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Probing Serpin Conformational Change Using Mass Spectrometry and Related Methods

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Abstract

The folding, misfolding and inhibitory mechanisms of serpins are linked to both thermodynamic metastability and conformational flexibility. Characterizing the structural distribution of stability and flexibility in serpins in solution is challenging due to their large size and propensity for aggregation. Structural mass spectrometry techniques offer powerful tools for probing the mechanisms of serpin function and dysfunction. In this chapter, we review the principles of the two most commonly employed structural mass spectrometry techniques – hydrogen/deuterium exchange and chemical footprinting – and describe their application to studying serpin flexibility, stability and conformational change in solution. We also review the application of both hydrogen/deuterium exchange and ion mobility mass spectrometry to probe the mechanism of serpin polymerization and the structure of serpin polymers.

Introduction

Conformational mobility is key to serpin function, dysfunction and regulation (Gettins, 2002). In order to inhibit their target proteases, serpins must undergo a massive conformational change in which the solvent exposed reactive center loop (RCL) inserts into the 5 stranded beta-sheet A, becoming a sixth strand. A similar rearrangement occurs when serpins such as PAI-1 spontaneously transition from the active to the latent form. Serpins also undergo subtler conformational changes, such as those responsible for the allosteric activation of antithrombin by heparin. Pathological serpin dysfunction is also mediated by conformational change: all of the currently proposed models for serpin polymerization require considerable remodeling of sheet A (Sivasothy, et al., 2000, Yamasaki, et al., 2008).

While nuclear magnetic resonance is the premier technique for characterizing the conformational dynamics of proteins in solution, the serpins' relatively large size (~45 kD) and propensity for aggregation make them poor candidates for high resolution NMR studies. Techniques such as fluorescence resonance energy transfer and site directed spin labeling can provide information on both the structure and dynamics of large proteins in solution. However, these techniques require engineering cysteins into the protein of interest and then attaching optical or paramagnetic probes that can potentially perturb native structure. Additionally, the structural information provided by these methods, while valuable, is limited to the probe site(s).

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Relative to other techniques for probing solution structure and dynamics, structural mass spectrometry methods offer a number of attractive features. They provide the ability to simultaneously monitor a large number of intrinsic, non-perturbing probes (amide hydrogens or reactive side chains) that are distributed throughout the structure. Mass spectrometry based methods require small amounts of sample (as little as nanomoles of protein) and, because the analysis step is separate from the labeling step, interrogation of protein structure and dynamics can be performed under a wide range of conditions. In this chapter, we review the principles and procedures of the most common structural mass spectrometry methods – hydrogen/deuterium exchange and oxidative footprinting – and describe their application to probing serpin structure, dynamics and misfolding.

Protein hydrogen/deuterium exchange exploits the fact that backbone amide hydrogen atoms will readily exchange with deuterium when a protein is incubated in D₂O (Bai, et al., 1993) (Figure 1). Exchange is catalyzed by both acid and base and is thus strongly pH dependent: a fact of critical importance for HXMS. The rate of exchange is at a minimum at pH 2.5, and increases by approximately a factor of 10 with each pH unit increase or decrease. The intrinsic exchange rate is also sequence dependent, and the exchange rates in unstructured peptides can vary by a factor of ~30 depending on the amino acid sequence. Intrinsic exchange rates can be predicted using data from extensive NMR studies of model peptides (Bai, et al., 1993).

In folded proteins, exchange rates depend on the local structural environment. Amide hydrogen atoms that are sequestered from solvent or involved in hydrogen bonding are protected from exchange. In order for exchange at these positions to occur, local and/or global fluctuations must disrupt the structure and expose amide hydrogens to solvent (Wales and Engen, 2006). The frequency with which such fluctuations occur determine the exchange rate for a given amide hydrogen. Rates of hydrogen exchange in folded proteins span 8 orders of magnitude, making them extremely sensitive probes of local stability and dynamics. Interpretation of measured H/D exchange rates depends on the relative rates of the underlying processes: the “breaking” of local structure that exposes an amide hydrogen for exchange, k_f ; the reformation of this structure, k_{-f} ; and the intrinsic, chemical rate of exchange for the exposed hydrogen, k_{int} . The process is described schematically as:

When the dynamics of protein fluctuations are fast compared with the chemical rate of exchange then $k_{-f} \gg k_{int}$, and the observed rate of exchange can be expressed as:

$$k_{obs} = \frac{k_f}{k_{-f}} k_{int} = K_{unf} k_{int} \quad (1)$$

Where K_{unf} is the equilibrium constant for the unfolding/refolding process(es) that expose the amide hydrogen in question for exchange. The case when $k_{-f} \gg k_{int}$ is referred to as the EX2 regime of exchange, and Eq. 1 reflects the fact that under these conditions unfolding/refolding will typically occur many times before exchange occurs, and thus the observed rate of exchange is proportional to the amount of time that the amide hydrogen spends in an exposed conformation. Hydrogen atoms in less stable regions that unfold frequently will exchange quickly while those in stable regions that rarely unfold will exchange slowly.

The conditions for EX2 exchange will typically hold for most proteins under physiological conditions. Denaturants such as urea or GuHCl will slow the rate of refolding, while alkaline

pH will dramatically increase the chemical rate of exchange. As a result, in the presence of denaturants or at high pH, $k_{-1} \ll k_{int}$ and

$$k_{obs}=k_1 \quad (2)$$

This is referred to as the EX1 regime of exchange, and the observed exchange rate is a direct read out of the rate of unfolding. EX1 exchange is especially significant for H/D exchange studies carried out using mass spectrometry, as it allows the direct quantification of different structural populations in solution. This will be described in more detail below.

