

A PROTEIN FAMILY UNDER ‘STRESS’ – SERPIN STABILITY, FOLDING AND MISFOLDING

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1. ABSTRACT

The native fold of inhibitory serpins (serpin proteinase inhibitors) is metastable and therefore does not represent the most stable conformation that the primary sequence encodes for. The most stable form is adopted when the reactive centre loop (RCL) inserts, as the fourth strand, into the A β -sheet. Currently a serpin can adopt at least four more stable conformations, termed the cleaved, delta, latent and polymeric states. The accessibility of these alternative low energy folds renders the serpin molecule susceptible to mutations that can result in dysfunction and pathology. Here, we discuss the means by which the serpin can attain and preserve this metastable conformation. We also consider the triggers for misfolding to these more stable states and the mechanisms by which it occurs.

2. INTRODUCTION

Serpins represent a superfamily of homologous proteins with a diverse range of functions including blood coagulation, fibrinolysis, programmed cell death, development and inflammation (1, 2). In the majority of these roles, serpins function by inhibiting proteinases. Approximately 800 serpins have been identified to date, with sizes ranging between 350–400 residues and molecular weights from 40 to 50kDa (3). Serpins are thought to have undergone divergent evolution over a period of approximately 600 million years and have recently been identified in prokaryotes (4, 5), confirming their presence in every taxonomic phylum (3).

Relatively few serpins are non-inhibitory, however these proteins perform a range of roles. For

instance, HSP47 functions as a molecular chaperone, assisting in the assembly of collagen (6), while cortisol binding globulin acts as a hormone transport protein (7). The vast majority of serpins act as proteinase inhibitors, most commonly inhibiting chymotrypsin-like serine proteinases. Some serpins, however, exhibit cross-class inhibition, acting as inhibitors of cysteinyl proteinases of the papain family (8). Inhibitory serpins utilise a unique inhibitory mechanism that involves large-scale conformational change resulting in a substantial rearrangement of their native structure and also that of the proteinase (9–11).

The native serpin fold consists of three β -sheets (A–C) and nine α -helices (A–I) and a reactive centre loop (RCL), traversing the fifth strand of the A β -sheet and the first strand of the C β -sheet (Figure 1). The RCL contains the scissile bond that acts as the cleavage site for the cognate proteinase, and undergoes a dramatic conformational change during inhibition. The initial indications that the RCL may be mobile came from the elucidation of the first crystal structure of α_1 -antitrypsin, which had been proteolytically cleaved within its RCL (12). It was observed that the N-terminal side of the cleavage site inserts in the middle of the A β -sheet between strands three and five to form the fourth strand, with the two residues of the scissile bond becoming separated by more than 65 angstroms. It is now apparent that a similar conformational change occurs during proteinase inhibition (10). Upon cleavage of the scissile bond by the cognate proteinase, the proteinase is translocated from one pole of the serpin to the other in conjunction with RCL insertion (10).

