and melittin, respectively, were used for these calculations. The results are reported to two significant figures.

Circular Dichroism Experiments

Stock solutions of bradykinin, α-msh, and melittin dissolved in deionized water (Milli-Q Water System, Millipore) were prepared. Sample solutions containing 10% to 100% water in each of the anhydrous organic solvents were prepared from the stock solutions of each peptide. The final peptide concentration ranged from 10^{-4} to 10^{-5} M for all the solutions. The anhydrous organic solvents, methanol (99.8%), isopropanol (99.5%), and acetonitrile (99.8%) were purchased from Aldrich (Milwaukee, WI) and kept inside the drybox. The circular dichroism (CD) spectra were acquired on an AVIV 62DS circular dichroism spectrometer (AVIV, Inc., Lakewood, NJ) at a constant temperature of 25 °C. Before every experiment, a calibration of the spectrometer detection system was performed by measuring the ellipticity ratio of the 192.5 to 290 nm bands of (1*S*)-(−)-10-camphorsulfonic acid (CSA). The measured ellipticity ratio was always between the acceptable range of 1.9 to 2.2 for all the experiments. The acquired data were averaged, smoothed, and converted from ellipticity to molar ellipticity before being plotted using KaleidaGraph 3.0 (Synergy Software, Reading, PA).

Results

MALDI H/D Exchange Experiments

Figures 1, 2, and 3 contain the MALDI mass spectra for bradykinin, melittin, and α-msh each dissolved in the three deuterated solvent systems used in this study. Note that the isotopic peak distribution shifts to higher masses as D_2O is added. Figure 4 contains histograms showing the extent of H/D exchange obtained for bradykinin in methanol-d_4, anhydrous acetonitrile, and isopropanol-d_8 solvents as D_2O is added. Although the exact amounts of H/D exchange differ for each solvent system, the general trends are similar. For example, at low percentages of D_2O the percent H/D exchange is low and as the amount of D_2O is increased, the percent H/D exchange increases. Figures 5 and 6 contain similar data for α-msh and melittin, respectively. The only significant difference among the results for the various solvent systems is that the exchange percentages for low D_2O (<10% D_2O) content solutions are lower in the acetonitrile/D_2O system than for methanol-d_4/D_2O. In isopropanol-d_8/D_2O the exchange percentages are even lower than in the acetonitrile/D_2O system. For example, in methanol-d_4/D_2O solvent, bradykinin (Figure 4a) has an exchange percentage of 87% at 10% D_2O, but in 10% D_2O/acetonitrile (Figure 4b) the exchange percentage is only 62%. Note that for isopropanol-d_8/D_2O solvent (Figure 4c), bradykinin reaches 87% exchange at 50% D_2O. Conversely, in methanol-d_4/D_2O and acetonitrile/D_2O solvent systems, bradykinin reaches approximately 90% exchange at 10% and 20% D_2O, respectively. Similarly, in isopropanol/D_2O, α-msh reaches 80% exchange at 40% D_2O, an exchange percentage that is observed at 5–10% D_2O in the methanol-d_4/D_2O solvent and 15%–25% in the acetonitrile/D_2O solvent (Figure 5). The only exception to this general trend is for melittin in the methanol-d_4/D_2O and acetonitrile/D_2O solvents (Figure 6a, b). The exchange percentages in these two solvent systems are very similar (accounting for experimental error) at all D_2O contents. Conversely, for melittin the exchange percentages are lower at the low water content solutions (<20% D_2O) in isopropanol-d_8/D_2O solvent (Figure 6c).

Circular Dichroism Spectra

CD spectroscopy is a frequently used technique to follow peptide and protein conformational changes in solution. Consequently, we examined the CD spectra for the three model peptides used in our studies to access the conformational changes that occur as solvent composition is changed. Figures 7, 8, and 9 contain CD

