Protein Conformational Stability Probed by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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Abstract: We have probed the conformational stability of cellular retinoic acid-binding protein I, a predominantly β-sheet protein, using hydrogen/deuterium (H/D) exchange in solution. Transiently populated intermediate states were detected using H/D exchange measurement under mildly denaturing conditions (pH 2.5 and room temperature). By inducing collisionally activated dissociation in the nozzle-skimmer region of the electrospray source of an FT ICR mass spectrometer (MS), residue-specific information was obtained as to the degree of protection of backbone amide hydrogen atoms as a function of exchange time. The measurements do not appear to be influenced by intramolecular proton mobility in the gas phase. Multiply charged fragment ions covering half of the protein sequence were readily assigned using the extremely high resolution of FT ICR, allowing in some cases protection at individual amide hydrogen atoms to be measured. The results reveal distinct structural regions featuring very different backbone protection patterns. The high data acquisition rate of the FT ICR MS results in significant improvement of temporal resolution over NMR spectroscopy.

Introduction

Use of H/D exchange in combination with multidimensional NMR (e.g., pulse labeling experiments) has become a powerful tool to probe protein structure and conformational stability.[1] Electrospray ionization mass spectrometry (ESI MS) is now emerging as a complementary tool to monitor the kinetics of isotope exchange reactions of polypeptides and proteins.[2−4] Mass spectrometry is unique in its ability to distinguish populations with differing hydrogen exchange properties, whereas NMR can only yield a population average of proton occupancy.[5,6] This invaluable feature allows us to probe the multiplicity of folding pathways and the energy landscape of protein conformation.[7] Over the past several years ESI MS has been employed to study events occurring during folding in a number of proteins. For instance, quenched flow techniques in conjunction with NMR or ESI MS allow detection of structure formation during kinetic refolding since those amides which are hydrogen bonded become protected against exchange with bulk solvent.[6,8] Recent advances have been made to enhance the abilities of mass spectrometry to localize sites of exchange by using proteolysis under conditions where further exchange is minimized. Site-specific information can be obtained by quenching the isotope exchange after a certain time by dropping the solution pH and temperature, and digesting the partly exchanged protein with pepsin. The deuterium content of each peptic fragment can then be measured directly by ESI MS.[9,10] Recently, for instance, Smith and co-workers have detected two intermediate states for urea-induced unfolding of aldolase, a 160 kDa protein.[11] The spatial resolution of this method can be further improved by using gas-phase fragmentation methods in conjunction with proteolysis.[12] One limitation of this method, however, is the possibility of back-exchange occurring during the purification and subsequent analysis of fragments. Nevertheless, this method has proved useful for probing the dynamic nature of proteins.[13,14]

In combination with electrospray ionization, Fourier transform ion cyclotron resonance (FT ICR) can be used to obtain single mass unit resolution with proteins exceeding 40 kDa in size.[15]

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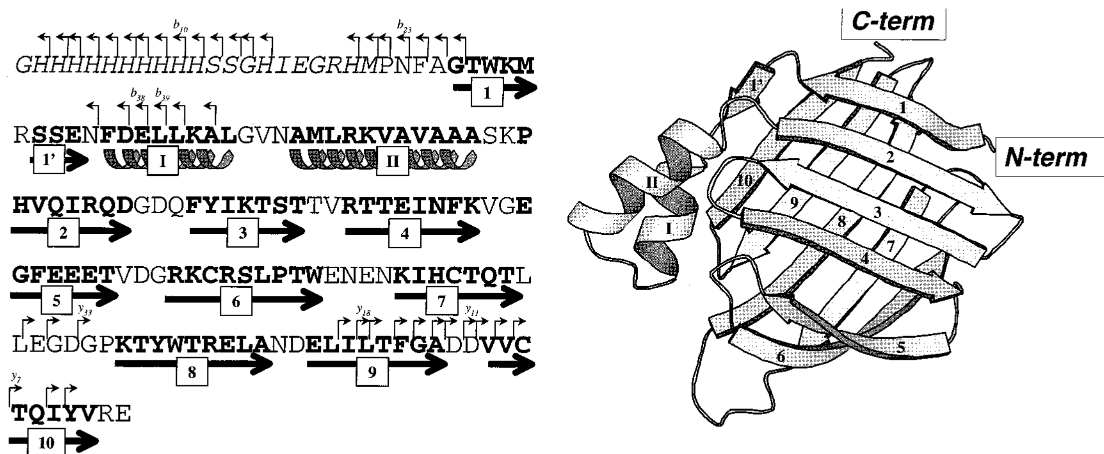