The basic HXMS experiment is illustrated schematically in Figure 2. Protein is diluted into D₂O under the desired conditions and incubated for a fixed time period. The exchange reaction is then “quenched” by dropping the pH to 2.5 and the temperature to 0°C. This slows the chemical rate of H/D exchange by approximately 5 orders of magnitude. Protein is then digested with the acid protease pepsin. This can be accomplished either by adding pepsin to the quenched solution and incubating at 0°C for 5 minutes, or by passing the quenched protein over a column of immobilized pepsin. The latter method has generally been reported to give more efficient digestion. The digest is then loaded on to a reverse phase HPLC column and eluted directly into an electrospray ionization mass spectrometer. As deuterium is one mass unit heavier than hydrogen, the extent of deuterium uptake in each peptic fragment can be determined from the mass shift relative to an undeuterated reference sample.

Even under quenched conditions, back exchange of deuterium with hydrogen occurs, albeit slowly, and it is therefore desirable to correct for this deuterium loss due to back exchange. This is accomplished by preparing a 100% deuterated reference sample and subjecting it to the identical procedures of digestion and analysis that are used on the experimental samples. A 100% deuterated sample can be prepared by incubation the protein in a solution of D₂O containing high concentrations of a denaturant such as guanidine deuteriochloride. By comparing the measured level of deuteration for this reference sample with the theoretical maximum deuterium level, the degree of back exchange for each peptide can be determined. When conditions are optimized, it is generally possible to achieve average back exchange levels of 15–20%. The reference sample can be used to correct for back exchange using the following equation:

$$D = \frac{m - m_{0\%}}{m_{100\%} - m_{0\%}} \times N$$

where D is the corrected level of deuterium incorporation, $m_{0\%}$ is the measured mass of an undeuterated reference sample, $m_{100\%}$ is the measured mass of the fully deuterated reference sample, m is the measured mass of the experimental sample and N is the total number of exchangeable NH's on the peptide of interest. Plotting this corrected deuterium level versus time for each peptic fragment reveals the local rates of H/D exchange in different regions of a protein's structure. It should be noted that fully deuterated reference samples can be difficult to prepare, because denaturants result in noisy spectra with the result that some peptides of interest may not be detectable. If the same protein is being compared under different conditions (for example, in the presence/absence of a ligand) then relative deuterium levels may be compared directly without correcting for the extent of back exchange, which should be identical within error provided that experimental conditions such as temperature, pH and ionic strength are held constant. The above description applies when exchange occurs in the EX2 regime. EX1 exchange results in characteristic “double isotopic envelopes” which will be discussed in more detail below when equilibrium unfolding studies of serpins are described.

Hydroxyl-radical-mediated ($\bullet\text{OH}$) protein footprinting combined with mass spectrometric detection has recently been developed to define protein structure, assembly, and conformational changes in solution based on measurements of the reactivity of amino acid side-chains (Takamoto and Chance, 2006). This method probes protein structure by monitoring solvent accessibility using $\bullet\text{OH}$ modification (Xu and Chance, 2007). Solvent exposed reactive side chains are readily oxidized by $\bullet\text{OH}$, while buried side chains resist oxidation. Oxidized side chains can subsequently be identified by characteristic mass shifts using mass spectrometry (Figure 3). $\bullet\text{OH}$ radicals can be generated by several methods including Fenton reagent, photo-oxidation of peroxide and radiolysis of water (Guan and Chance, 2005). The intrinsic reactivity of side chains with $\bullet\text{OH}$ radicals has been extensively studied, and their relative susceptibility to oxidation is Cys > Met > Trp > Tyr > Phe > His > Leu, Ile > Arg, Lys, Val > Pro, Ser, Thr > Gln, Glu > Asp, Asn > Ala > Gly (Takamoto et al., 2007). Due to either low reactivity or low detectability of the resulting modified species, Gly, Ala, Asp, Asn, Arg and Glu make for poor probes in footprinting experiments, leaving the remaining 14 standard amino acids as potential probes.

Hydroxyl radical footprinting has both advantages and disadvantages compared with HXMS. Footprinting does not provide information on entire polypeptide chain as HXMS potentially does (excluding prolines). However, the 14 usable side chains are usually well distributed throughout the sequence, and for these side chains footprinting provides single amino acid resolution. Perhaps more importantly, side chain oxidation is a stable covalent modification that is not subject to rapid back exchange like H/D exchange. This allows for larger and more complex protein systems to be studied, because time consuming post-labeling purification procedures can be employed.

Applications of HXMS

Native state dynamics

Dynamics of a canonical serine protease inhibitor, α_1 -antitrypsin studied by H/D exchange and radiolytic footprinting—Because the serpins require extensive conformational mobility when carrying out their functions, the dynamical properties of the native state are of interest. Tsutsui et al. applied HXMS to probe the native state dynamics of the canonical serpin human wild-type α_1 -antitrypsin (WT α_1 -AT) in the metastable form (Tsutsui, et al., 2006). This study employed a version of the standard protocol for HXMS of native proteins:

1. Purified protein (5 μg , 565 nM) in H_2O , 10 mM phosphate buffer, 50 mM NaCl at pH 7.8 was diluted 24 fold into D_2O , 10 mM phosphate buffer pD 7.8 and incubated for different time periods at room temperature. For preparing D_2O buffers recall that pD is the reading on a standard pH meter +0.4.
2. At time intervals ranging 5 seconds to 3000 seconds, the reaction was quenched by the addition of an equal volume of cold 100 mM phosphate pH 2.4 and immediately frozen by immersion in a dry ice ethanol bath. Samples were stored at -80°C .
3. Pepsin was dissolved at 1 mg/ml in H_2O + 0.05% TFA, pH 2.5. Frozen samples were thawed and pepsin was added to give a final mass/mass ration of 1:1. Digestion was allowed to proceed on ice for 5 minutes.
4. The digest was loaded on to a reverse phase C18 column immersed in ice and eluted directly into an electrospray ionization mass spectrometer with a water-acetonitrile gradient from 10 to 45% acetonitrile in 12 minutes. The acetonitrile contained 0.05% TFA.