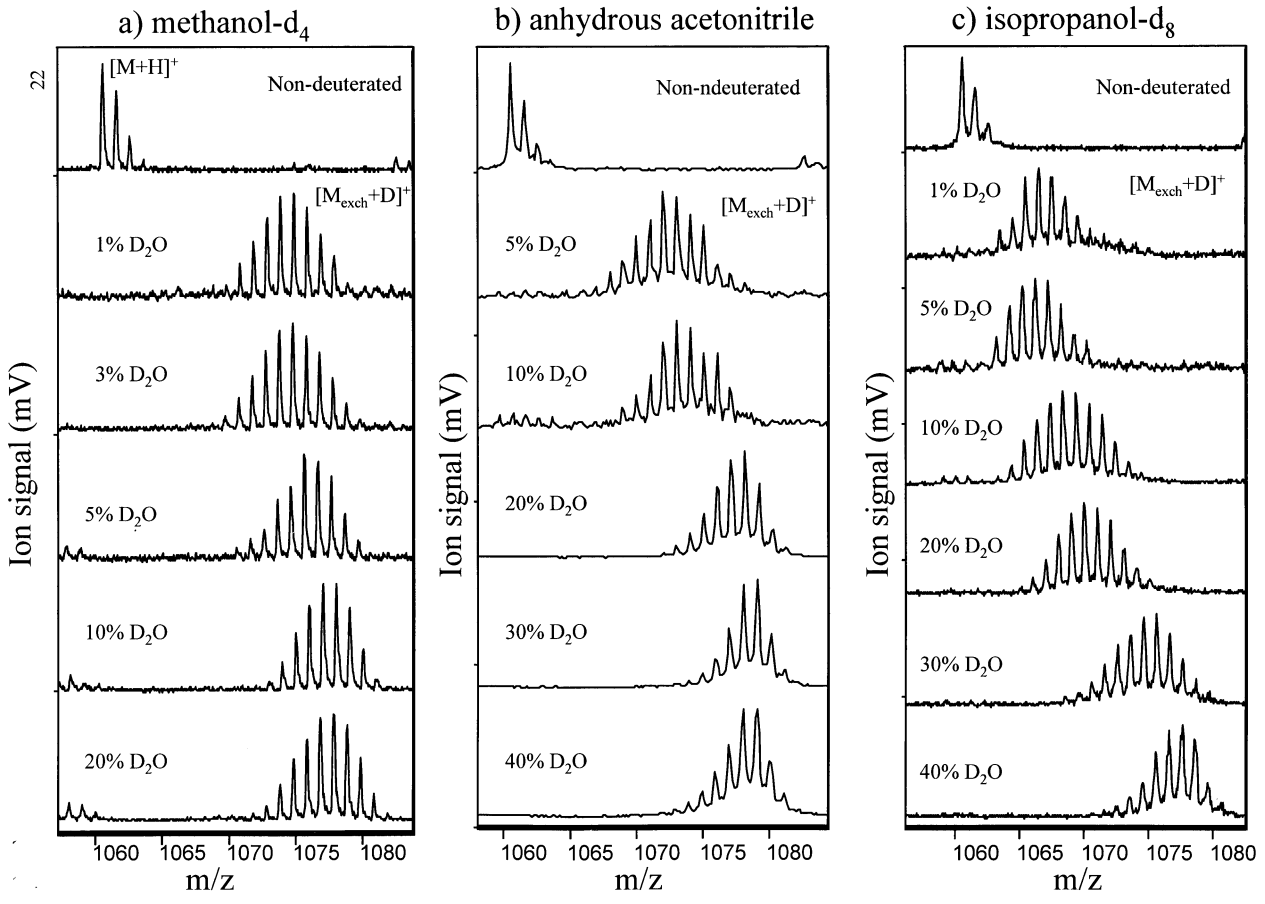

Figure 1. MALDI H/D exchange spectra for bradykinin as water is added in (**a**) methanol-d_4, (**b**) anhydrous acetonitrile, and (**c**) isopropanol-d_8. The MALDI matrix was α-cyano-4-hydroxycinnamic acid.

spectra for each peptide dissolved in methanol, acetonitrile, and isopropanol. The spectral changes that occur as water is added to the solutions are quite evident. For example, the CD spectrum for bradykinin in aqueous solution (Figure 7) is composed of an intense negative trough at 205 nm, a positive peak at 222 nm, and a weak negative trough around 235 nm (this peak is absent in the acetonitrile/H_2O solvent system). As water is added to each of the solvent systems, some common changes occur. For example, the negative peak at 205 nm intensifies and slightly shifts to shorter wavelengths as the water content is increased. Also, as the percentage of water is increased, the positive peak at 222 nm intensifies slightly but it is less dramatic in the methanol/H_2O system as compared to changes observed in the acetonitrile/H_2O and isopropanol/H_2O solvent systems.

The CD spectra for α-msh undergo dramatic changes as water is added to the solutions (Figure 8). The spectral transitions are very similar for the three solvent systems used in this study. The CD spectrum obtained at low water content for all three solvent systems is composed of a negative peak around 208 nm with a slight positive hump around 225 nm. As the water content is increased, the 208 nm peak intensifies and moves to shorter wavelengths, and the peak at 225 nm becomes more intense as water is added.

Figure 9 contains the CD spectra for melittin in methanol/water, acetonitrile/water, and isopropanol/water solutions. In solutions containing low amounts of water, the CD spectrum for melittin contains two minima, a small peak at 224 nm, and a more intense peak at 208 nm. As water is added to the organic solvents, both peaks lose intensity and the 208 nm band shifts to shorter wavelengths. The spectral changes in each solvent system are different for melittin. For instance, the spectrum obtained in 10% water/methanol smoothly converts into the spectrum for 100% water solution as the amount of water is increased (Figure 9a). In acetonitrile solution, the 80% water spectrum closely resembles the spectra obtained at low percentage water (Figure 9b). Conversely, the spectrum for the 90% water/methanol solution is very similar to the spectrum at 100% water. The CD spectra of melittin in isopropanol (Figure 9c) do not change significantly up to 60% added water. The transition from 60% to 80% and then to 90% water is more abrupt if compared to the spectra from methanolic solutions.

Discussion

The studies described herein follow our earlier studies examining the effects of solvent on MALDI ion yields