Figure 1. Primary and secondary structure of CRABP I and its fragmentation pattern (in-source collisional activation). The regions of secondary structure are indicated in bold with β-sheets numbered in Arabic, α-helices in Roman numerals. Small arrows indicate the CAD fragments assigned, and fragments described in the text are labeled.

Isotopic enrichment or depletion strategies to obtain narrower isotopic distributions may extend this mass limit even further.[16] In the present work, we assess the applicability of controlled collisionally activated dissociation (CAD) of protein ions with analysis by FT ICR MS to obtain residue-specific information about hydrogen exchange processes. Since dissociation occurs directly in the ESI interface region, fragment ions can be immediately separated and analyzed in the mass spectrometer, avoiding the time required for sample workup. The extremely high resolution of FT ICR MS allows relatively trivial assignments of overlapping multiply charged fragment ions and has the potential to measure H/D exchange at individual amide sites.

In this paper, we investigate the conformational stability of individual structural elements in cellular retinoic acid-binding protein (CRABP I), a predominantly β-sheet protein (Figure 1). By altering the solution pH we can populate species in which secondary structure elements become labile to exchange with solvent,[17−19] and rapidly measure their exchange properties by CAD and FT ICR MS. These data allow us to begin to form a high-resolution picture of the dynamic opening events occurring within CRABP I, furthering our understanding of the unfolding process and the events which enable ligand binding in this protein.

Experimental Section

Pseudo-wild-type CRABP I (containing a 22 residue N-terminal His-tag[20] to facilitate purification and a point mutation, R131Q, to enhance stability[21]) was prepared and purified as described previously.[20] All labile hydrogen atoms were replaced with deuterium by dissolving lyophilized protein in D_2O and incubating the solution at 45 °C for 15 min. The protein was then extensively exchanged and desalted at 4 °C with D_2O/CD_3COOD (Cambridge Isotopes, Andover, MA) using Centricon-10 micro-concentrators (Amicon, Beverly, MA), followed by lyophilization. Dry protein samples were dissolved to a concentration of ∼1 mg/mL in d_4-acetic acid/D_2O adjusted to pD_{read} 2.5. Back-exchange was initiated by diluting the initial protein solution 1:50 in acetic acid/H_2O, pH 2.5 at room temperature (∼25 °C), followed by continuous infusion into the electrospray ionization source of a mass spectrometer at 20 μl/min with a syringe pump (Cole-Parmer, Vernon Hills, IL). pD meter readings were uncorrected for isotope effects.

Mass spectral data were obtained using an Apex II (Bruker Daltonics, Billerica, MA) Fourier transform ion cyclotron resonance mass spectrometer equipped with a thermally assisted electrospray ionization source (Analytica of Branford, CT) and a 4.7 T magnet. Collisionally activated dissociation was induced by increasing the skimmer potential from 80 to 300 V.

Results and Discussion

Measurement of Individual Amide Exchange Rates by CAD. We have previously shown that it is possible to perturb the unfolding equilibrium of CRABP I under mildly denaturing solvent conditions induced by altering the solution pH or adding small amounts of chaotropic agent.[22,23] At least one marginally stable unfolding intermediate was identified by monitoring the isotope exchange kinetics of *d*-CRABP I in protiated solvent at pH 2.5 (Figure 2). These experiments clearly demonstrate populations of CRABP I with differing degrees of exchange protection, yielding a low-resolution picture of the dynamic processes which allow exchange to occur.