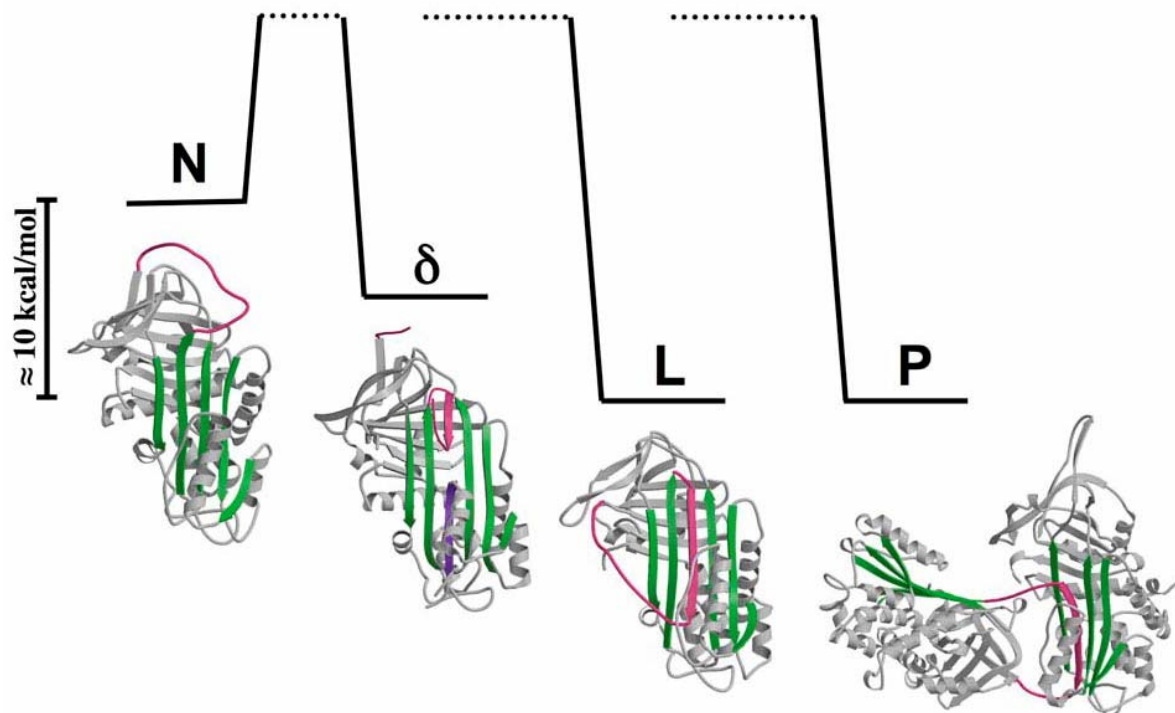


Figure 1. Energy Diagram Illustrating Alternative Serpin Conformations. A minimal thermodynamic scheme is presented for serpin misfolding. Here, the native state (N) represents the least stable conformation, followed by the intermediately stable delta conformation. The difference in stability between the latent (L) and polymeric (P) states is unknown, and they are presented here as being equally stable. Kinetic barriers exist between the native and misfolded states, and these are illustrated as being capped by dotted lines as the energies of these barriers are unknown, and can be manipulated by mutation and solvent conditions. The structures shown are for native and latent α_1 -antitrypsin, delta α_1 -antichymotrypsin and a loop A-sheet model of an α_1 -antitrypsin dimer. The A β -sheets are shown in green and the RCLs in pink. The A β -sheet inserted portion of helix-F in the delta structure is in purple. These images were produced with Molscript (106).

3. THE NATIVE SERPIN FOLD IS A KINETICALLY TRAPPED INTERMEDIATE

A high degree of structural flexibility is required to facilitate the large-scale conformational change that accompanies the inhibitory activity of the serpin. However, this flexibility only provides the means by which the serpin can undergo structural rearrangement, it does not provide the energy for the transition. The thermodynamic basis of this is inextricably linked to the fact that serpins do not fold to their most stable state.

In general, the driving force for protein folding and the accurate, and often independent, attainment of a precise tertiary structure is the minimisation of free energy in the reaction system (13). The so-called 'new view' of protein folding describes this process in terms of a stochastic movement towards the native state from the unfolded state along energy landscapes, that involves ensembles of conformations. Here, the unfolded ensemble is representative of a vast array of random coil like structures, each of which can approach the native fold via distinct pathways. Most proteins have a sizeable difference in free energy between the native and unfolded states, averaging between 5-15 kcal mol⁻¹, with serpins typically residing in middle this range (≈ 10 kcal mol⁻¹) (14, 15). As such, the difference in free energy sufficiently biases the conformational space, so that a random

search through all possible conformations is not required. The searching through conformational space is limited by the formation of native contacts, which are presumably more energetically favorable than non-native ones, and result in a decrease in free energy (16).