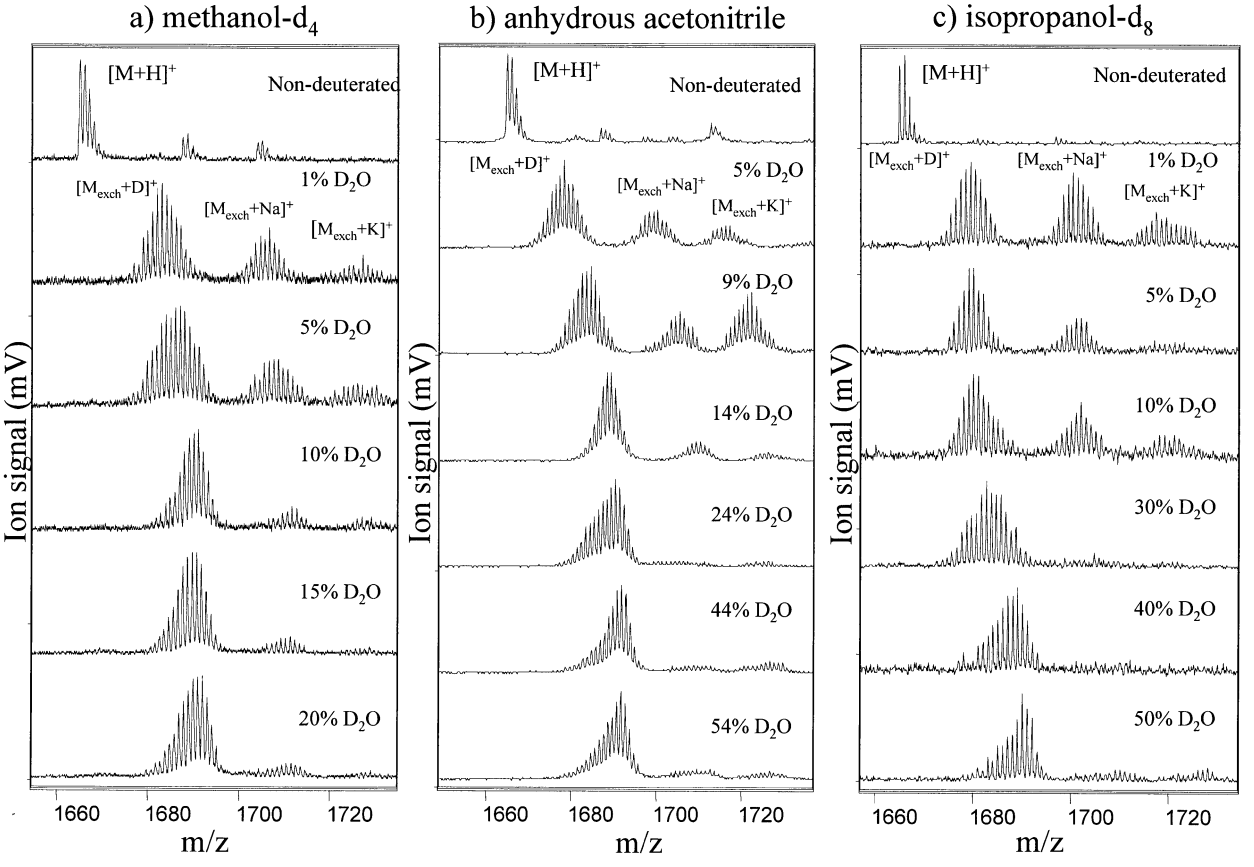

Figure 2. MALDI H/D exchange spectra for α-melanocyte stimulating hormone as the water percentage was increased in (**a**) methanol-d_4, (**b**) anhydrous acetonitrile, and (**c**) isopropanol-d_8. The MALDI matrix was α-cyano-4-hydroxycinnamic acid.

[23]. The results from earlier studies were interpreted in terms of peptide conformation having a significant influence on MALDI ion yields. In the studies described here we present MALDI H/D exchange and CD data for bradykinin, α-msh, and melittin that clearly shows conformational changes of the peptide as the solvent environment is altered. Studies by Rohl and co-workers [28] show that for a 26-residue α-helical peptide (same length as melittin) 25% of the exchangeable hydrogens are protected after a period of 20 h (1200 min). These results show that it is realistic to expect some degree of protection for structured peptides even after long periods of time. Although these studies were performed in aqueous solution, we can expect similar behavior in organic solvents due to their ability to strengthen intramolecular hydrogen bonding [29, 30]. We were unable to locate references in which similar studies were performed in nonaqueous solvents. The following discussion centers on demonstrating the utility of MALDI along with H/D exchange as a probe for solution peptide conformation. In this paper we do not attempt to address questions concerning the positional integrity of the deuterium labels of the partially deuterated peptide ions. We are currently evaluating postsource decay methods and photofragment spectrometry to determine the position of the deuterium labels.

Alcohols such as methanol and trifluoroethanol are known to be structure promoting solvents for peptides, although they do not have the same effects on globular proteins [29, 31–33]. The denatured state of globular proteins is stabilized by methanol, due to the preferential binding of nonpolar solvent to the exposed hydrophobic residues in the denatured conformation [33]. Ishii attributed the stabilizing effect of methanol on the secondary structure of coiled-coil tropomyosin in terms of the availability of the hydrophobic residues of this protein to the solvent environment in its folded conformation [34]. Methanol stabilizes the folded tropomyosin conformation because the hydrophobic residues are not buried in the interior of the structure (as is the case of globular proteins). Therefore, methanol does not have to penetrate and disrupt the hydrogen bonds to reach the hydrophobic residues causing denaturation. The stabilization of peptide secondary structure in alcohols has also been explained in terms of a favorable effect on the intramolecular hydrogen bonding interactions [29, 30]. The decrease in dielectric constant of the organic solvents promotes intramolecular hydrogen bonding due to a decreased ability to stabilize dipoles and charges as compared to water. In addition, these solvents do not compete as efficiently as water for the hydrogen bonds.