This basic protocol can be varied in a number of ways. To reduce the final volume to be injected onto the HPLC column, concentrated acid may be added to lower the pH rather than 100 mM phosphate buffer. The elution gradient can also be varied in order to optimize the separation of peptic fragments, although it is recommended to keep the total elution time < 20 minutes.

In Figure 4, the flexibility of native α_1 -AT is depicted as follows: for each peptic fragment, the ratio of the number of slowly exchanging amide hydrogens (measured by HXMS) to the number of amides participating in hydrogen bonds (determined from the crystal structure) is calculated. A ratio close to 1 indicates stable hydrogen bonding and therefore rigid structure. As expected from the crystal structure of WT α_1 -AT in the metastable state (Elliott, et al., 2000), amide hydrogen atoms in the reactive center loop (the RCL) underwent rapid exchange with deuterium, reaching 80 % deuterium uptake within 10 seconds of labeling time due to solvent-exposed amide hydrogen atoms (Figure 3). This lack of protection in the RCL also indicates the absence of protein aggregation during the experiment since the formation of the loop-sheet polymers requires burial of amide hydrogen atoms in the RCL region between β -strands 3A and 5A. HXMS data further indicate that not only the formation of the loop-sheet polymers but also a spontaneous conformational change to the latent form do not occur under the experimental conditions. For example, structural rigidity in β -strand 1C hinders “peeling-off” of the strand from the adjacent β -strand 4C; thereby, restricting the translational mobility of the RCL. Furthermore, slow deuterium uptake of amide hydrogen atoms in β -strands 3A and 5A indicate stable hydrogen bonds between these strands. These observations suggest that a conformer with a partial unfolding of β -strands 3A and 5A is not significantly populated in the native state ensemble.

Comparable deuterium uptake to the RCL region was observed in the N-terminal half of helix-A and the C-terminal half (the top half) of F-helix. Moderate flexibility was observed in the C-terminal half of helix A, the N-terminal half of F-helix, and helices D and E. Although these regions appear to form stable helices in the crystal structure (Elliott, et al., 2000), HXMS data indicate structural flexibility in these regions. These findings highlight the strength of hydrogen-deuterium exchange, allowing one to characterize transiently populated conformers that cannot be detected by many other techniques. Flexibility in these regions appears to be important for the inhibitory function. Since F-helix is situated nearby the RCL insertion site, a conformational change to form a serpin-protease covalent complex requires either rigid-body movement of F-helix away from β -sheet A or unfolding of F-helix to avoid a steric clash between the protease and the F-helix. Rapid deuterium uptake in the top of F-helix suggests a partial unfolding of this region; therefore, the conformation of F-helix in the metastable state is amenable to undergo the conformational change prior to the protease binding. Flexibility in helices A and D are suggested to play an important role in functional regulation of other serpins such as antithrombin and plasminogen activator inhibitor-I through allostery (Gettins, 2002). Interestingly, viral and thermophilic bacterial serpins completely lack helix D or have their N-terminal half of helix A or D truncated (Gettins, 2002). Since evolution tends to exploit flexible regions to optimize protein function, structural fluctuations in helices A and D hint an intimate relationship between evolution of allostery and function.

The metastable form of α_1 -AT was also studied using radiolytic footprinting. Footprinting indicates that, as expected, solvent exposed side chains are readily modified while most buried side chains are protected (Zheng, et al., 2008). There are however, several exceptions. Tyr 160, which is located near the C terminus of the F-helix and is largely buried, is readily oxidized, indicating that this region is highly labile in solution. This finding is consistent with results from H/D exchange. Met 374 and Met 385 are both nearly totally buried, and

their sulfur atoms are completely inaccessible. Nevertheless, both are oxidized during footprinting. These residues are located near cavities that have been identified as contributing to inhibitory efficiency through cavity filling mutagenesis studies (Lee, et al., 2001). Facile oxidation of these putatively buried side chains suggests that the structure near these cavities is highly dynamic, and this is supported by molecular dynamics simulations, which indicate that structural fluctuations transiently expose the sulfur atoms of both Met 374 and Met 385. The structural environment around these cavities is thus highly dynamic, and such structural lability may contribute to inhibition.

The effects of a stabilizing cavity-filling mutation on the native state dynamics of α_1 -antitrypsin

Numerous mutagenesis studies have been performed to investigate a relationship between the inhibitory function and the thermodynamic stability of α_1 -AT. It has been proposed that the metastability of α_1 -AT arises from inefficient structural packing, and such structural defects in the metastable state facilitate the conformational change to form a thermodynamically more stable protease-serpin complex during inhibition (Lee, et al., 2000). Therefore, mutations that improve side-chain packing are expected to increase the thermodynamic stability of α_1 -AT relative to wild-type and result in decreased inhibitory activity. In particular, G117F represents such a mutation that fills a cavity between β -sheet 2A and F-helix (Lee, et al., 2000). In the crystal structure of G117F, no obvious structural difference was observed compared to wild-type except a shift in the position of the F-helix (Gooptu, et al., 2009). The downward shift of the F-helix in G117F introduces repacking of residues at an interface between F-helix and β -sheet 2A, and these local interactions at the interface were proposed to contribute to the stabilization of the mutant in the metastable state.