Detailed structural information cannot, however, readily be obtained using standard mass spectrometry techniques alone. To obtain high-resolution details about the individual amides which undergo exchange in these intermediate states we used CAD in the electrospray interface ("in-source", or cone fragmentation).[24−26] The extremely high resolving power (exceeding 50 000) of the FT ICR analyzer allows isotopic resolution of each fragment and, hence, unambiguous assignment of charge states. Structurally informative data can then be deduced from the fragmentation spectra of multiply charged protein ions with molecular weights up to 50 kDa.[26−33] Fragment ions derived from different parts of the protein exhibit significant

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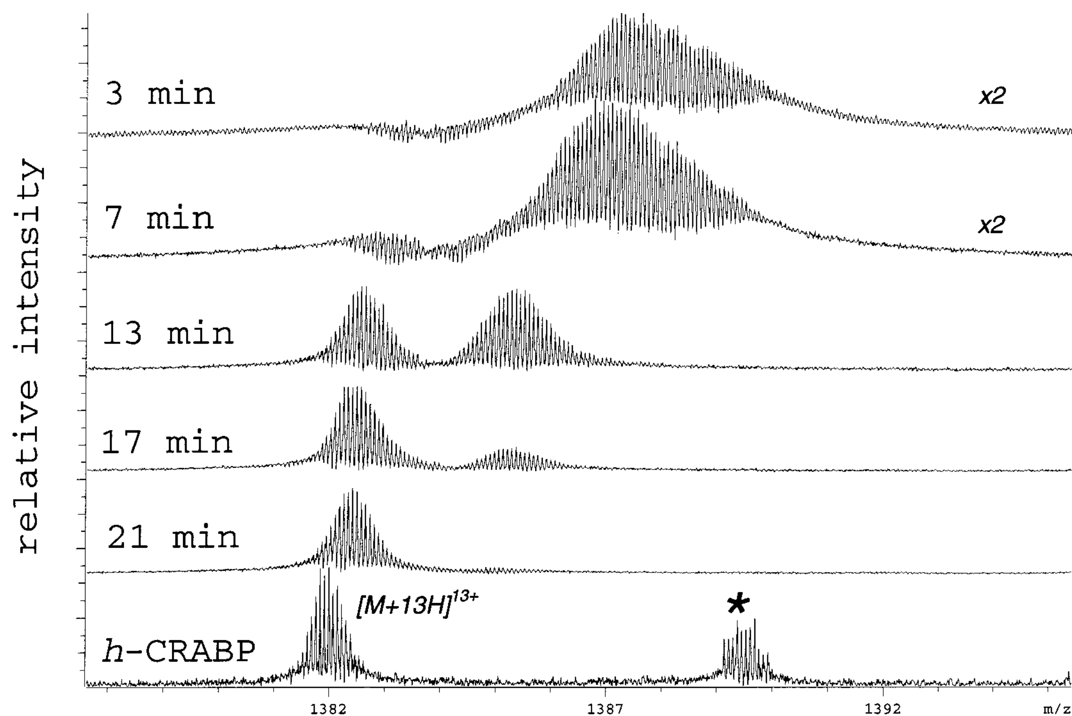

Figure 2. Time course of exchange of intact d-CRABP I in 98% H_2O: 2% ^2H_2O at pH 2.5, as measured by FT MS. The +13 charge state is shown, with the scale expanded by ×2 for the first two time points measured. The bottom panel shows a typical spectrum for fully protonated CRABP I in H_2O. The peak marked with (∗) corresponds to a protein−Tris adduct. The Tris adduct is not present in any other spectra due to extensive buffer exchange prior to measurements.

variation in deuterium content and time evolution. These data provide very accurate site-specific information on the solvent accessibility of various structural elements within the protein as a function of time and, therefore, characterize conformational stability in a site-specific fashion. To ensure the validity of such data, one has to prove that the isotope exchange is effectively quenched in the gas phase so that the deuterium content at individual amides is not altered (vide infra).

