Protein Folding Mechanisms Studied by Time-Resolved Electrospray Mass Spectrometry

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Introduction

Mass spectrometry (MS) owes its remarkable rise as a bioanalytical tool to the development of two “soft” ionization techniques: matrix-assisted laser desorption/ionization (MALDI) (1,2) and electrospray ionization (ESI) (3). The salient feature of soft ionization is that a wide range of analytes, from small metabolites to very large biomolecular systems, can be transferred intact into the gas phase, thus making them amenable to mass spectrometric analysis. For MALDI-MS, the sample is embedded into a crystalline matrix prior to analysis. For ESI, in contrast, ionization is initiated directly from the liquid phase by spraying analyte solution from the tip of an electrically charged capillary. The small droplets formed by the ESI emitter undergo rapid evaporation and fission, ultimately releasing the analyte as multiply protonated (or deprotonated) species (4).

Due to the seamless coupling to the liquid phase, ESI-MS is a powerful tool for studying chemical and biochemical processes in online experiments. The composition of a reaction mixture can be monitored continuously by direct infusion into the mass spectrometer, while the process of interest occurs in solution. The high selectivity of ESI-MS offers the unique opportunity of tracking a multitude of reactive species simultaneously. The result is a global view that provides both accurate kinetic parameters and detailed insights into reaction mechanisms (5).

The ESI charge state distribution is a highly sensitive probe for detecting changes in the solution-phase conformation of proteins. In the commonly used positive ion mode, unfolded polypeptide chains acquire a larger number of protons and will thus appear at lower mass-to-charge ratios (m/z) than their folded counterparts. Presumably, this effect is related to the changes in overall compactness upon unfolding, the larger surface area of an unfolded protein, and the fact that in the native state some protonation sites may be buried inside the interior of the protein (6). Also, possible charge compensation mechanisms are being discussed (7). In addition to providing information on the protein conformation, ESI-MS allows tracking the occurrence of protein-ligand and protein-protein interactions. This is due to the gentle nature of the ionization process that often preserves the composition of noncovalent complexes, such that the ligand binding state of a protein can be deduced from the mass of the corresponding ions (Figure 1).

For many proteins, folding and unfolding are reversible processes that occur spontaneously upon exposure to a suitable solvent environment, typically within a time range of milliseconds to seconds. The mechanisms of these conformational transitions continue to be a hotly debated research topic (8). Many proteins have been characterized as apparent two-state folders, while others fold through transient intermediates. Studies on folding intermediates are complicated by their short lifetimes and by the fact that they often do not accumulate to a significant extent. Nonetheless, the detection and structural characterization of these elusive species represents a very important approach for deciphering the mechanisms of folding and unfolding. While deviations from two-state behavior can sometimes be inferred from equilibrium experiments, direct information on transient folding intermediates can be gained only through kinetic investigations. Optical stopped-flow spectroscopy is one of the classical approaches for studies of this kind. Additionally, pulsed hydrogen exchange quench-flow methods, combined with off-line analysis by nuclear magnetic resonance (NMR) or MS have proven to be extremely useful (9). Limitations of these well-established methods include the poor selectivity of optical detection and the very laborious nature of quench-flow experiments, where kinetic data must be pieced together from numerous single time point measurements. Online ESI-MS methods represent an interesting alternative for mechanistic investigations on protein folding and unfolding, especially in cases where structural transitions involve changes in the ligand binding state or quaternary structure. Following a brief summary of some technical aspects, we will highlight two examples that demonstrate the use of ESI-MS for kinetic and mechanistic studies on protein conformational changes.

![Figure 1. Detecting changes in protein conformation and ligand binding state by electrospray ionization mass spectrometry (ESI-MS), using myoglobin as example.](image-url) (A) Native myoglobin is a tightly folded heme-protein complex, exhibiting relatively low ESI charge states. The degree of protonation for the various peaks is indicated [e.g., 9+ represents (myoglobin + 9H)+]. (B) Acid-unfolded myoglobin exhibits much higher charge states and a significantly broadened distribution. The mass of the protein ions generated under these conditions is lower, due to the loss of heme upon acid denaturation (16952 versus 17568 Da). The resulting shift in mass-to-charge ratio (m/z) is highlighted for the 9+ charge state (vertical dashed line). Free heme in panel B is denoted as H.
Time-Resolved ESI-MS

The continuous-flow rapid-mixing setup depicted in Figure 2 is capable of performing kinetic experiments with ESI-MS detection on a time scale of milliseconds to minutes (10). This system represents a concentric capillary mixer with adjustable reaction chamber volume. Reactant solutions are continuously expelled from both syringes. Syringe 1 is connected to the inner capillary, whereas the solution delivered by syringe 2 flows through the outer capillary. Diffusive mixing occurs at the end of the inner capillary, thereby initiating the reaction of interest. The reaction proceeds while the mixture flows towards the outlet of the apparatus, where ESI takes place. For an average solution flow velocity \( v_a \), the reaction time \( t \) is given by \( t = l/v_a \), where \( l \) is the distance between mixing point and outer capillary outlet. A detailed analysis of the measured kinetics has to take into account laminar flow effects in the reaction zone. A discussion of this subject is given in Reference 10.