Sengupta et al. investigated the dynamics of G117F mutant by HXMS (Sengupta, et al., 2009). Several regions that are involved in the conformational change during the protease inhibition showed reduced exchange with deuterium compared to that of the same regions in wild-type (Figure 5). As expected from the crystal structure of G117F, significant changes in deuterium uptake were observed in the F-helix. In wild-type, the C-terminal region of the F-helix underwent rapid exchange with deuterium, with rates comparable to the RCL region; in contrast, the same region in G117F was found to be structurally more rigid as judged by reduced deuterium uptake. However, the effects of the mutation were not confined locally. Instead, the effects of the mutation were propagated to distant key regions involved in inhibition. A key region for the RCL insertion, β -strand 5A, also showed slower deuterium exchange than wild-type. Decreased structural fluctuations in these regions indicate a difficulty to access to a conformer that can readily form the protease-serpin complex since structural flexibility reflects frequency of structural deviations from the ensemble averaged structure represented by the crystal structure. Therefore, the underlying mechanism of reduced inhibitory activity seen in G117F is due to an inability to access to a conformer that readily undergoes a global conformational change during the inhibition. Reduced exchange was also seen in β -sheet C and helix D. When introduced into the pathological Glu342→Lys (Z) mutant of α_1 -AT, the G117F mutation leads to decreased polymerization and increased secretion from cultured cells. It has been shown that removal of strand 1C from the rest of sheet C is required for polymer formation, and one potential mechanism for resisting polymerization is strengthened interactions between strands 1 and 2C, as evidenced by decreased H/D exchange rates in this region in G117F. (Meagher, et al., 2000).

Effects of glycosylation on the native state dynamics of human α_1 -antitrypsin

—Glycosylation, a common post translational modification, plays a number of crucial roles, including modulating intermolecular interactions, helping prevent aggregation, increasing the lifetime of circulating proteins by conferring resistance to proteolytic degradation, and intrinsic stabilization of protein structure (Shental-Bechor and Levy, 2009, Varki, 1993). The

majority of previous bio-physical studies of α_1 -AT have employed a recombinant form that is produced in *E. coli* and is therefore unglycosylated. Glycosylation usually increases the global stability of proteins, and further, global stabilization is often accompanied by reduced flexibility in the native state (Shental-Bechor and Levy, 2009). It has been shown previously that glycosylation increases the stability of α_1 -AT against both thermal and chemical denaturation (Kwon and Yu, 1997).

Human plasma α_1 -AT (HP α_1 -AT) is normally fully glycosylated at three different Asn residues (Asn46, Asn83 and Asn247) with a mixture of bi- and tri-antennary complex glycans (Mills, et al., 2001). HXMS revealed that, as with recombinant α_1 -AT (RC α_1 -AT) the regions containing significant α -helical and β -sheet content exchanged more slowly than the regions consisting largely of turns or loops. Residues 62–77 (helix C), 100–118 (helix D and strand 2A), 227–240 (strands 1B, 2B and part of 3B), 318–329, 325–338 (strand 5A) and 374–384 (strands 4B and 5B) and 266–275 (helix H) all showed relatively slow exchange. Also as expected the RCL and other surface loops showed rapid exchange. While HXMS patterns in recombinant and plasma α_1 -AT were similar overall there were several differences. Peptides 188–205, containing a loop and a portion of strand 4C, and 318–329, containing a loop between helix I and strand 5A, both showed increased exchange in HP α_1 -AT. Peptides 208–227, 227–240 and 353–372 showed reduced exchange in HP α_1 -AT relative to RC α_1 -AT. Peptide 208–227 contains residues that are spatially adjacent to the glycan at position 46, and might be stabilized by direct interactions with the sugar moieties. Such stabilization could propagate to neighboring residues in β -sheets B and C. Stabilization of residues in peptide 227–240 by interactions with the glycan moieties at position 83 is also possible. There is no obvious structural explanation for the destabilization seen at the bottom of strand 5A, as it is not close to either a site of glycosylation or a region that is affected by glycosylation.

The H/D exchange rates in the key regions which play important roles in the metastable→stable transition were not affected by glycosylation. Flexibility was retained in the F-helix, while rigidity in the “breach” and “shutter” regions was likewise unaltered. This study did identify one difference in the native state dynamics of recombinant and glycosylated α_1 -AT that has potential functional significance. It demonstrated that the release of β -strand 1C from the rest of β -sheet C is required for the formation of pathological serpin polymers (Chang, et al., 1997). In recombinant α_1 -AT, strand C showed substantial protection from H/D exchange indicating a stable interaction with the rest of sheet C. This interaction would clearly deter polymer formation. Strand 1C showed increased protection from H/D exchange in HP α_1 -AT. Previous work has shown that HP α_1 -AT is more resistant to aggregation/polymerization than RC α_1 -AT (Kwon and Yu, 1997). These results suggest that increased stability of strand 1C is one factor contributing to increased resistance to the formation of pathological polymers.

Determination of thermodynamic stability using hydrogen-deuterium exchange combined with mass spectrometry

Protein flexibility and stability are not interchangeable. Flexibility in folded proteins reflects the structure of the free energy landscape near the free energy minimum that defines the native state. Observed flexibility will generally be dominated by non-native, possibly partially unfolded, structures that are close in energy to the native structure. In contrast, thermodynamic stability reflects the free energy difference between the native state ensemble and the denatured state ensemble. Both quantities – flexibility and thermodynamic stability – are clearly relevant to understanding the behavior of inhibitory serpins, which rely on both metastability and conformational mobility for their function.

