ACCELERATED COMMUNICATION

Detecting equilibrium cytochrome *c* folding intermediates by electrospray ionization mass spectrometry: Two partially folded forms populate the molten-globule state

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Abstract

Nanoelectrospray ionization mass spectrometry (nano-ESI-MS) is applied to the characterization of ferric cytochrome *c* (cyt *c*) conformational states under different solvent conditions. The methanol-induced molten-globule state in the pH range 2.6–3.0 is found to be populated by two distinct, partially folded conformers I_A and I_B . The more compact intermediate I_B resembles that induced by glycerol in acid-unfolded cyt *c*. The less compact one, I_A , also can be induced by destabilization of the native structure by trifluoroethanol. I_A and I_B can be detected, in the absence of additives, around the midpoint of the acid-induced unfolding transition, providing direct evidence for involvement of equilibrium folding intermediates in cyt *c* conformational transitions at low pH. This study shows that mass spectrometry can contribute to the characterization of molten-globule states of proteins by detection of distinct, although poorly populated, conformations involved in a dynamic equilibrium.

Keywords: Protein folding intermediates; methanol-induced molten globule; trifluoroethanol; cytochrome *c* acid-induced unfolding; nanoelectrospray ionization mass spectrometry

The characterization of protein folding intermediates can shed light on the mechanisms underlying conformational transitions. Techniques based on optical spectroscopy and nuclear magnetic resonance (NMR) allowed description of several, partially folded protein conformations in both kinetic and equilibrium experiments (Brockwell et al. 2000; Englander 2000). This kind of information contributes to our understanding of problems such as modularity of protein structures, hierarchy of events in protein folding, and mechanisms of molecular evolution (Baldwin and Rose 1999). However, protein folding intermediates are difficult to characterize because of their highly dynamic nature and low thermodynamic stability. Therefore, a major effort is

being dedicated to improving the tools for detecting and characterizing these labile states in solution.

Mass spectrometry is a relatively new technique in protein science (Chait and Kent 1992). Its growing impact is because of the development of mild measurement conditions that preserve noncovalent interactions, allowing investigation of folding and binding phenomena (Miranker et al. 1996; Pramanik et al. 1998; Last and Robinson 1999). By electrospray ionization mass spectrometry (ESI-MS), single protein ions are isolated from protein solutions and detected in the gas state on the basis of their mass-to-charge (m/z) ratio (Gaskell 1997). It is well documented (Katta and Chait, 1991; Le Blanc et al. 1991; Loo et al. 1991; Fligge et al. 1998; Konermann and Douglas 1998a, 1998b; Babu et al. 2001) that the multiple-charge distributions recorded by ESI-MS report on the conformation of the protein in the sprayed solution, reflecting the influence of solvent accessibility on the ionization state of side chains. Compact conformations typically result in protein envelopes shifted to-

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ward higher m/z values, as compared with unfolded conformations of the same molecule. Although not directly sensitive to changes in protein secondary structure (Koner-mann and Douglas 1997; Grandori et al. 2001b), ESI-MS can detect even minor changes in tertiary structure, such as compression of lysozyme induced by polyols (Grandori et al. 2001a). Compactness therefore seems to be the feature that ESI-MS monitors with high specificity and sensitivity. These properties make ESI-MS an interesting new tool for analysis of folding intermediates.

Cytochrome (cyt) *c* is a single-domain, mainly helical globular protein containing 104 amino acids and one covalently attached heme group (Bushnell et al. 1990). Horse cyt *c* undergoes a highly cooperative, acid-induced unfolding transition between pH 3.0 and pH 2.0 at 25°C (Goto et al. 1993). Both equilibrium (Goto et al. 1993) and kinetic (Mines et al. 1996) measurements suggested involvement of an intermediate state in cyt *c* folding and unfolding at low pH. Cyt *c* partially folded states can be induced by stabilization of compact conformations at pH ~2.0 by salt (Goto et al. 1993) or glycerol (Kamiyama et al. 1999), or by destabilization of the native structure at pH ~3.0 by methanol (Kamatari et al. 1996). Independently folding units in cyt *c* structure have been identified by hydrogen-exchange (Roder et al. 1988; Jeng and Englander 1991) and peptide-complementation (Wu et al. 1993) experiments. In this work, nano-ESI-MS is applied to the investigation of equilibrium folding intermediates of ferric cyt *c* in previously characterized and novel solvent conditions.