Metastability, as observed in the serpins, arises when the conformational landscape of a given protein contains troughs of lower free energy than that of the native state itself (17). In such cases, the native state does not represent the thermodynamically most stable conformation. Rather, it is akin to a kinetically trapped intermediate. For a number of proteins, metastability is considered to be an important regulator of their biological functions, including the membrane fusion protein, hemagglutinin (18-20), from the influenza virus, the prokaryotic serine proteinase, alpha-lytic proteinase (21), subtilisin (22, 23), luciferase (24, 25), and inhibitory members of the serpin superfamily (26).

In the case of serpins, insertion of the RCL into the A β -sheet of the molecule results in a substantial decrease in the free energy of the serpin and an increase in its stability by more than 10 kcal/mol (27-33). In the literature this structural transition is commonly referred to as the 'stressed' to 'relaxed' transition (32) in that the native metastable serpin fold is a high energy state that relaxes to lower free energy when cleaved in the RCL. The

first study to identify this behaviour by Carrell and Owen in 1985, demonstrated that both native α_1 -antitrypsin and antithrombin precipitated from solution when heated at 60°C. However, upon cleavage of the RCL of both proteins, precipitation was not observed even after two hours incubation at 80°C (32). In this study, ovalbumin provided a convenient control, as it is incapable of RCL insertion. In similar experiments, ovalbumin was not observed to undergo equivalent stabilization by proteolysis (32). Subsequently, analysis of a variant of ovalbumin with the P14 arginine replaced by threonine, demonstrated that RCL cleavage did result in its insertion with a concomitant increase of 16°C of its temperature of denaturation as measured by differential scanning calorimetry (28). Interestingly, serpins also undergo a similar increase in thermostability when complexed with peptides analogous to the RCL (34). This suggests that increased stability is a product of hydrogen bond formation and hydrophobic interactions made by the addition of a sixth strand to the A β -sheet.

3.1. The molecular basis of serpin metastability

The sheer number of inhibitory serpins that have been identified and the fundamental importance of the biological processes that they regulate imply that the metastable serpin architecture is incredibly successful from an evolutionary perspective. Extensive studies on alpha-lytic protease and members of the serpin superfamily have yielded an understanding of how nature engineers metastability into protein folds. In the case of alpha-lytic protease, the metastable native state is considerably less stable than both its molten globule-like folding intermediate and even its unfolded state (35). The native state can only be formed in the presence of a pro-region that lowers the kinetic barrier between these two species.

In the case of serpins, the metastability of the native state correlates strongly to non-ideal interactions that impart strain within the native fold that can be alleviated by the adoption of alternative conformations. In extensive studies by the laboratory of Yu and colleagues, such interactions have been observed throughout the entirety of the native serpin molecule, seemingly without localisation to specific structural elements or regions. These include the presence of hydrophobic pockets on the surface of the protein (36), overpacking of side-chains (37), the burial of polar groups (38) and cavities in the hydrophobic core of the protein (39). Such aberrations are energetically unfavorable. Mutational modification of these sites results in increased stability of the native state of the serpin (36-42). However, at a number of sites, a penalty of this can be a concomitant decrease in inhibitory activity (36) as the structural flexibility afforded by such non-ideal interactions, and the free energy difference between the native and cleaved conformations, are obligate for initiation and propagation of the serpin inhibitory mechanism.

4. LOW ENERGY ALTERNATIVE SERPIN CONFORMATIONS

The metastable nature of the native serpin fold renders the protein sensitive to mutations that can result in

aberrant structural rearrangement into dysfunctional alternative conformations with increased stability. A physical characterisation of these conformations has recently been discussed in the review by Dafforn et.al. (43). These alternative states are implicated in disease processes such as liver cirrhosis, thrombosis, angioedema and dementia and generally arise due to single amino acid substitutions within the primary sequence of the given serpin.