Kinetic experiments are performed by initially positioning the mixer directly at the ESI source. This represents the earliest time point that can be monitored (\( t = 5 \text{ ms} \)) for a total flow rate of 100 \( \mu \text{L/min} \). The assembly consisting of syringe 1, inner capillary, and mixer is then slowly pulled back by a step-per motor-driven mechanism, while solutions from the two syringes are continuously passed through the system. High voltage is applied directly to the stainless steel outer capillary, and ESI occurs at the outlet of the device, which is mounted in a custom-designed ion source. The mass spectrometer monitors the ions emitted from the source, thereby providing data at successively later time points. These experiments generate data in three dimensions: (i) the time axis is determined by the mixer position; (ii) the \( m/z \) axis provides information on the identity of the species in the reaction mixture; and (iii) the intensity axis is related to the concentration of each of the various solution-phase species. The setup depicted in Figure 2 can be interfaced with any type of ESI mass spectrometer. For the examples discussed in the following sections, a time-of-flight (TOF) instrument was used. The software routines required to operate the mass spectrometer during these kinetic experiments are analogous to those used during the acquisition of liquid chromatography-MS data. Mass spectra for specific reaction times and intensity-time profiles for selected ionic species can be extracted from the measured total ion current (TIC).

Unfolding Kinetics of Large Noncovalent Protein Complexes

The core oxygenase domain of inducible nitric oxide synthase (iNOS\(_{\text{COD}}\)) is a homodimeric protein complex of 103 kDa. Each of the two subunits binds a heme cofactor. In addition, two tetrahydrobiopterin (\( \text{H}_4\text{B} \)) moieties are sequestered at the dimer interface. Time-resolved ESI-MS was used to study the acid-induced unfolding of iNOS\(_{\text{COD}}\) (11). Following a pH jump from 7.5 to 2.8, the ionic signals corresponding to the intact protein dimer show a rapid decay that is characterized by a relaxation time of 360 ms (Figure 3A). This process occurs concomitantly with the formation of monomeric heme-bound proteins that appear in a wide range of different charge states, representing solution-phase structures from tightly folded conformers all the way to significantly unfolded species (Figure 3B). The disruption of the protein-protein interactions is accompanied by loss of \( \text{H}_4\text{B} \). The heme-bound monomers represent short-lived intermediates that are depleted with a relaxation time of 620 ms. The main products of this process are highly unfolded monomeric proteins in their heme-free (apo) form (Figure 3C). In simple terms, the denaturation of iNOS\(_{\text{COD}}\) can be described as a sequential process. Disruption of the native protein dimer generates...
monomeric species that subsequently undergo unfolding with concomitant loss of heme. A detailed global analysis of the kinetic data reveals a number of additional steps and parallel processes that cannot be discussed here due to space constraints (11). When considered in the context of other recent studies (12), the iNOS COD kinetics shown here suggest that the occurrence of complex reaction mechanisms involving short-lived intermediates is a common feature for the denaturation of large noncovalent protein complexes.

Detection of Hidden Folding Intermediates

An extension of the experimental approach described above is the use of time-resolved ESI-MS in conjunction with online hydrogen/deuterium exchange (HDX). For this type of experiment, a second mixer is added to the outlet of the device depicted in Figure 2, which allows an approximately 25-ms pulsed HDX labeling step to take place immediately prior to ESI (13). This continuous-flow double-mixing approach represents a powerful tool for the detection of short-lived intermediates during folding (9). In a typical experiment, the first mixing step exposes an initially denatured protein to refolding conditions for a variable amount of time. Subsequently, HDX is initiated by mixing the protein with deuterium oxide (D₂O) under rapid exchange conditions (i.e., at slightly basic pH). During this step, labile hydrogens in amide groups and amino acid side chains undergo isotope exchange. Every single HDX event increases the protein mass by 1 Da, such that the extent of labeling can be determined directly from the mass shifts of the protein ions in the spectrum. Importantly, this isotope labeling occurs in a structurally sensitive manner. Largely unfolded proteins have a considerable number of exchangeable hydrogen atoms exposed to the solvent and will therefore show high exchange levels. In contrast, more tightly folded protein structures experience a lower number of exchange events, resulting in a smaller mass shift. The HDX level and ESI charge state distribution represent complementary structural probes; the former reports on the intactness of the hydrogen bonding network and the accessibility of exchangeable sites, whereas the latter monitors the overall compactness of the protein.