Results and Discussion

The methanol-induced molten-globule state

Figure 1 shows nano-ESI-MS spectra of cyt *c* in the pH range 3.0–2.6, in the presence and in the absence of 25% methanol. As reported previously (Koner-mann and Douglas 1997; Grandori et al. 2001a, 2001b), cyt *c* acid-induced unfolding can be monitored by ESI-MS. The folded and unfolded protein envelopes are well resolved and centered, respectively, on the 9+ and 18+ ion peaks. The broadness of the multiple-charge distributions reflects the different conformational freedom of the two states. The highly dynamic unfolded conformations result in a wider spectrum of different charge states. Addition of 25% methanol drastically destabilizes the native structure, resulting in a larger fraction of the population detected in the unfolded state at each pH value. Besides shifting the equilibrium between the folded and the unfolded states, methanol causes appearance of intermediate m/z distributions. A new maximum corresponding to the 14+ ion is well resolved at pH 3.0 and pH 2.8. Another one, at the position of the 11+ ion, is detectable at pH 2.8 and pH 2.6. Both intermediate protein envelopes are resolved in the spectrum recorded at pH 2.8, resulting in a

multimodal m/z distribution having four distinct components. The distribution centered on the 11+ ion at pH 3.0 and that centered on the 14+ ion at pH 2.6 seem to be masked by the prominent adjacent peaks and appear as shoulders in the spectra.

These intermediate multiple-charge distributions, detectable between those of the folded and the unfolded protein, strongly suggest the existence of two distinct, partially folded conformers populating the previously described methanol-induced molten-globule state of cyt *c* (Kamatari et al. 1996). Also in the case of myoglobin, methanol-induced conformational changes at pH 4.0 could be detected by formation of bimodal multiple-charge distributions in ESI-MS spectra (Babu and Douglas 2000). Addition of 25% methanol does not alter the multiple-charge distribution of unfolded cyt *c* at pH 2.2 (data not shown), further indicating that the effects observed at slightly higher pH are caused by protein conformational changes and not to interference of the cosolvent with the electrospray. It is relevant that a multiple-charge distribution centered on the position of the 11+ ion had been detected by ESI-MS analysis of cyt *c* in another condition known to stabilize the molten-globule state, that is, pH 2.0 plus 10% glycerol (Grandori et al. 2001a). Nanospray techniques allow relative mild temperature and voltage conditions for protein ESI-MS analysis. This feature motivated the choice of this technique for the present work and could explain why intermediate distributions had not been detected in previous investigations of methanol effects on cyt *c* ESI-MS spectra by regular sample interfaces (Babu et al. 2001). The two partially folded forms described here will be referred to as I_A (maximum corresponding to the 14+ ion) and I_B (maximum corresponding to the 11+ ion).

Stabilization of I_A by trifluoroethanol

Trifluoroethanol (TFE) has been used extensively as cosolvent for conformational studies on peptides and proteins (Nelson and Kallenbach 1986; Buck et al. 1993; Luo and Baldwin 1998; Hamada et al. 2000) because of its property of stabilizing secondary structure, particularly α -helices and β -hairpins. TFE-induced oligomerization of an amphipathic helix peptide has been investigated by ESI-MS (Fermendjian et al. 2001). Like methanol, TFE concomitantly strengthens hydrogen bonds and weakens hydrophobic interactions, but it is more effective than methanol in both regards. Because of its complex mechanism of action, its effects in protein folding experiments can be difficult to interpret (Main and Jackson 1999). A recent report suggested induction of a molten-globule state in acid-unfolded cyt *c* at pH 1.9 by low concentrations of TFE (Konno et al. 2000). On the other hand, nano-ESI-MS does not detect TFE-induced compact conformations under similar condi-