Fragmentation of CRABP I was induced by collisionally activating the ions in the electrospray source of the mass spectrometer (by increasing the skimmer potential from 80 to 300 V). The charge state of each fragment ion is readily determined by measuring the spacing between isotopic peaks; even overlapping peaks of different charge states can be easily resolved. Overall, 62 fragment ion peaks (mostly N-terminal *b*- and C-terminal *y*-ions, following the nomenclature by Biemann[34]) were assigned on the basis of the known sequence of the protein. Since most fragments form a series which differ in mass by a single amino acid unit, unambiguous assignment was readily possible. We saw no evidence of internal fragmentation, presumably because the central structure of the protein is more stable in the gas phase. In all, cleavage at 44 out of a total of 156 peptide linkages was detected in the CAD process under the conditions employed here (Figure 1). Using continuous infusion and CAD, exchange occurring in solution was measured in fragment ions as a function of exchange time (see Figures 3 and 4). The extent of deuterium incorporation into each fragment was then measured using the centroid mass for each isotopic cluster.

Regions of the protein which are unstructured in solution would not be expected to retain any deuterium label even at the earliest time of measurement. The construct used in these experiments includes a supposedly unstructured 22 residue N-terminal His-tag (hereinafter residue numbering includes the His-tag such that the wild-type sequence begins with Pro[22]). All fragment ions derived from this region, and indeed the first four residues of the wild-type CRABP I sequence [Pro[22]→Ala[25]] undergo complete exchange following 2 min of exposure of *d*-CRABP I to protiated solvent at pH 2.5.[35] Representative examples (b_{10}^{2+} and b_{23}^{4+}) are shown in Figure 3a. These data confirm that the His-tag region of the protein contains no persistent structural interactions and is completely unprotected against exchange, as are the first four residues in the native conformation of CRABP I, which do not form intramolecular hydrogen bonds in the folded state.[36]

By contrast, peptide fragments derived from regions of stable secondary structure show significant protection against exchange. The two N-terminal β-strands **1** and **1′**, [Gly[26]→Met[30]] and [Ser[32]→Gly[34]], as well as helix **I** [Phe[36]→Leu[43]] are known from NMR analysis to be quite stable against amide exchange in the native state.[36] The isotope exchange kinetics measured within these segments is slow, with less than half of the amide deuterium atoms exchanged following 13 min of exposure of the protein to the protiated solvent at pH 2.5 (Figure 3b). The isotopic distribution of fragment ions derived from the helical

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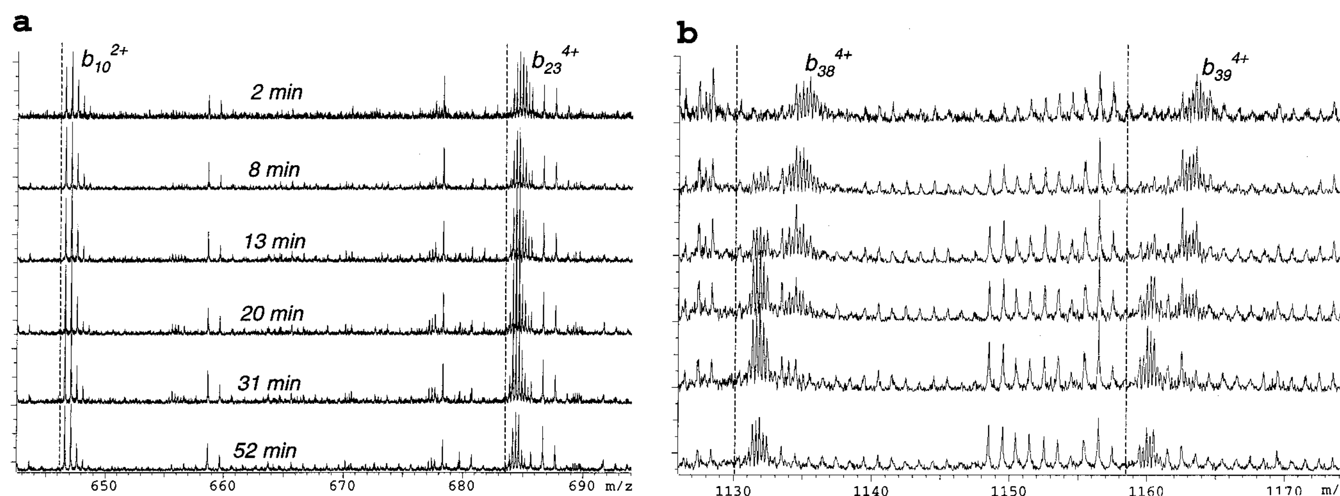

Figure 3. (a) Time evolution of exchange in isotopic clusters of b_{10}^{2+} and b_{23}^{4+} ions in aqueous solution, pH 2.5, corresponding to the His-tag region of *d*-CRABP I. Rapid exchange is consistent with the lack of structure in this region of the protein. (b) Time evolution of isotopic clusters of b_{38}^{4+} and b_{39}^{4+} fragment ion peaks, corresponding to polypeptide segments from the N-terminus, including strands **1** and **1′**, and part of helix **I**. This region of secondary structure is stable under these conditions and hence exchanges slowly.