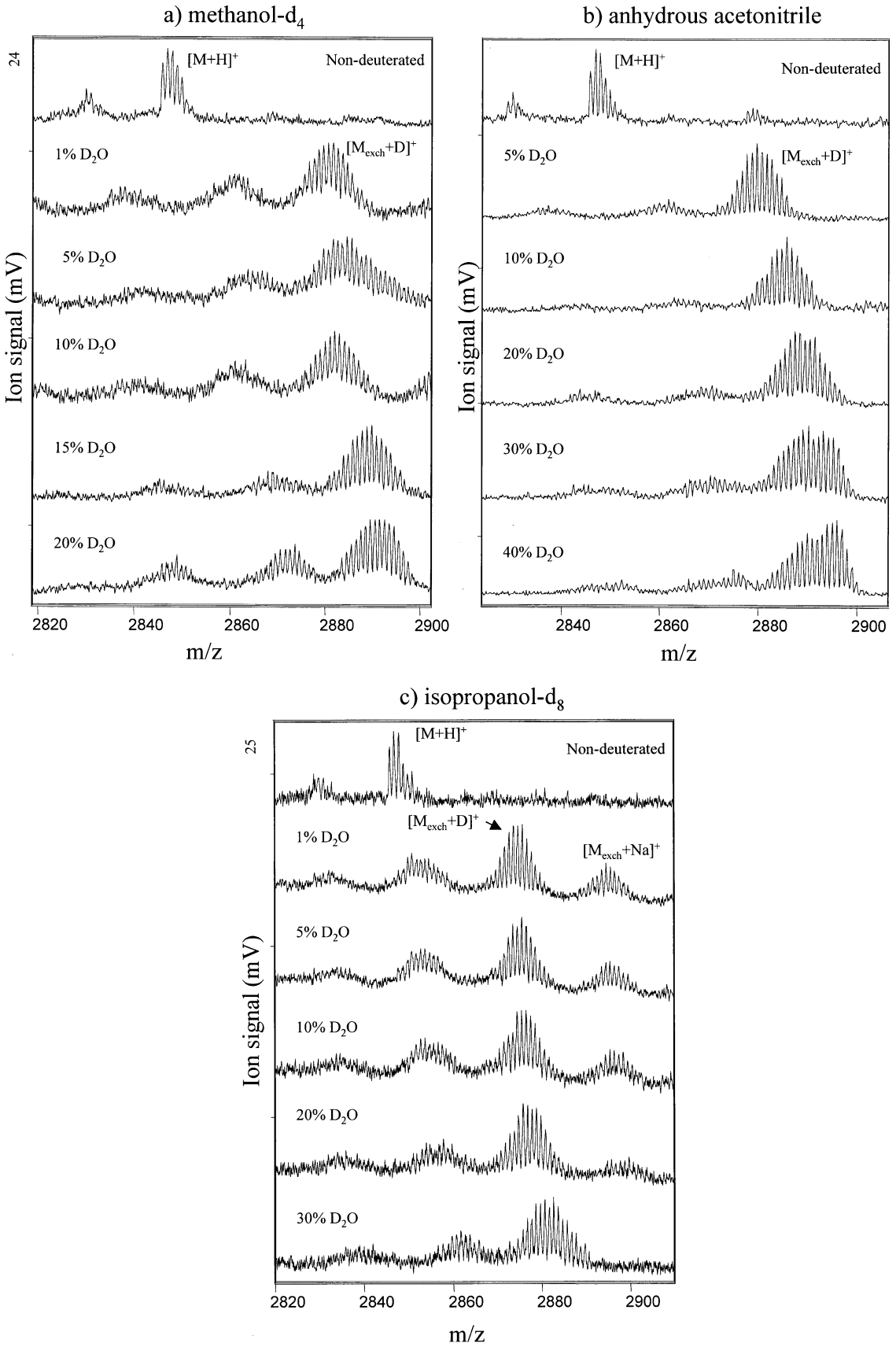

Figure 3. MALDI H/D exchange spectra for melittin as water is added in (**a**) methanol-d_4, (**b**) anhydrous acetonitrile, and (**c**) isopropanol-d_8. The MALDI matrix was α-cyano-4-hydroxycinnamic acid.

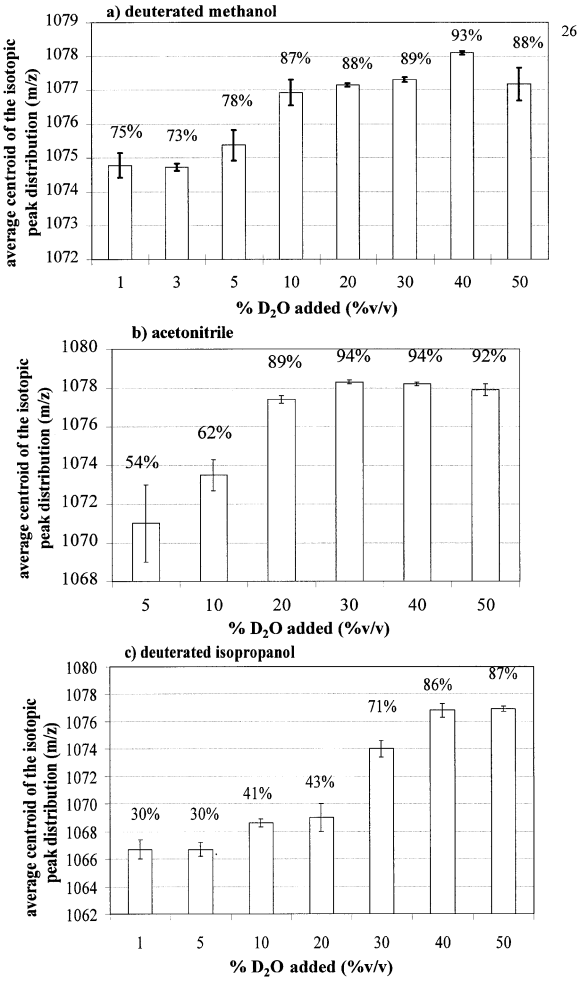

Figure 4. Plots of average centroid of the isotopic peak distribution vs the amount of D_2O added to solutions of bradykinin in (**a**) methanol-d_4, (**b**) anhydrous acetonitrile, and (**c**) isopropanol-d_8. The percentages on top of the bars correspond to the calculated exchange percentages. The error bars are the standard deviation of the average centroid for five replicate spectra.

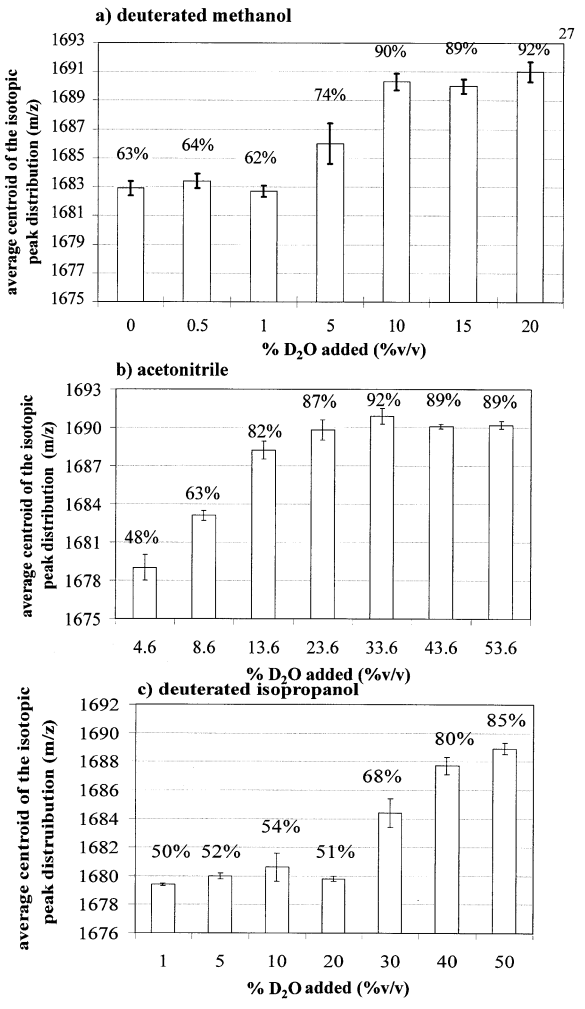

Figure 5. Plots of average centroid of the isotopic peak distribution vs the amount of D_2O added to solutions of α-melanocyte stimulating hormone (α-msh) in (**a**) methanol-d_4, (**b**) anhydrous acetonitrile, and (**c**) isopropanol-d_8. The percentages on top of the bars correspond to the calculated exchange percentages. The error bars are the standard deviation of the average centroid for five replicate spectra.