The question whether the folding kinetics of ubiquitin (molecular weight 8.5 kDa, 144 exchangeable hydrogen atoms) follow a simple two-state mechanism or whether there is evidence for the occurrence of a folding intermediate is currently being debated in the literature (13). Our group studied the refolding of this protein (initially denatured in methanol/water at pH 2.0) using time-resolved ESI-MS with online pulsed HDX. Prior to refolding, the protein shows an ESI mass spectrum exhibiting a broad charge state distribution with a maximum around 11+ (Figure 4A). The transition to the native state was triggered by a change in solution conditions to pH 10.0. Mass spectra acquired during refolding have a bimodal appearance. They exhibit a relatively broad charge state distribution centered at 9+ and a more narrow one encompassing the 6+ and 5+ charge states. Refolding of the protein is reflected in a gradual intensity decrease of the protein ions in charge states around 9+ and a concomitant increase of the 6+ and 5+ ions (Figure 4, B and C).

Figure 4. Ubiquitin folding monitored by time-resolved electrospray ionization mass spectrometry (ESI-MS) with online pulsed hydrogen/deuterium exchange (HDX). (A) Spectrum of the unfolded protein prior to triggering the conformational transition. Spectra for reaction times of 40 ms and 3.3 s are shown in panels B and C, respectively. (D and E) Mass shift distributions resulting from HDX for the 9+ (blue dashed line) and 5+ (red solid line) charge states for reaction times of 40 ms and 3.3 s. (F) Proposed three-state folding mechanism of ubiquitin. D, denatured ubiquitin; D*, folding intermediate; F, folded state. The charge state distributions (CSDs) of D* and F are indistinguishable; D and D* exhibit the same HDX behavior. Only the combination of HDX and CSD as nonredundant structural probes allows the folding intermediate D* to be detected. m/z, mass-to-charge ratio; t, time. Adapted with permission from Reference 13.
After pulsed HDX, the mass distributions observed for charge states around 9+ exhibit a large shift of 83 (±4) Da relative to unlabeled ubiquitin (Figure 4, D and E, blue dashed curves). For t = 3.3 s, the 6+ and 5+ ions exhibit a much smaller shift of 58 (±2) Da (Figure 4E, red curve). Interestingly, however, for t = 40 ms the HDX behavior observed for the 6+/5+ charge states coincides with that observed for the 13+ to 7+ signals (Figure 4D). It is concluded that the refolding of ubiquitin under the conditions studied here involves three kinetic species: (i) the denatured state D (charge states around 9+, large mass shift); (ii) a folding intermediate $D^*$ (charge states 6+/5+, large mass shift); and (iii) the folded state F (charge states 6+/5+, small mass shift). The fact that D and $D^*$ show indistinguishable HDX characteristics suggests that these two species undergo rapid interconversion during the labeling pulse. This implies that D and $D^*$ are separated by a relatively low energy barrier. In contrast, the transition to F occurs on a slower time scale, which is indicative of a major barrier. A schematic depiction of a possible reaction mechanism is given in Figure 4F.

In summary, the refolding of ubiquitin under the conditions used here is not a simple two-state (D $\rightarrow$ F) process. Time-resolved ESI-MS with online HDX reveals the presence of an intermediate species. This result is in line with the notion that the occurrence of folding intermediates is more widespread than commonly thought, especially in cases where a cursory analysis indicates two-state behavior (13).

**Conclusions**

The examples discussed above highlight the versatility of time-resolved ESI-MS as a tool for studying protein folding and unfolding on the time scale of milliseconds to seconds. The high selectivity of ESI-MS allows the kinetic behavior of multiple conformations and ligand binding states to be monitored simultaneously, thereby providing detailed insights into reaction mechanisms that may involve short-lived intermediates and parallel pathways. The inclusion of an online pulsed HDX step adds a further dimension to the experiment that greatly facilitates the detection of transiently populated species. Time-resolved ESI-MS is not restricted to studies on protein folding and unfolding; other interesting applications include the exploration of enzymatic and bio-organic reaction mechanisms (14). One current limitation of all online ESI-MS techniques is their relatively low tolerance for nonvolatile salts and some other solvent additives. However, the use of ultrarapid microfluidic desalting devices and other recently developed strategies will help to resolve this issue (15). In any case, it seems certain that MS-based techniques are about to become an indispensable tool for a wide range of kinetic applications in biological chemistry.

**Acknowledgments**

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canada Foundation for Innovation (CFI), the Province of Ontario, and the Canada Research Chairs Program.

**References**


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