The addition of denaturants such as guanidine hydrochloride (GuHCl) or urea will slow the rate of protein folding and thus give rise to EX1 exchange. In the case of EX1 exchange, all amide hydrogens in a given region exchange when that region unfolds. If unfolding is slow ($t_{1/2} > 1$ s), this leads to characteristic bimodal isotopic envelopes in the mass spectra of peptides from that region, with the relative areas of the upper and lower mass-to-charge ratio (m/z) peaks corresponding to the populations in solution that have sampled the unfolded state (upper m/z peak) or remained folded (lower m/z peak). After a short (~10 seconds) pulse with D₂O, the relative areas under the low and high m/z envelopes are often directly proportional to the equilibrium populations of the folded and unfolded states. It is not guaranteed that the relative areas under the peaks give an accurate estimate of the relative equilibrium populations. However, in the presence of low to moderate concentrations of denaturant, the rates of both unfolding and refolding are frequently slow enough that the peak areas do accurately reflect the equilibrium. Excellent agreement between folding/unfolding equilibrium constants estimated from pulse-labeling HXMS and more traditional methods has been found in many cases (Deng and Smith, 1999). While small proteins typically unfold in a concerted 2-state manner, larger proteins often contain distinct cooperatively folding domains. In proteins that unfold in a multistate manner, such cooperative domains are readily identified by HXMS.

Equilibrium unfolding study of WT- α_1 -AT using the pulse labeling technique—

Wild-type α_1 -AT was incubated in different concentration of guanidine-H₂O at pH 7.8 for one hour at room temperature to equilibrate followed by 10 fold dilution with pD 7.8 D₂O buffer containing the same concentration of guanidine as in the pre-incubated sample (Tsutsui and Wintrode, 2007). In GuHCl concentrations ranging from 0.5 to 1.2 M, double isotopic envelopes were clearly evident for all peptides, indicating the coexistence of folded and unfolded populations. For example, two mass envelopes observed in a peptic fragment covering residue 64–77 correspond to a population with the region 64–77 folded and another population with the same region unfolded (Figure 6a). Thereby, populations with a particular region folded and unfolded at different guanidine concentrations can be quantified by assessing the area under each of the two mass envelopes using a Gaussian equation, and the unfolded fraction for each peptic fragment can be plotted against guanidine concentration to obtain ΔG_{U-N} (Figure 6b).

ΔG_{U-N} values for different regions within WT- α_1 -AT structure was found to be in a range of 2.6–5.6 kcal/mol (Tsutsui and Wintrode, 2007). Although the C-terminal region of F-helix was found to be as flexible as the RCL region in the continuous labeling experiment, this region was not the least stable region. Therefore, structural flexibility does not always correlate with the local thermodynamic stability of a protein. For all regions analyzed, the denaturation midpoint ($[\text{GuHCl}]_{1/2}$) was found to be 0.8–1 M guanidine chloride. Denaturation curves for several representative peptic fragments from different structural regions are shown in Figure 7. Chemical denaturation studies using circular dichroism (CD) spectroscopy found 2 unfolding transitions in α_1 -AT; the first with a midpoint $[\text{GuHCl}]_{1/2} = 0.8$ M resulted in the loss of ~30% of the native signal, while the remaining ~70% was lost during a broad transition with a midpoint $[\text{GuHCl}]_{1/2} = 2.8$ M (James, et al., 1999). The transition observed by HXMS corresponds very well with the first transition observed by CD. The presence of clear double isotopic envelopes indicates that unfolding is cooperative, while the fact that the unfolding curves are near superimposable for all but one peptide indicates, surprisingly, that the whole α_1 -AT structure acts as a single cooperative unit, despite the appearance of multiple transitions in the CD unfolding curve. The fact that this cooperative transition coincides very well with the first transition observed by CD spectroscopy indicates that the transition is not well described as “unfolding” per se, since nearly 70% of secondary structure is retained after the transition. Further, gel chromatography shows that α_1 -AT at 1.5 M GuHCl is somewhat expanded relative to the

native state but still significantly more compact than the fully denatured state. Finally, Trp 194 has a significant fluorescence signal above 1.2 M GuHCl (Cabrita, et al., 2002), while the nearby peptide 241–251 shows persistent protection against H/D exchange even above 2 M GuHCl (the only peptide in this study to do so). This suggests that, while there is a cooperative loss of stable hydrogen bonding throughout most of the molecule, there is nonetheless considerable persistent structure. Taken together with differential scanning calorimetry data, these observations indicate that the first denaturation transition at 0.8–1 M guanidine is cooperative and involves an accumulation of a molten globule form that has solvent accessible amide hydrogen atoms while retaining considerable secondary structure and perhaps residual side chain packing. HXMS detected only the first transition from the metastable to the molten globule form. The second transition detected by CD and fluorescent spectroscopy involves unfolding of the molten globule form. Protease inhibition requires partial disruption of β -sheet A (Lee, et al., 2001, Seo, et al., 2000, Seo, et al., 2002). The fact that the entire α_1 -AT molecule unfolds as a single cooperative unit suggests that local unfolding in β -sheet A may induce unfolding in other regions of the molecule. Therefore, an alternative molecular mechanism of the protease inhibition involves a formation of the molten globule form to allow RCL insertion between β -strands 3A and 5A. In support of this hypothesis, extensive transient unfolding of α_1 -AT structure during the inhibition was observed by another HXMS study, described below (Baek, et al., 2009). As described in the next section, an inability to accumulate the molten globule form appears to be correlated with the loss of the protease inhibitory activity.