Fig. 1. Methanol-induced partially folded states. Nano-ESI-MS spectra of 5 μ M cyt *c* in water/acetate. (A) pH 3.0, 0% methanol; (B) pH 2.8, 0% methanol; (C) pH 2.6, 0% methanol; (D) pH 3.0, 25% methanol; (E) pH 2.8, 25% methanol; (F) pH 2.6, 25% methanol. Peaks are labeled by the corresponding charge states.

tions and, rather, points out destabilization of cyt *c* tertiary structure by TFE at low pH (Grandori et al. 2001b).

Exploring the effect of TFE on cyt *c* conformations by nano-ESI-MS at different pH values, it was found that 5% TFE at pH 2.8 stabilizes an intermediate form characterized by the same multiple-charge distribution as I_A (Fig. 2). Comparison with the spectrum obtained at 0% TFE under the same experimental conditions (Fig. 1) also indicates TFE-induced protein unfolding. The same concentration of TFE does not affect the spectrum of the protein at pH 3.0 (data not shown) and results in just a higher fraction of unfolded protein at pH 2.6 (Grandori et al. 2001b). These results show that TFE, like methanol, can induce formation of a molten-globule-like state by destabilization of cyt *c* native tertiary structure at low pH. In agreement with previous evidence (Grandori et al. 2001b), these data suggest that the reduced solvophobic effect in the presence of TFE is the predominant factor affecting cyt *c* tertiary structure at low pH, consistent with the view of an already destabilized native conformation due to repulsion among positively charged groups.

Although the main charge state in the distribution of the unfolded protein fluctuates between 18+ and 19+, the envelope corresponding to the denatured state in the presence of 5% TFE is characterized by predominance of the 20+ ion (Figs. 1 and 2). That TFE does not shift the envelope of the folded protein in the same spectra argues against the hypothesis of a solvent effect on the technique. Induction of higher charge states by TFE in a short peptide and denatured cyt *c* suggest that the coil-to-helix transition can indirectly affect ESI-MS spectra by increasing side chain solvent accessibility (Grandori et al. 2001b). The minor shift in the unfolded protein envelope induced by TFE can be inter-

preted as a loss of residual tertiary contacts in the denatured state upon stabilization of highly helical, extended conformations. Structural characterization of proteins in the denatured state is an interesting but technically challenging problem. However, these data suggest that conformational properties of denatured proteins and their dependence on solvent conditions can be monitored by ESI-MS.

Formation of I_A and I_B during acid-induced unfolding

The data reported in Figure 3 show that I_B can be detected, in the absence of additives, as resolved maximum corresponding to the 11+ ion, in nano-ESI-MS spectra of cyt *c* recorded at pH 2.5. A form similar to I_A appears as a shoulder on the unfolded protein envelope. Detection of I_A and I_B at pH 2.5 is strongly dependent on the experimental conditions. These components disappear from the spectra at higher curtain-gas flow or spray-tip potential (data not shown), typically resulting in a higher fraction of unfolded protein. Thus, it seems that intermediate conformations are easily denatured during the analysis under these solvent conditions. At pH 2.4 (Fig. 3), I_A and I_B are barely detectable. Equilibrium titration of cyt *c* solutions between pH 3.0 and pH 2.0 monitored by Soret absorption suggested that cyt *c* acid-induced unfolding follows a three-state mechanism (Goto et al. 1993). The results described here bring direct evidence for the existence of a marginally stable, partially folded state of cyt *c* at low pH and show that this is characterized by two distinct, albeit dynamic, conformers, similar to those populating the methanol-induced molten-globule state.

The sum of the intensities of the peaks belonging to each protein envelope in ESI-MS spectra provides an approximate estimate of the relative amounts of the detectable species. Using data recorded at pH 2.5, as in Figure 3A, the fraction of molecules populating both intermediate states is calculated to be 13% (± 3). This estimate is based on the simplifying assumptions that the 14+ and 13+ ions represent I_A only and the 12+ and 11+ ions I_B , that the m/z distribution of each species is symmetric, and that the contributions of the $i+2$ and $i-2$ peaks of I_A and I_B are negligible, where i is the main charge state. Although possibly underestimated, this value suggests that the experimental conditions for detection of cyt *c* partially folded states at low pH by ESI-MS might be further improved, because the maximal accumulation of folding intermediate during equilibrium titrations has been estimated to be ~25% (Goto et al. 1993).