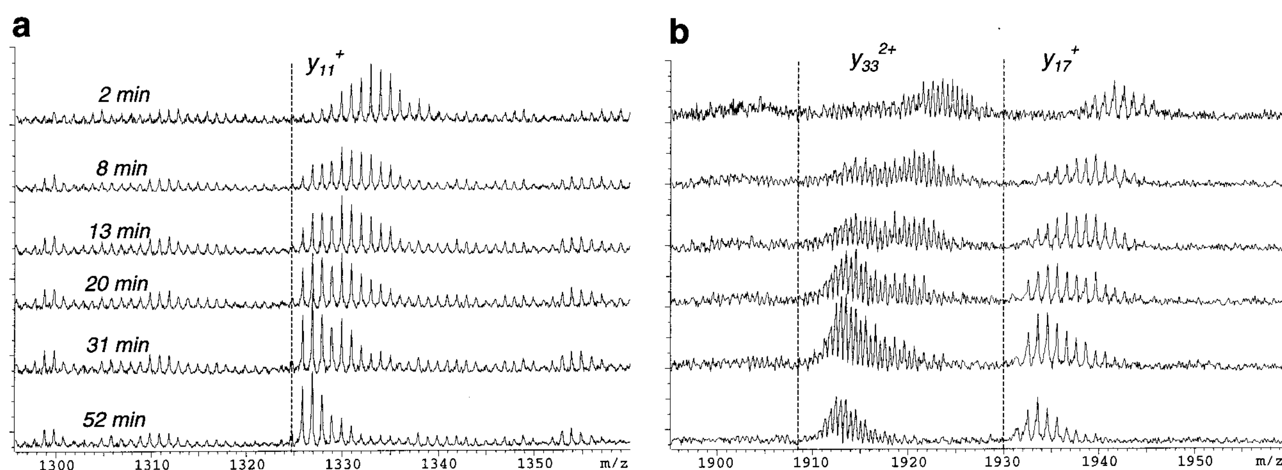

Figure 4. (a) Time evolution of isotopic distribution of y_{11}^{+} fragment ion peak derived from strand **10** and the β-turn **9/10** in 98% H_2O:2% ^2H_2O, pH 2.5. The gradual shift to lower *m/z* over time is indicative of noncooperative exchange. (b) Time evolution of isotopic distribution of y_{33}^{2+} fragment ion peak (**7/8** β-turn region of the protein). The corresponding polypeptide segments includes strands **8**, **9**, and **10**, as well as the β-turns **8/9** and **9/10**, and a part the β-turn **7/8**. The exchange behavior of this fragment is a convolution of the mass shift exhibited by the y_{11}^{+} ion with a bimodal exchange pattern in strand **8**.

region of the protein (represented by b_{38}^{4+} and b_{39}^{4+} in Figure 3b), exhibits clearly bimodal character beyond 8 min. The *m/z* difference between the maxima for b_{38}^{4+} is 13 $\pm$ 1, consistent with the length of the segment [Gly^{26}→Glu^{38}]. Similar data were obtained for all fragment ions derived from the helical region of the protein (b_{35}^{n+} through b_{42}^{n+}). Using this method of studying sequential overlapping fragments we can potentially identify and characterize the proton occupancy at an individual amide. The accuracy of these measurements is currently somewhat limited by the uncertainty in determining the centroids of each isotopic cluster. However, a combination of this method with ^{13}C depletion strategies[16] will clearly eliminate these uncertainties. In the case above the sequence [Gly^{1}→Ala^{25}] exchanges rapidly, but subsequent fragments which contain sequences extending further into the protein structure show that from Gly^{26} to Ala^{42} there is significant stable structure. The pattern of exchange of the amide groups within the [Gly^{26}→Ala^{42}] segment suggests that this region of the protein become unprotected simultaneously and all of the exposed amides then exchange cooperatively, which argues that unfolding and exchange of this region is a highly concerted process. These observations indicate that the isotope exchange is occurring in the so-called EX1 exchange regime,[1] in which the rates of exchange at each amide are governed by the closing rate of the structural region which fluctuates to expose the amide. If the exchange were EX2 (rate of exchange dependent on the opening/closing equilibrium and the intrinsic chemical exchange rate) then this would lead to a smearing of the bimodal peak distributions since exchange would be noncooperative.