Equilibrium unfolding of G117F mutant studied by HXMS—An equilibrium unfolding study of a cavity-filling mutant G117F by *Sengupta et al.* provided an important insight into the mechanism of the mutant stabilization and decreased inhibitory activity (Sengupta, et al., 2009). The denaturation mid-point ($[\text{GuHCl}]_{1/2}$) determined by CD spectroscopy and HXMS agreed at 1.4 M guanidine, 0.6 M higher than that found in wild-type unfolding. In HXMS data, co-existence of two populations was observed at 1.4 M guanidine, one corresponding to the folded and the other to the unfolded form. Subsequently, the mass envelop corresponding to the folded form disappeared above 3 M guanidine indicating the two state cooperative transition. The degree of stabilization relative to wild type ($\Delta\Delta G$) for peptic peptides analyzed in the HXMS study was found to be clustered around 4.2 kcal/mol, in a good agreement with $\Delta\Delta G$ of 5 kcal/mol found in a previous fluorescent spectroscopic study (Lee, et al., 2000). However, unlike wild-type, the second transition was absent in the equilibrium unfolding curve monitored by CD spectroscopy. A loss of tertiary and secondary structures was detected above 1.5 M guanidine by fluorescent spectroscopy and above 3 M guanidine by CD spectroscopy respectively (Lee, et al., 2000, Sengupta, et al., 2009). Although non-coincidence of two denaturation curves obtained by spectroscopic methods implies the existence of an intermediate, it is not a direct evidence for the three state protein unfolding (Dill and Shortle, 1991). Non-overlapping denaturation curves are observed in the variable two state model where properties of one state such as the radius of the molecule change with external variables such as denaturant concentration and temperature (Dill and Shortle, 1991). Equilibrium unfolding studies by HXMS provide a direct evidence of a two state transition through an observation of populations. Thus, the unfolding of G117F involves only a single transition, from the metastable to the unfolded state without accumulating the molten globule form observed in the wild-type unfolding. Based on the crystal structure of the mutant, favorable aromatic side chain interactions around the mutation site were thought to contribute to the stabilization of the mutant (Gooptu, et al., 2009). However, there was no significant difference in local $[\text{GuHCl}]_{1/2}$ determined by HXMS between F-helix region and other regions indicating similar local stability in all regions analyzed. Instead, HXMS along with spectroscopic data suggested that the mutant stabilization is achieved by changing the

unfolding mechanism through destabilization of the molten globule form. According to the lattice model for protein denaturation, a single mutation can have a large effect on the stability of a protein by changing the distribution of conformations in the denatured state (Shortle, et al., 1992). Therefore, the effect of a mutation is not always evident in crystal structures if mutations affect the thermodynamic stability of proteins by altering sampling in the denatured state ensemble.

Based on the equilibrium unfolding behavior of wild type α_1 -AT, it was proposed that the molten globule form is a possible intermediate for the RCL insertion during the inhibition (Tsutsui and Wintrobe, 2007). This leads to the suggestion that the decreased inhibitory activity of G117F could be due to its inability to access and accumulate the molten globule form during the inhibition. That partial denaturation is required during the inhibitory conformational change is supported by the detection of “functional unfolding” during inhibition, described below. Together these results reveal that the folding and functional energy landscapes of inhibitory serpins are intimately related.

Effects of glycosylation on the equilibrium unfolding of α_1 -AT observed using HXMS—Glycosylation generally thermodynamically stabilizes the native states of proteins (Shental-Bechor and Levy, 2009), and spectroscopic studies have shown that glycosylated α_1 -AT is more stable than unglycosylated recombinant α_1 -AT (Kwon and Yu, 1997). It has also been found that significant stabilization of α_1 -AT can lead to impaired activity, and yet glycosylated α_1 -AT is fully active. To gain further insight into the relationship between stability and activity in glycosylated α_1 -AT, pulse labeling HXMS was used to monitor the equilibrium unfolding of glycosylated human plasma α_1 -AT (HP α_1 -AT).

The most surprising result of this study was the finding that glycosylation appears to stabilize the molten globule state of α_1 -AT rather than the native state. CD spectroscopy shows that the midpoint of the GuHCl induced unfolding transition is shifted from ~0.8 M (for recombinant α_1 -AT) to 2.3 M. Further, the transition appears to be 2 state for glycosylated α_1 -AT rather than 3 state as observed for recombinant. Pulse labeling HXMS, however, reveals a very different picture of the unfolding transition. All peptides in glycosylated α_1 -AT displayed cooperative unfolding with a transition midpoint at ~1 M GuHCl, and were fully unfolded by 1.2 M GuHCl, similar to recombinant (Figure 8).

It therefore appears that glycosylation stabilizes the compact denatured form of AT, but not the native state. Further, glycosylation appears to alter the properties of the compact denatured state of α_1 -AT. Unlike recombinant, which loses ~30% of its native ellipticity above 1 M GuHCl, plasma AT retains near native levels of ellipticity. It has been argued that compaction of the polypeptide chain induces secondary structure (Yee, et al., 1994), and our results suggest that HP α_1 -AT at 1.2 M GuHCl is more compact than RC α_1 -AT under the same conditions. Despite the increased secondary structure content of plasma AT at low GuHCl concentrations, it retains virtually no protection against H/D exchange under these conditions, indicating that whatever secondary structure is present must be marginally stable and subject to frequent and significant fluctuations. α_1 -AT is thus highly unusual in that glycosylation does not appear to stabilize the native state. In the case of inhibitory serpins, this peculiar behavior may reflect functional requirements since significant stabilization of the native metastable form of AT could lead to compromised inhibitory activity. Previous studies on the effects of glycosylation on protein folding and stability have found that the primary effect of glycosylation is to destabilize the denatured state (Shental-Bechor and Levy, 2008). In the case of α_1 -AT, the effects of glycosylation on the unfolded state appear to be decoupled from the stability of the native state. In a protein with a simple 2-state folding mechanism, such destabilization would naturally drive the protein to the native state. The equilibrium folding/unfolding mechanism of α_1 -AT, however, is not 2-state. Instead,

AT unfolds through a complex mechanism involving a molten globule intermediate. These results suggest that glycosylation does destabilize the unfolded state of α_1 -AT at mild GuHCl concentrations, but this drives α_1 -AT into the compact denatured state rather than to the native state.