In a MALDI experiment the analyte experiences several environmental changes before it is transformed into a gas-phase ion. Initially, the analyte exists as a solute in a solvent and as the solvent evaporates, the analyte precipitates to form a solid. Because the matrix is present at much higher concentrations (molar ratios of 1000/1 and 10,000/1 are common) and probably reaches supersaturation before the analyte, it is generally assumed that the analyte forms an inclusion in the matrix crystal lattice. The solid matrix-analyte is introduced into the mass spectrometer for laser desorption and ionization. As a direct result of laser irradiation, the matrix evaporates, carrying with it analyte molecules, and some fraction of the gas-phase matrix and analyte are ionized. We interpret the MALDI H/D exchange data as evidence that the peptide retains the solution-phase conformations during the precipitation process. The major underlying assumption that we make is that as the solvent evaporates forming the sample droplet, the matrix "locks" the solution peptide conformation. Therefore, according to our view, no major conformational changes occur as the solvent evaporates. Since deuterated gases are not introduced into the mass spectrometer, the deuterium-labeled gas phase ions are exclusive products of the solution phase H/D exchange processes.

The effects of organic solvents on the amide hydrogen exchange rates were investigated by Englander et al. [35]. They reported that organic solvents have only a small effect on the acid catalyzed exchange rates, but the base catalyzed exchange rate is considerably decreased. They also observed a shift of the minimum exchange rate to higher pH values for 50% D_2O/methanol-d_4 and 50% D_2O/acetonitrile solutions. According to their results, the organic solvents used in our experiments should not affect the H/D exchange kinetics because the H/D exchange process is expected to be

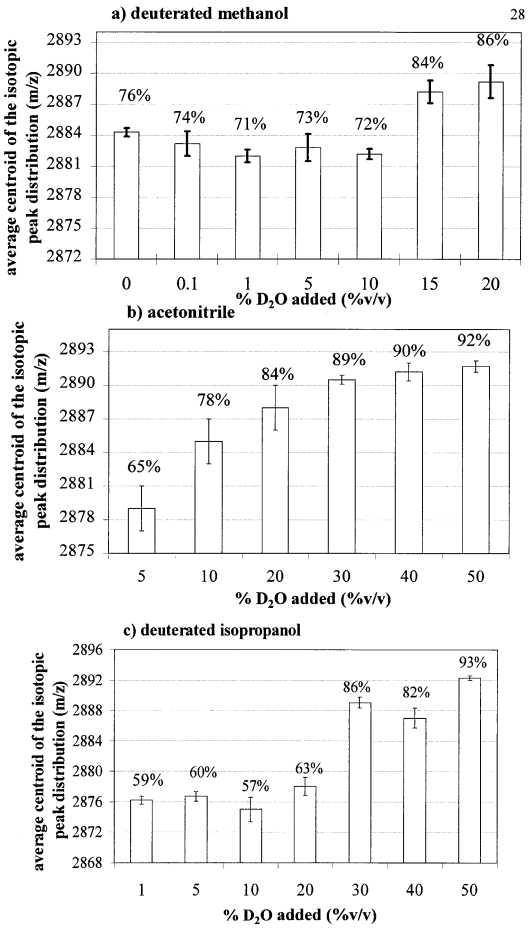

Figure 6. Plots of average centroid of the isotopic peak distribution vs the amount of D_2O added to solutions of melittin in (**a**) methanol-d_4, (**b**) anhydrous acetonitrile, and (**c**) isopropanol-d_8. The percentages on top of the bars correspond to the calculated exchange percentages. The error bars are the standard deviation of the average centroid for five replicate spectra.

acid catalyzed (due to the presence of matrix in the solution). To examine this assumption, H/D exchange percentages in the solvent systems utilized in this study were measured for the unstructured peptide poly-D,L-alanine. We found that for the 10-residue peptide in 1, 5, 10, and 20% D_2O/methanol-d_4 solutions, the centroid of the isotopic peak distribution was close to 100% exchange for all solutions and the peak corresponding to the 100% exchange was always observed. These results suggest that organic solvents are not slowing the exchange kinetics in our experiments; however, due to the difficulties associated with accurate determination of pH (or pD) in organic/water solvents we did not attempt to closely examine the effects of pH on H/D exchange in this study. In addition, pKa values for the MALDI matrix are affected by the solvent composition (organic/water) and studies are under way to determine whether such equilibria affect the MALDI H/D exchange process.

One can argue that matrix present in solution (mM concentrations) might disrupt the solution conformation of the peptides, and CD cannot be used to ascertain conformational changes caused by the matrix compound due to the high matrix UV absorbance. We did, however, measure the methanol solution CD spectrum of melittin (1×10^{-4} M) in 3 M urea and found no significant changes in the helical conformation. The urea to analyte ratio in this case was approximately 30,000/1, an order of magnitude higher than the matrix to analyte ratio used in our MALDI H/D exchange experiments. Although we have not extensively studied the effect of utilizing a different matrix, preliminary results with 2,5-dihydroxybenzoic acid do not show any significant differences in the H/D exchange. This further supports our assumption that the changes in H/D exchange with changes in solvent system are due to solvent effects. Based on these results we assume that the matrix does not strongly influence the peptide conformation.

Bradykinin is a nine-residue peptide hormone and is a potent pain-producing agent. Its conformation in aqueous solution has been extensively studied by both NMR and CD [36–39]. In aqueous solution, bradykinin possesses a high degree of flexibility and lacks any constant elements of secondary structure. The conformation of bradykinin in aqueous trifluoroethanol, dioxane, and dimethyl sulfoxide solutions has also been studied with CD and NMR [38, 40–42]. All of these studies agree that bradykinin adopts a more compact conformation going from aqueous to hydrophobic solvent environments, and it was proposed that this conformation resembles the one bradykinin adopts in the neuron receptor site.

Our H/D exchange studies suggest that bradykinin adopts a more compact conformation in organic solvents than in water. For example, the H/D exchange data for bradykinin reveal more elements of secondary structure in acetonitrile than in methanol. Furthermore, the isopropanol conformation seems to be the most compact of the three. Note that the percent H/D exchange at 10% D_2O in the three solvent systems are quite different (Figure 4). There are approximately 3.5 (20%) more protected labile hydrogens in acetonitrile/D_2O than in methanol-d_4/D_2O solvent system. The hydrogen exchange percentage in isopropanol-d_8/D_2O (Figure 4c) is even lower, e.g., 41% (seven hydrogens), corresponding to a difference of close to 50% more protected labile hydrogens in isopropanol-d_8/D_2O than for methanol-d_4/D_2O. The CD data for bradykinin (Figure 7) support this interpretation of the H/D exchange experiments results. Changes in the conformation of bradykinin are observed as water content increases in the three solvent systems. The minima at 202 nm increase in intensity as water is added. The transitions between the two extremes, namely, 10% water and 100% water solution, are similar for the three solvent systems.