Exchange kinetics of the C-terminal portion of the protein contrast sharply with the negligible protection of amide groups within the [Gly^{1}→Ala^{25}] segment of the protein and high protection of the following segment [Gly^{26}→Ala^{42}]. Time evolution of the isotopic distributions of fragment ion peaks y_4^{+} through y_{18}^{+} suggests that the hydrogen/deuterium exchange is mostly completed within 30 min (illustrated by y_{11}^{+} in Figure 4). It is also clear that exchange in this segment of the protein occurs in a noncooperative manner, since the isotopic distribution shifts gradually to lower *m/z* region over time. However, fragment ions derived from the adjacent β-strand **8** and from the turn preceding strand **8** [Leu^{120}→Pro^{126}] (e.g., y_{33}^{2+}, y_{35}^{2+} and y_{37}^{2+}) exhibit isotopic distributions with clearly bimodal

character (Figure 4). These data suggest that β-strand **8** only exchanges in a highly cooperative fashion involving all of the amides on this strand. Although the distributions are somewhat obscured by the contribution from the C-terminal less-structured region (see Figure 4a), it is still possible to estimate the number of protected sites by measuring the m/z difference between the two maxima in the isotopic distributions (14 ± 2). This estimate is surprisingly close to the number of amides within the protein segment [Gly^{125}→Leu^{139}], i.e., 14 peptide bonds, which is not covered by fragmentation (see Figure 2). This observation suggests that the segment [Gly^{125}→Leu^{139}] (e.g., strand **8** and the β-turn **8/9**) is significantly more protected than strands **9** and **10**, and exchanges cooperatively. This is consistent with the NMR data suggesting that β-strand **9** of the protein is somewhat dynamic in the native conformation, although the number of amides which can be probed by NMR is limited in this region of the protein [S.J.E. and L.M.G., unpublished data].

Proton Mobility in the Gas Phase. A major concern in using CAD to study protein folding and unfolding dynamics, however, is the possibility of hydrogen scrambling within the activated protein ions. Proton mobility within fragment ions (which occurs following the dissociation process) will not affect the mass or isotope distribution observed in the mass spectrum. However, if hydrogen scrambling occurs within the activated intact protein ion before fragmentation, or if there is proton exchange between fragment ions and molecules of evaporated solvent, then this would lead to fragment masses which do not reflect the true local deuterium content in solution. Ions are activated in the electrospray interface by undergoing multiple collisions with molecules of nebulizing gas (nitrogen) and evaporated solvent. Furthermore, it appears that due to the large size of protein ions (and thus, a large number of vibrational degrees of freedom) the activated ions should have lifetime sufficient to undergo exchange in the gas phase[39] (msec time scale). Indeed, when the CAD of CRABP I ions was attempted in the electrospray source of a magnetic sector instrument [I.A.K., unpublished data], no fragment ions could be detected in the mass spectrum. Only the use of trapping mass analyzers, for example, FT ICR or quadrupole trap [I.A.K., unpublished data], allowed the fragment ions to be observed. This suggests that the lifetime of activated protein ions exceeds μs time scale very significantly, and that the dissociation process is likely to take place in the trapping hexapole ion guide, rather than in the electrospray source itself. Given the elevated internal energy and prolonged lifetime of protein ions, it is not unreasonable to expect that there may be the possibility of hydrogen scrambling under these conditions. On the other hand, stable secondary structure in the gas phase certainly would provide efficient protection against hydrogen scrambling. Moreover, it has been demonstrated previously that many peptide ions, even "hot" metastable ions which decompose on the μs time scale, retain their secondary structure in the gas phase.[37,38] However, there are no conclusive experimental data to indicate that the collisionally activated peptide or protein ions can maintain their secondary structure on the msec time scale of trapping analyzers.