4.1. The latent conformation

The latent serpin conformation was first observed in plasminogen activator inhibitor-1 (PAI-1). Once purified, the activity of PAI-1 was demonstrated to diminish over time at 37°C, with a half life of approximately 2.5 hours (44), unless bound to vitronectin, as it exists in the circulation (45-48). The crystal structure of the deactivated form of this protein revealed that the RCL of the molecule was fully inserted into the A β -sheet without cleavage (49), which resulted in increased thermostability (50) (Figure 1). Termed 'latent', this conformation has subsequently been observed in other members of the serpin superfamily including α_1 -antitrypsin (51), α_1 -antichymotrypsin (52) and antithrombin (53, 54).

4.2. The delta conformation

The delta structure has been observed for a naturally occurring variant of α_1 -antichymotrypsin (Figure 1). Purification of the Leu⁵⁵-Pro mutant of α_1 -antichymotrypsin led to the isolation of three different conformational states, which eluted from ion-exchange chromatography with different characteristics. These states were native, and two inactive species, latent and delta α_1 -antichymotrypsin. The delta form of α_1 -antichymotrypsin demonstrated characteristics atypical of any other known serpin conformation in that it was more stable than the native state as measured by chemical and thermal denaturation, however the protein aggregated readily (55). Elucidation of the crystal structure of delta α_1 -antichymotrypsin to a resolution of 2.5 angstroms revealed a conformation that is suggested to be an intermediate between the native and latent states (55). In the structure, three residues of the RCL (P14-P12) had inserted into the top of the A β -sheet, between strands three and five. Furthermore, the last turn of helix-F and the loop connecting it to s3A had rearranged to insert eight residues into the bottom the A β -sheet.

4.3. The polymeric conformations

Serpin polymerisation forms the basis for the great majority of all serpin related disorders or serpinopathies (56, 57) (Figure 1). Like the other alternative serpin conformations discussed above that result in heightened stability and a concomitant decrease in free energy, serpin polymerisation can be attributed to the inherent flexibility and metastability of the native serpin fold. In the case of serpin polymers the conversion to a lower energy fold is satisfied by the formation of inter-molecular contacts instead of intra-molecular ones. Observation of serpin polymers formed *in vitro* and those isolated from diseased tissues are similar as observed by electron microscopy (51), revealing structures akin to

‘beads on a string’. More detailed analyses, however, have demonstrated that the precise nature of the inter-molecular linkages between monomeric units can vary, dependent on the protein involved and the conditions used during polymer formation (58-60). The polymer structures that have been observed to date are detailed below.

4.3.1. Loop A-sheet polymers

As the name implies, loop A-sheet polymers arise via the insertion of the RCL of one serpin molecule into the A beta-sheet of another, between strands three and five (61) (Figure 1). The first evidence for this mode of intermolecular linkage came from experiments in which α_1 -antitrypsin polymerisation could be blocked by incubation with excess concentrations of peptide analogous to the RCL (58, 61). In these experiments the serpin formed a binary complex with the peptide in preference to self-associating into polymeric species. This mode of polymerisation has also been supported by fluorescence resonance energy transfer (FRET) data which demonstrated conclusively that the α_1 -antitrypsin polymers were formed by the insertion of the RCL into the A β -sheet (62). Currently, there is no evidence to suggest that naturally occurring serpin deficiency variants polymerise via this mechanism.

4.3.2. Loop C-sheet polymers

Loop C-sheet dimers have been observed in the crystal structure of dimeric antithrombin, solved to 3 angstroms in 1993 (54), and subsequently at higher resolution in 1997 (63). In both cases, one of the antithrombin molecules is in the latent conformation in which strand one of the C β -sheet has peeled away to allow for full self-insertion of the RCL. The position left vacant by s1C has been filled by the RCL of the second molecule in these crystal structures. The naturally occurring M_{malton} variant of α_1 -antitrypsin (Phe⁵²-deleted) has been proposed to form polymers via this mechanism rather than loop A-sheet polymerisation as described above (64), whilst both wild-type α_1 -antitrypsin and antithrombin have also been shown to form polymers of similar morphology when heated in the presence of citrate (51, 59, 65, 66).