In earlier studies, Katta and Chait examined electrospray H/D exchange data of bradykinin from a 100% D_2O solution and reported exchange of all labile hydro-

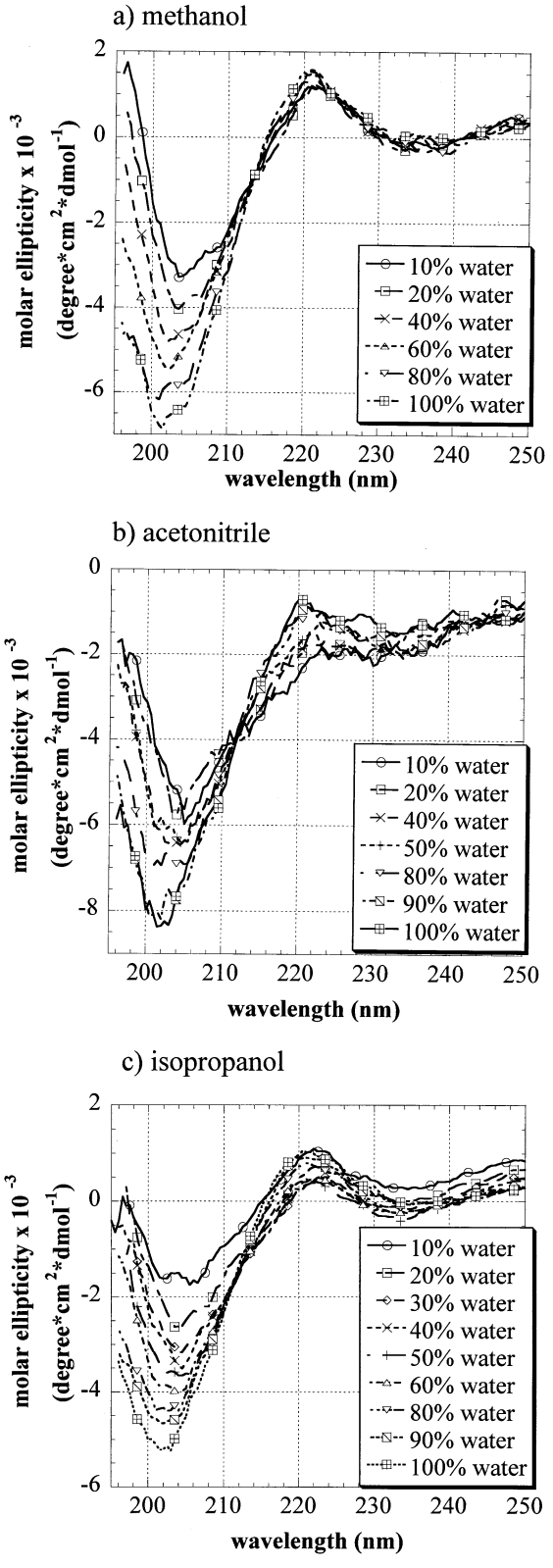

Figure 7. Far UV solution-phase circular dichroism spectra for bradykinin dissolved in (**a**) methanol, (**b**) acetonitrile, and (**c**) isopropanol as the water content in solution in increased.

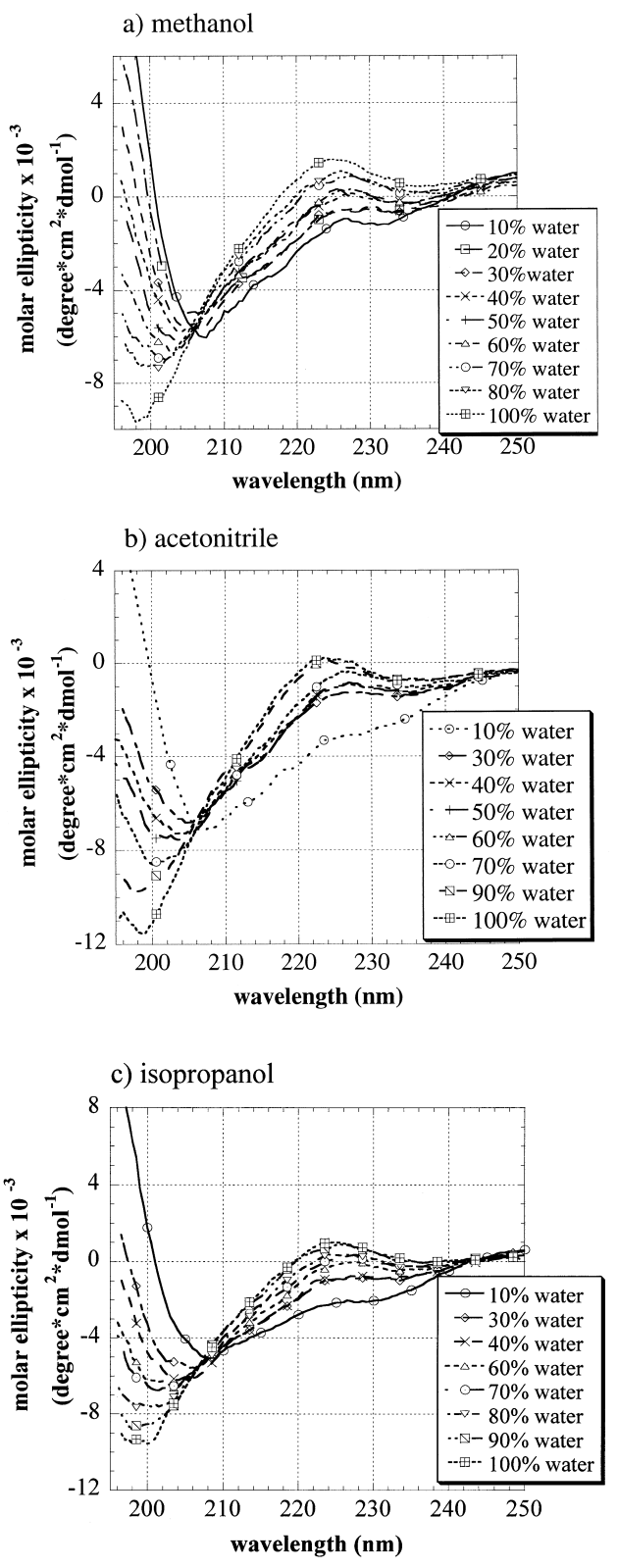

Figure 8. Far UV solution-phase circular dichroism spectra for α-melanocyte stimulating hormone (α-msh) dissolved in (**a**) methanol, (**b**) acetonitrile, and (**c**) isopropanol as the water is added to the organic solvent.