Recently, McLafferty and co-workers reported that significant hydrogen scrambling takes place within the ions of cytochrome *c* activated by SORI in the ICR cell of a mass spectrometer similar to one used in our work.[39] On the other hand, Anderegg[40] and later Waring[41] have reported that scrambling was minimal for short helical peptides under typical CAD conditions of a triple quadrupole instrument. More recently, Smith and co-workers obtained similar results for unstructured peptides using an ion trap analyzer.[12] Clearly, the extent of hydrogen scrambling depends on several experimental variables. It appears that the collisional activation energy is a very important determinant. Sufficiently elevated internal energy of peptide ions in the gas phase is known to decrease the H/D exchange rates very significantly.[42−44] Ion activation by SORI is a very slow heating process,[45,46] and therefore leads to extensive hydrogen scrambling (e.g., intramolecular H/D exchange). Other mass analyzers employ significantly higher activation energies.[45] Perhaps, more rapid heating prevents hydrogen scrambling, as the ion reaches the energy range where direct dissociation reactions are the dominant ones, fast enough to avoid any internal rearrangements. Although "in-source" collisional activation is also a rapid heating process, a convincing control measurement is necessary to ensure that any hydrogen scrambling within proteins in the gas phase does not alter the deuterium content locally. Alternatively, the extent of scrambling may depend on peptide size: proton mobility has been shown to be minimal in small peptides,[12] but the increased conformational flexibility of larger polypeptides and proteins may promote hydrogen scrambling, as has been observed in a number of cases (Miranker, A.D and Robinson, C. V., personal communication). Here we analyze the mobility of labile hydrogens in CRABP I under conditions of "in-source" CAD.

We know from the solution structure of this region that there is no persistent local structure in the His-tag region of CRABP I [Gly^{1}→Met^{21}] as judged by ^{15}N relaxation parameters and very rapid amide hydrogen exchange measured by NMR. If intramolecular scrambling of hydrogens occurred in the gas phase, then one might expect a significant incorporation of deuterons from elsewhere in the protein molecule prior to fragmentation, but we observe only completely protiated[35] fragments from this region of the protein from the earliest time point onward. Likewise, fragments formed from other more structured regions of the protein retain a significant deuterium content, and this proportion changes consistently over the time course of the experiment. These observations indicate that both inter- and intramolecular H/D exchange reactions in the gas phase are too slow to affect the measurements under the conditions employed.

It is clear that full structural characterization of the dynamic events which lead to formation of transient unfolding intermediates will require more extensive fragmentation of the protein, either with higher skimmer voltages, or else by using limited proteolysis using the methodology of Smith et al.[8,10,11] A large part of the protein, namely Leu^{43}→Leu^{121}, remains intact and could not be fragmented under the conditions employed in this experiment. However, initial tests of fragmentation efficiency

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using hexapole trapping,[47] suggest that by using long ion accumulation times we can obtain much more extensive fragmentation, covering almost the entire structure. We also can observe complementary fragments, that is, both the N- and C-terminal portions of a dissociated protein molecule, allowing even more corroborative data to be obtained about the exchange events occurring in the ensemble of molecules. We are investigating this further to determine whether this method is generally applicable to H/D exchange studies.

Conclusions

Using the protein CRABP I, we demonstrate that CAD of entire protein ions undergoing H/D exchange in solution provides a rapid method to localize hydrogen exchange patterns, using minimal quantities of protein. Time evolution of isotopic distributions of each peptide fragment ion characterizes conformational stability of the local structural elements that comprise the fragment. Since many fragments form continuous ladders, protection of individual amides can be determined for the corresponding segments of the protein. The measurements do not appear to be influenced by hydrogen scrambling in the gas phase. Further improvements in fragmentation efficiency (e.g., by using hexapole trapping of ions[47] or electron capture dissociation[48]) will lead to more detailed structural analysis of the internal motions in protein molecules.

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