4.3.3. Strand 7A polymers

To date, strand 7A polymers have only been formed by PAI-1 (67). In the only published crystal structure of PAI-1 in a non-latent conformation, the RCL formed loop-sheet interactions with neighboring molecules, generating an infinite chain within the crystal (67). This interaction was formed by the hydrogen bonding of the RCL of one molecule with the sixth strand of the A β -sheet of the next, whereby the donor RCL becomes the seventh strand of the acceptor molecule's A beta-sheet. Subsequent investigation demonstrated that PAI-1 could polymerise readily under conditions of low pH (68).

4.3.4. Disulfide linked polymers

Recently, a novel mode of polymerisation of α_1 -antitrypsin has been observed with initial dimerisation occurring via the formation of a disulfide bond upon incubation of the protein in 1.4M guanidine (69). α_1 -Antitrypsin has a single cysteine residue located on the B

β -sheet. Subsequently, this dimeric species goes on to form higher order polymers by interactions that are not well characterised (69). The use of high concentrations of peptide analogous to the RCL of α_1 -antitrypsin was unable to prevent the extension of the dimer (69). This suggests that the RCL itself does not form the basis for further linkage as it does in the other polymerisation models described above. The disulfide-linked polymers, however, have similar linear morphology to both loop A- and C-sheet polymers as judged by electron microscopy (69).

5. HOW DO SERPINS ATTAIN A NATIVE FOLD AND AVOID MISFOLDING

The potential for folding to the alternative serpin conformations detailed above brings into question, how is the native state attained at all by serpins? Or how does a folding serpin molecule avoid these other thermodynamically more favourable conformations? Although the answer to these questions remains elusive, the folding studies that have been carried out on members of the serpin superfamily point the way towards our understanding. Combined these studies suggest the following minimal pathway for serpin folding:



where ‘U’ is the unfolded ensemble, ‘I’ represents a transiently stable, partially folded ensemble, and ‘N’ represents the natively folded protein.

A number of these studies indicate the presence of multiple intermediate (I) species in the pathway, however an intermediate ensemble which populates at equilibrium in low denaturant concentrations appears to be common to most serpins (5, 14, 15, 70, 71). Ovalbumin, a non-inhibitory serpin, is an intriguing exception as CD analysis of its unfolding is clearly two state (33). Biophysical analysis in our laboratory suggest that this intermediate ensemble contains approximately 80% of the native secondary structure, with partially formed A and C β -sheets, a well formed B β -sheet and a non-native F helix (15, 72, 73). We have previously suggested that the first three strands of the B β -sheet along with the G and H helices serve as a nucleus for folding (72), and this is supported by its substantial structure in the intermediate ensemble.

Controversy exists as to whether such folding intermediates are productive and requisite, or whether they exist off the folding pathway and serve as kinetic traps that hinder the process (74, 75). Intermediates often slow the folding process as they contain non-native contacts within their structure. As such, the disruption of these contacts establishes a kinetic barrier to the native state that must be overcome. In the case of serpins, however, we have proposed that such non-native contacts may be productive and indeed obligate to the attainment of the native state. Evidence from this comes from our fluorescence studies in which we identified non-native interactions around the top of the A β -sheet and the F-helix (72, 73).

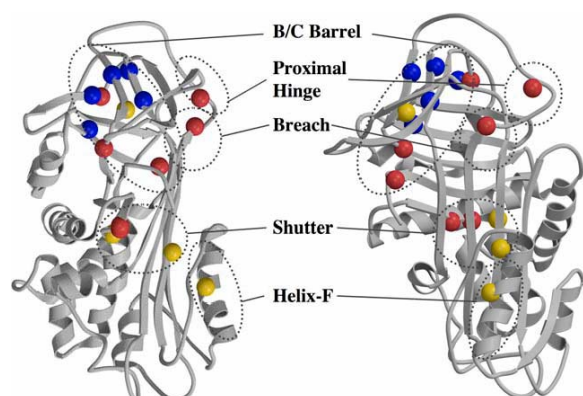


Figure 2. The Molecular Switches of Serpin Misfolding. Residues known to promote the formation of alternative serpin conformations are highlighted as spheres on the α_1 -antitrypsin scaffold as viewed from the side (left) and front (right) of the molecule. Residues highlighted in red promote polymerisation, those in blue promote latency and those in gold promote a bifurcation of the misfolding pathway into both polymeric and latent species. These images were produced with Molscript (106).