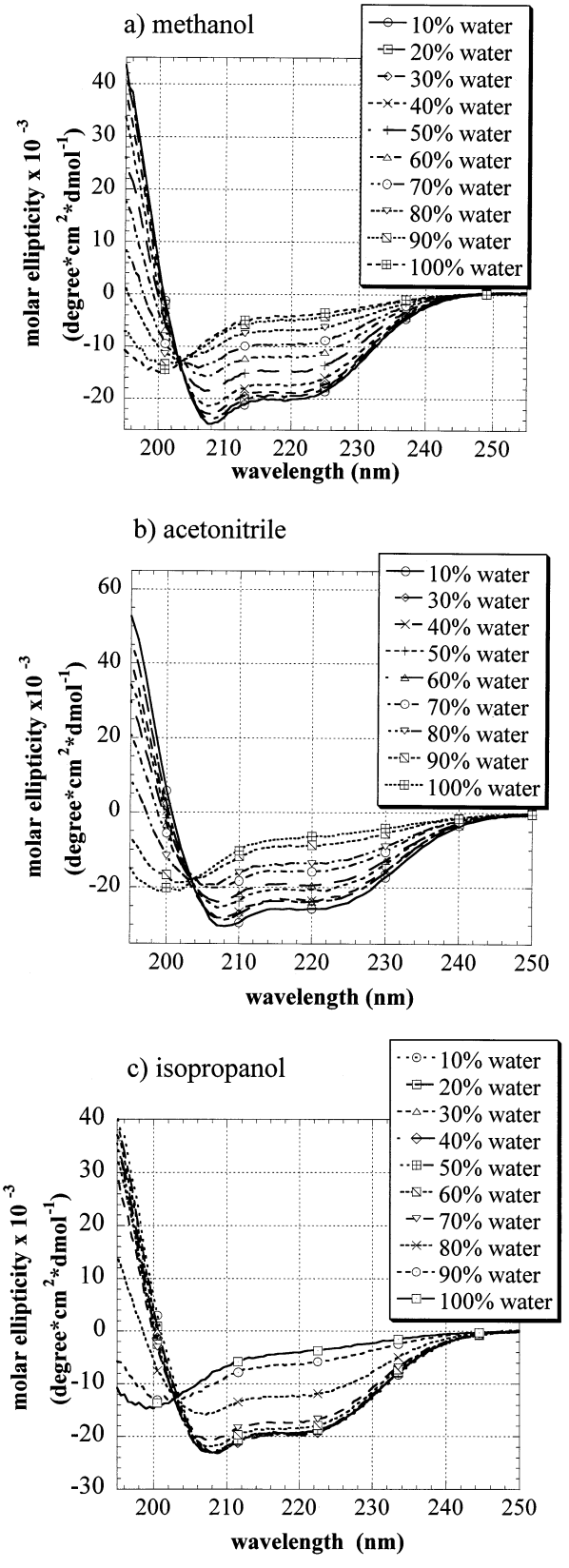

Figure 9. Far UV solution-phase circular dichroism spectra for melittin dissolved in (**a**) methanol, (**b**) acetonitrile, and (**c**) isopropanol as the water is added to the organic solvent. Melittin goes from a helical conformation in organic solvent to a random coil conformation in water.

gens [14, 43]. Our results for the high water content solutions, where bradykinin is expected to assume a random coil conformation, are in agreement with their results. To compare our MALDI H/D exchange results herein, electrospray H/D exchange experiments for the three peptides in the various D_2O/deuterated organic solvent systems are under way. It is possible that gas phase H/D exchange processes occurring during the ionization step might cause differences in the observed results for both experiments.

The 13-residue peptide pituitary hormone, α-msh, is involved in the physiological mechanism of organism pigmentation changes [44]. The conformation of α-msh in low pH aqueous solution is random coil; however, in 1.25 mM sodium dodecyl sulfate the peptide acquires a β-form [45, 46]. We were unable to find any reports of the conformation of α-msh in organic solvents. The H/D exchange data can be interpreted as the peptide acquiring a more folded conformation in organic solution. As the solvent environment is made increasingly aqueous, the peptide structure exposes more labile hydrogens as evidenced by the increase in H/D exchange percentages. For example, in 5% D_2O/methanol-d_4 the H/D exchange reaches 74%, as compared to 48% and 52% in acetonitrile and isopropanol-d_8, respectively. Although the exchange percentages for the 5% D_2O acetonitrile and isopropanol-d_8 solution are approximately the same (within experimental error), it takes more D_2O in the isopropanol-d_8 system to reach 85% exchange than for acetonitrile. Thus it appears that isopropanol helps to stabilize a more folded structure at higher amounts of water. The CD spectra for α-msh in the three solvent systems also supports an explanation based on water induced conformational changes (Figure 8). A striking observation from the MALDI H/D exchange spectra for α-msh (Figure 2) is that as the D_2O content in solution is increased (for all the three solvent systems), the relative abundance of the ions formed by alkali metal ion attachment to the peptide, e.g., $[M + Na]^+$ and $[M + K]^+$, decrease. This effect was observed and discussed previously [23]. Briefly, we interpret these observations in terms of a higher affinity of the Na^+ and K^+ ions for the peptide relative to the organic solvent. As the water content in solution increases, the alkali ions have a higher affinity for water than for the peptide. Note that the relative abundances of the Na^+ and K^+ adduct ions are more pronounced in the isopropanol-d_8 solvent and this effect might be due to the lower solvent polarity of isopropanol, relative to methanol and acetonitrile. It is quite possible that the increase in abundance of the $[M + Na]^+$ and $[M + K]^+$ ions in isopropanol solvent systems reflects solvent dependent changes in alkali metal ion affinities of the peptides, e.g., changes in the peptide conformation. We are currently examining this further using tandem mass spectrometry, both postsource decay and photodissociation.