“Functional unfolding” during the native $\rightarrow$ cleaved transition

It is clear from crystal structures that large scale structural rearrangements are required for the translocation of trapped protease and the insertion of the RCL into β -sheet A to proceed. However, crystal structures of the native and cleaved states provide limited information on the extent of the structural rearrangements that occur during the transition. The apparent correlation between inhibitory efficiency and the ability to populate a compact denatured state upon mild perturbations to solvent conditions, discussed above, suggest that it may be necessary to visit partially unfolded conformations during the inhibitory conformational change.

Baek et al. tested this hypothesis using HXMS (Baek, et al., 2009). They incubated α_1 -antitrypsin in D₂O under three conditions: (1) native α_1 -antitrypsin alone, (2) the covalent complex between cleaved α_1 -antitrypsin and inhibited trypsin, and (3) α_1 -antitrypsin diluted into D₂O containing an equimolar amount of trypsin. By comparing local H/D exchange rates under these three conditions, Baek et al. were able to identify regions that transiently unfold during the native $\rightarrow$ cleaved transition as well as regions that are stabilized in the inhibitory complex.

Baek et al. found multiple regions that showed increased deuterium uptake under condition (3), indicating transient local unfolding during the native $\rightarrow$ cleaved transition (Figure 9). Some of these transient unfolding regions are readily predicted from the crystal structures of the native and cleaved forms. The very top of β -strands 3 and 5 A show evidence of unfolding (although they are both part of larger peptides that include additional structural elements), as does the C terminal portion of the F helix. However, transient unfolding also occurs in regions that would not be easily predicted from crystal structures, including regions quite distant from the RCL insertion site. β -strands 2, 3 and 6 B, 4C and 6 A, as well as most of α -helices A and G, all appear to undergo at least partial transient unfolding during the conformational change. These results support the contention that extensive structural disruptions throughout the molecule are required during the native $\rightarrow$ cleaved transition. Interestingly, there is not evidence for unfolding in most of β -strands 3 and 5 A, despite the fact that these two must clearly separate in order to accommodate the RCL. This indicates that excepting the tops of these strands (forming part of the “breach” region where RCL insertion begins) the “opening” must be very transient and lead quickly to either re-closing or RCL insertion. Baek et al suggest that rapid displacement of strand 3 and 5 A interactions by the RCL in a “zipping” type mechanism could accomplish RCL insertion without significant solvent exposure in strands 3 and 5 A.

Additionally, HXMS identified several regions that were stabilized in the inhibitory complex relative to the metastable state. Notably, β -strand 5 A is stabilized in the complex. This is consistent with the observation that stabilizing mutations in strand 5 A lead to impaired inhibitory activity, which suggested that instability of strand 5 A was important for inhibitory function. β -strand 2 C was also stabilized in the complex, perhaps due to improved interactions with strand 1 C after the “strain” imposed by the RCL is removed.

Investigating the Polymerization Pathway and Polymer Structure of α_1 -AT by HXMS and Ion Mobility MS

The formation of serpin polymers *in vivo* is associated with a variety of diseases including dementia, emphysema, and liver cirrhosis collectively known as serpinopathies (Lomas and Carrell, 2002). Due to its medical significance, the mechanism of serpin polymerization has been vigorously investigated primarily by spectroscopic techniques (Dunstone, et al., 2000, Lomas, et al., 1995, Dafforn, et al., 1999). *In vitro*, the formation of polymers is facilitated at acidic pH, mild denaturant concentration, or elevated temperature above 45°C (Dafforn, et al., 1999, Devlin, et al., 2002). The presence of an aggregation-prone intermediate, often denoted as M^* , has been inferred from the kinetics of polymerization, and possible structural features of M^* may be gleaned from a crystal structure of α_1 -antichymotrypsin in which β -strands 3A and 5A are separated and both the RCL and part of helix F are inserted to stabilize the separation (Gooptu, et al., 2000). The classical serpin polymerization pathway proposes an accumulation of an intermediate with separated β -strands 3A and 5A prior to the polymer formation (Ekeowa, et al., 2010). Subsequent annealing of the RCL of one serpin molecule to the opening between β -strands 3A and 5A of another serpin molecule leads to the formation of polymers (Lomas and Carrell, 2002). Recently, an alternative polymerization pathway was proposed based on a crystal structure of antithrombin polymers (Yamasaki, et al., 2008). This β -hairpin polymerization pathway requires complete expulsion of β -strand 5A from β -sheet A of one serpin and subsequent insertion of both strand 5A and the RCL into sheet A of another serpin. Both proposed polymerization mechanisms require the formation of an intermediate in which β -sheet A is substantially disrupted and therefore increased H/D exchange should be observed in this region if the intermediate(s) accumulate to a significant degree prior to polymerization.

Incubation at slightly elevated temperatures such as 45°C leads to polymerization of wild type α_1 -AT, and HXMS studies were performed under these conditions (Tsutsui, et al., 2008). At 45°C, polymerization is very slow compared with the time scale of the hydrogen-deuterium exchange reaction so that dynamics of the intermediate can be investigated. Wild-type α_1 -AT was incubated at 45°C for different time periods up to 5,000 seconds in pre-warmed D_2O buffer, and deuterium uptake of different regions at 45°C in the monomeric α_1 -AT intermediate structure was compared to that of the monomeric metastable state at 25°C. The deuterium labeling time at 25°C was corrected for temperature-dependence of the hydrogen-deuterium exchange reaction by incubating the sample in D_2O buffer for six times longer than corresponding labeling time at 45°C. Surprisingly, deuterium uptake in β -strand 3A and 5A at 25°C and 45°C was comparable. Because the separation of β -strands 3A and 5A would certainly lead to increased deuterium uptake, this result suggests that either the strand separated M^* conformation is not populated to a significant extent or that it is a transient species that quickly proceeds to the polymeric form. A substantial increase in deuterium uptake was observed in β -sheet C. These observations led to the suggestion that mild heating initially weakens interactions in sheet C, and that subsequently, an encounter with a second serpin molecule leads to the displacement of strand 1 C and transient formation of M^* , followed quickly by the formation of an intermolecular loop-sheet linkage.