In particular, the non-native interactions made by residues of the F-helix may play an important role in preventing misfolding. Previously we have demonstrated that the F-helix is disrupted in the intermediate ensemble (73). Taken together with the X-ray structure of delta α_1 -antichymotrypsin (55), these data suggest that the top of helix-F may be inserted between strands 3A and 5A in the intermediate ensemble. Such a conformation may be protective, as it would prevent self-insertion of the RCL and the transition to latency, as well as preventing the formation of s4A polymers. As such, the existence of a stable intermediate on the serpin folding pathway may be essential to the attainment of a metastable native conformation.

6. THE MOLECULAR SWITCHES OF SERPIN MISFOLDING

The accounts of naturally occurring serpin variants associated with polymerisation and latency, as well as those observed from mutagenic studies, have allowed many of the molecular switches for serpin misfolding to be mapped onto the tertiary scaffold. These mutations appear to cluster to the mobile structural elements, as illustrated in Figure 2, and summarised in Table 1.

The proximal hinge (RCL residues P15-P9), the breach (top of β -sheet A), the shutter (middle of β -sheet A) and helix-F all require substantial flexibility in order for the RCL to insert as the fourth strand of the A β -sheet during proteinase inhibition. The possible exception to this statement is the B/C barrel which comprises the juncture of the A and C β -sheets, which are generally considered to remain relatively static during proteinase translocation, as cleavage of the RCL by the cognate proteinase removes any potential strain on s1C. However, a substantial cluster of variants in this region results in transitions to polymer or latency, or both. Taken together with data implicating this

region as a folding nucleus (72), these data suggest this region serves to anchor the RCL against inappropriate conformations during the folding process, and to maintain the RCL in a native conformation in the fully folded protein.

The mechanisms by which these mutations result in misfolding are not well defined. Based on the free-energy diagram in Figure 1, the simplest explanations centre on either destabilisation of the native state in regards to alternative folds, or reducing the energy of the kinetic barriers between these conformations. In the study by Jung *et al.*, the thermodynamic stability of the natively folded Z (Glu³⁴²-Lys) deficiency variant of α_1 -antitrypsin, appears to be very similar to that of the wild-type protein as determined by transverse urea gradient PAGE (76). However, in another study, the thermal denaturation of Z α_1 -antitrypsin showed an 8°C decrease in the melting temperature compared to wild-type (77). Potentially, certain variants may promote misfolding by reducing the stability of the native state as well as the energy of the kinetic barriers. Such a rationalisation has been implied to explain the rate of conversion to the latent state of α_1 -antitrypsin containing B/C barrel mutations (42).

Solutes can also substantially modify the serpin conformational landscape. Previous studies have shown that the osmolytes trimethylamine N-oxide and sarcosine can increase the stability of the native serpin fold and protect against misfolding events (78, 79). Osmolytes favour the folding of proteins to their thermodynamic minima by forming adverse interactions with the polypeptide chain (80). Essentially, this raises the free energy of the unfolded state of a given protein in relation to its native state and augments the impetus to fold. Interestingly, when α_1 -antitrypsin was refolded in the presence of the osmolytes, only misfolded species were formed (78, 79). This supports the notion that the native serpin fold is not representative of the thermodynamic minimum. Citrate and glycerol have also demonstrated stabilising effects, however, unlike the global stabilisation afforded by osmolytes, these compounds appear to bind specifically to the serpin scaffold (81, 82). Whilst the binding site(s) of citrate are currently unknown (82), crystallographic evidence has demonstrated that glycerol binds to and stabilises a hydrogen bonding network centred on the conserved His³³⁴ residue in the shutter region of the serpin (81).