Formation of the cyt *c* folding intermediates I_A and I_B has been explored by nano-ESI-MS in the pH range 2.2–3.0 and methanol concentration range 0–25% (Fig. 4). The results illustrate the strong pH dependence of the effect of methanol on cyt *c* conformational states and thus further support the hypothesis that the effects of solvent conditions on cyt *c* ESI-MS spectra reported here reflect their actual influence

Fig. 2. Trifluoroethanol-induced partially folded state. Nano-ESI-MS spectrum of 5 μ M cyt *c* in water/acetate at pH 2.8, 5% TFE. Peaks are labeled by the corresponding charge states.

Fig. 3. Acid-induced partially folded states. Nano-ESI-MS spectra of 5 μM cyt *c* in water/acetate. (A) pH 2.5; (B) pH 2.4. Peaks are labeled by the corresponding charge states.

on the protein structure. It is also relevant that the same charge states characterize the intermediate distributions obtained by ESI-MS under different solvent conditions, as described here and previously (Grandori et al. 2001a). This fact strongly suggests that these intermediate distributions report on protein-specific structural properties. Two partially folded conformations of cyt *c* have been identified in kinetic folding experiments (Roder et al. 1988; Chan et al. 1997). More work is required to establish the relationship

between those and the equilibrium intermediates described here.

Concluding remarks

Mass spectrometry is unique among the available techniques for molecular spectroscopy in that it sorts molecules while detecting them, without averaging the output signal over the molecular ensemble. At the same time, it provides structural information on each isolated ion. These features are at the basis of its potential as a tool for protein structural studies that can improve the detection of poorly populated states in conformationally heterogeneous samples. Moreover, its high sensitivity to changes in protein tertiary structure is of particular relevance for the detection and characterization of folding intermediates, because these can be effectively distinguished from both the folded and the unfolded states. The present study shows that MS technologies can help increase the resolution at which we describe dynamic equilibria involving different conformational states. The complementarity of MS to the other methods for protein structural characterization is a promising feature for the development of new experimental approaches in protein science.

Fig. 4. Methanol and pH dependence of cyt *c* conformational states. The conformational states detectable in nano-ESI-MS spectra of 5 μM cyt *c* in water/acetate are summarized in this figure. (open square) Unfolded state; (square with slash) folded and unfolded states; (square with plus) *I_A*, *I_B*, folded and unfolded states. The latter symbol refers to conditions in which at least one intermediate is detectable as resolved maximum in the spectra. One form might be detectable as shoulder of the folded or unfolded protein envelopes.

Materials and methods

Mass spectra were recorded on a Mariner time-of-flight, ESI mass spectrometer (Perkin Elmer) in the positive-ion mode using a nanoelectrospray sample interface. The spray-tip potential was set at 1600 V, the nozzle potential at 20 V, and the nozzle temperature at 80°C. The experimental parameters were optimized by tuning the instrument on the peak of cyt *c* 9+ ion at pH 3.0. Samples were sprayed at ~25°C with a curtain-gas flow of ~0.6 L/min. The

capillaries were purchased from Protana (inner diameter of 0.69 mm and medium length of the outermost tip). The software for data recording (Mariner Workstation version 4.0) and data processing (Data Explorer version 3.2) were supplied by Applied Biosystems. Spectra were recorded with an integration time of 3 sec and averaged over at least 30 sec. TFE and horse heart type VI cyt *c* were purchased from Sigma and methanol from Merck. Protein samples were prepared in water/acetic acid mixtures without further purification. The pH was adjusted to the desired value with diluted acetic acid (a pH value of 2.4 corresponds to an acetic acid content of ~4%).

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