Melittin is a peptide containing 26 residues and is one of the major components of the venom from the bee

Apis mellifera [47]. The conformation of mellitin in solution has been extensively studied and is a complex function of solvent, concentration, pH, and ionic strength [48–56]. In water, an equilibrium between the tetrameric form of the peptide and the unfolded monomer has been observed. The solution conditions that favor the random coil conformation in water are low ionic strength, acidic pH, and submillimolar peptide concentrations. Conversely, high ionic strength, basic pH, and higher than millimolar peptide concentrations favor the folded tetrameric conformation. The folded or helical monomeric form of the peptide has not been observed in water. The secondary structure of the folded monomers that compose the tetramer has been described by Terwillinger and co-workers from data obtained by X-ray crystallographic analysis of melittin crystals grown from aqueous solution [51, 52]. The overall structure of the monomers that constitute the tetramer resemble a bent helical rod, composed of two α-helical regions joined by a bend with helical character between residues Thr-11 and Gly-12.

Bazzo and co-workers studied the conformation of melittin in methanol solution using ^{1}HNMR nuclear Overhouser enhancement (NOE) data utilizing distance geometry and restrained molecular dynamics analyses [57]. They reported that melittin exits as a helical monomer in methanol solution. The conformation of the monomers in methanol resembles the one obtained from the X-ray crystallography data of the tetramer crystals grown from aqueous solution. H/D exchange ^{1}HNMR studies of the conformational stability of melittin in methanol solution further confirms the results reported by Bazzo and co-workers [58].

Our MALDI H/D exchange results for melittin in deuterated methanol, anhydrous acetonitrile, and deuterated isopropanol/water systems are consistent with earlier reports (Figure 6). The H/D exchange results show that the conformation melittin acquired in organic solvents protects the labile hydrogens more than the conformation in water. Furthermore, increases in the exchange percentages are interpreted as solvent-induced conformational changes as water is added to the organic medium. Monitoring these changes in the exchange percentages gives information on the flexibility of peptide conformation in binary solvent systems. For example, H/D exchange percentages for 10% D_2O in the three solvents is approximately 15% to 20% less in isopropanol-d_8 as compared to methanol-d_4 and acetonitrile. Also, the exchange percentage reaches 85% at 20% water in both methanol-d_4/D_2O and acetonitrile/D_2O system. To reach this same degree of exchange in the isopropanol-d_8/D_2O system requires 30% water. Thus, it appears that melittin prefers to remain folded in lower polarity solvents. Methanol and acetonitrile have very similar dielectric constants, $\epsilon = 33.0$ and $\epsilon = 36.64$, respectively [59]. Thus, in solvent systems of similar polarity, the conformation that melittin adopts in the two solvents should be similar. On the other hand, the dielectric constant of isopropanol, $\epsilon = 20.18$ [59], is lower and we would expect a folded conformation to be more stable at higher water content solutions.

The CD data obtained for melittin in the different solvents systems clearly demonstrate changes in conformation as the water content in the solution is increased (Figure 9). For example, the CD spectrum in 10% water/90% methanol solution is typical for a peptide with helical conformation [60]. The spectra in 100% water correspond to a peptide having a random coil conformation. These data are in agreement with the known conformation of melittin in methanol and deionized water discussed above. The spectra for 10% water/acetonitrile and water/isopropanol are similar to the spectrum obtained for the 10% water solution in methanol (Figure 9). Coupling these results with the MALDI H/D exchange results obtained for melittin suggests that the conformation of melittin in the acetonitrile and isopropanol solvents are very similar to the methanol system, probably helical. The transitions between the helical folded state and the random coil conformation in the three solvents are different, and the observation of an isodichroic point at 203 nm in all three solvent systems supports a two-state transition between the conformations. For instance, in the methanol/water solvent system the transition between the folded helical conformation and the random coil is smooth. The transition in the acetonitrile/water system is not as smooth as for the methanol/water. That is, we observe a gradual change in conformation in contrast to the isopropanol/water system. In isopropanol/water, the CD spectra suggest that melittin remains folded up to 50% water and this result contradicts the result obtained by MALDI H/D exchange. The MALDI H/D exchange is consistent with >90% exchange at 50% D_2O/isopropanol-d_8; however, the conflicting CD and H/D exchange data could possibly be explained by an absence of secondary structure that provides protection against H/D exchange. This discrepancy could also be explained in terms of the aggregation state of melittin and by differences in peptide concentration for the two experiments. The concentration of melittin in the stock solution prepared for the MALDI H/D exchange experiment is micromolar as compared to millimolar for the CD experiment. As discussed above, the aggregation state of melittin is highly dependent on concentration. Hence, the higher concentrations required for CD are sufficient for aggregation of monomers in isopropanol and this could cause the peptide to remain folded in solutions containing as much as 50% water. This is consistent with the observation of an isodichroic point because the conformation of the helical monomer is very similar to the conformation of the monomers forming the tetramer (*vide supra*). CD in the wavelength range we used is not sensitive to changes in quaternary structure, unless these changes are accompanied by changes in secondary structure. Therefore, the CD spectra of the helical monomers and of the tetramer should be very similar. Thus, it appears that melittin undergoes

the following transitions as water is added to organic solvents:

$$\text{helical monomers} \overset{\text{four}}{\rightleftarrows} \text{tetramer} \rightleftarrows \text{four random coils}$$

100% organic solvent → 100% water

Validation of the proposed mechanism requires careful control of the solution conditions (pH, concentration, and ionic strength) as well as a specific method to detect the tetramer. Electrospray ionization can potentially be used to address this issue and such studies are currently underway.

Conclusion

This article demonstrates the utility of MALDI-TOF mass spectrometry to monitor solution phase H/D exchange of peptides. The interpretation of our H/D exchange and CD results coincide in that peptides acquire a folded conformation in the organic solvents used in this study. As water is added to the solutions, the three peptides unfold and eventually acquire a random conformation in aqueous solutions. H/D exchange in combination with MALDI-TOF mass spectrometry provides a simple and straightforward method for studying conformational changes that occur as the solvent environment of the peptide is changed. This methodology can be applied to the study of protein folding and noncovalent interactions with only few modifications. These results also support the notion that one of the factors affecting the MALDI ion yields is conformation. It is reasonable to expect that when a peptide is folded, the basic sites are not as available for protonation by the matrix as compared to the unfolded conformation. This can lead to a decrease in the ion yields for MALDI samples prepared using a solvent composition that promotes the folded conformation, e.g., low water contents.

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