In contrast, a recent ion mobility mass spectrometry (IM-MS) result argues against the notion that the M^* intermediate is not populated to a significant degree. Ion mobility MS measures the drift time of protein ions in a tube filled with a carrier buffer gas. Drift time can be converted into a collision cross section, which depends on the protein ion's shape and size. IM-MS can thus separate and characterize proteins with different oligomerization states and/or conformations. Performing IM-MS on α_1 -AT polymers formed by heating, Ekeowa et al. detected native monomers, polymers, and a monomeric intermediate with an enlarged collision cross section, indicating an expanded structure (Figure 10) (Ekeowa, et al., 2010). This indicates that a non-native monomeric species does in fact accumulate during

polymerization. There are several possible explanations for the apparent discrepancy between the HXMS and IM-MS results. Polymers for IM-MS were formed by heating at 60°C, while polymers for HXMS were formed by heating at 45°C. The increased temperature may have increased the population of M^* to detectable levels. Additionally, the length of the incubation prior to measurement was different in each case.

The structure of polymers formed by extended incubation at 45°C was probed using HXMS (Figure 11) (Tsutsui, et al., 2008). Compared with monomeric α_1 -AT, dramatically reduced exchange was seen in the RCL, the C terminal portion of helix F and helix E. Smaller but still significant decreases were seen in strands 3A and 5A, strand 3C and helix D. There are currently 2 major structural models for serpin polymers: a long standing “loop-sheet” model, in which the RCL of one serpin is inserted between strands 3 and 5A of another serpin, and a more recent “domain swap” model in which strand 5A is extracted from sheet A of one serpin, and both it and the RCL insert into Sheet A of another serpin becoming strands 4 and 5 A (expelling the other serpin’s strand 5 A in the process). The pattern of decreased H/D exchange in the polymer relative to the monomer is generally consistent with both models. Decreased exchange in either model would be expected in the RCL, due to its sequestration from solvent, and in strands 3 and 5 A, where the conversion from parallel interactions with each other to antiparallel interactions with the newly introduced strand 4 A is expected to result in increased stability. Decreased exchange in helix F could result from burial in the serpin-serpin interface, as might be expected from some of the loop-sheet models, but it could also result from improved packing against the 6 stranded β -sheet A.

Future Prospects

There are several areas in which structural mass spectrometry techniques can shed light on serpin structure and function. As mentioned above, the structure of pathological serpin polymers remains a subject of debate, and it is possible that polymers formed under different conditions (temperature, pH, denaturant) may adopt distinct structures. The application of HXMS to different polymer preparations and to polymers of serpins other than α_1 -AT may clarify this. Additionally, the application of radiolytic footprinting to serpin polymers would provide single residue resolution information on which side chains become sequestered from solvent upon polymerization. Such information would provide valuable constraints for attempts to construct plausible structural models of serpin polymers.

In addition to the mechanism of polymerization, the mechanism of serpin folding remains to be elucidated. A detailed view of the serpin folding pathway would help explain the remarkable fact that serpins do not fold to the global free energy minimum, but are trapped in a metastable conformation. The relatively slow rates of serpin folding (Kim and Yu, 1996) combined with the ability of HXMS to directly quantify the populations of folded and unfolded species in a structurally resolved manner suggests that HXMS is a promising technique for resolving the kinetic folding pathways of serpins in detail. Finally, serpins *in vivo* interact with and are regulated by a variety of proteins and other molecules. Examples include the allosteric activation of antithrombin by heparin and the regulation of PAI-1 by vitronectin (Gettins, 2002). Structural mass spectrometry provides an array of powerful methods for defining the mechanisms of serpin regulation by proteins, lipids and small molecules.

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Figure 1.
Hydrogens in proteins. The amide hydrogens monitored by H/D exchange are circled.

Measuring hydrogen exchange rates by mass spectrometry

Figure 2.
Overview of the experimental procedure for measuring local H/D exchange rates using mass spectrometry.

Figure 3.
Overview of the experimental procedure for monitoring side chain solvent accessibility using radiolytic footprinting.

Figure 4. Distribution of conformational flexibility in the metastable form of α_1 -AT determined by HXMS.

Figure 5. Allosteric rigidification resulting from the cavity filling G117F mutation in α_1 -AT. F117 is shown as magenta spheres.

Figure 6.

A) Example of a double isotopic envelope observed during GuHCl denaturation of α_1 -AT. The peptide shown corresponds to residues 64–77. B) Equilibrium unfolding curve for peptide 64–77 constructed from the relative areas under the low (native) and high (denatured) mass peaks in the mass spectra at different GuHCl concentrations.

Figure 7.
HXMS derived denaturation curves for peptic fragments derived from different regions of α_1 -AT.

Figure 8.
Mass spectra for peptides derived from different regions of glycosylated α_1 -AT at 0, 1.0, 1.2 and 4 M GuHCl.

Conformational changes in α_1 AT during complex formation with β -trypsin.

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Figure 9. Patterns of deuterium uptake in different regions of α_1 -AT during the inhibitory conformational change. Red and orange indicate regions that undergo “functional unfolding” during inhibition.

Figure 10.

Ion mobility MS of α_1 -AT monomer and polymers. After heating, an additional monomeric species with a slightly increased collision cross section (indicating a somewhat expanded structure) is evident.

Figure 11.
Changes in deuterium uptake in different regions of α_1 -AT upon polymerization.