7. THE KINETIC PATHWAY OF SERPIN MISFOLDING

Although the structures of the polymeric species discussed above vary, they share a commonality in their formation. All of the polymers mentioned form under partially denaturing conditions such as elevated temperature, altered pH or incubation with low concentrations of chemical denaturant (51, 65, 68, 69, 83). Alternatively, the serpins in question may contain destabilising mutations that lead to polymer formation (84). This suggests that the adoption of a non-native protein conformation is a prerequisite for polymerisation, as has

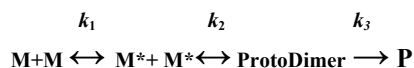
Table 1. The Locations on the Serpin Scaffold of Key Residues for Misfolding

Residue	Location	Misfolded State	Serpin	Reference
52	Shutter	P	α_1 -Antitrypsin, C1-Inhibitor	84, 97
53	Shutter	P	α_1 - Antitrypsin	91, 98
55	Shutter	P/L	α_1 -Antichymotrypsin	99
158	Helix-F	P/L	ATIII	100
187	Shutter	P/L	ATIII	100
228	B/C Barrel	P	α_1 -Antichymotrypsin	99, 101
229	B/C Barrel	L	α_1 - Antitrypsin	42
254	B/C Barrel	L	α_1 - Antitrypsin	42
286	B/C Barrel	L	α_1 - Antitrypsin	42
288	B/C Barrel	L	α_1 - Antitrypsin	42
342	Breach	P	α_1 - Antitrypsin	102
349	Proximal Hinge	P	C1-Inhibitor	103, 104
364	B/C Barrel	L	α_1 - Antitrypsin	42
366	B/C Barrel	P/L	C1-Inhibitor	105
370	B/C Barrel	P	C1-Inhibitor	95, 105
391		P	C1-Inhibitor	95, 105

(P) Polymer, (L) Latent

been suggested for a number of unrelated proteins associated with conformational disease (57). Conformational change and polymerisation have been characterised most extensively for the serpins, α_1 -antitrypsin (77, 85), α_1 -antichymotrypsin (86) and PAI-1 (68).

Analysis of the early spectroscopic changes during polymerisation of these serpins reveal the presence of a fast, concentration independent phase, corresponding to the formation of a non-native species (M^*) (68, 77, 83, 86). Subsequently, these polymerisation processes demonstrate slower kinetic phases whose rates correlate positively with increasing protein concentration (68, 77, 83, 86). This corresponds to a bimolecular process in which monomeric units associate with one another. Recently we observed a second concentration dependent kinetic phase during the polymerisation of α_1 -antitrypsin under acidic conditions (83). Here the first concentration dependent phase represented the formation of a dimeric species that could dissociate to active monomers upon neutralisation of the reaction pH. This step was followed by an irreversible formation of oligomers via stabilisation and elongation of the dimers as described in the following kinetic scheme using the notation of Dafforn *et al.* (77):



where 'M' represents the natively folded monomeric form of the serpin, ' M^* ' represents the polymerogenic, conformationally altered monomeric serpin, 'ProtoDimer' represents the dissociable dimeric species and 'P' represents the polymeric species.

Here, polymerisation appears to involve the sequential addition of monomers to form a chain in an essentially one-way process. The polymerisation of α_1 -antichymotrypsin, however, appears to be more

complicated, in that it has been shown to be nucleation dependent (86, 87). Nucleation dependent aggregation is typically characterised by the rate limiting and dynamic formation of high-energy aggregation intermediates prior to the consolidation of these species as stable aggregates. These aggregation intermediates are generally thought to be small oligomers, however recent studies have demonstrated protein aggregation systems in which the nucleus is monomeric (88). Currently, the precise nature of the α_1 -antichymotrypsin nucleus is not known.

7.1. The polymerogenic intermediate (M^*)

Elucidation of the high-resolution structure of the monomeric serpin polymerogenic precursor (M^*) represents an important hurdle in the understanding of serpin polymerisation and is essential for the rational design of therapeutics to block this process. However, structural characterisation of such intermediate species is inherently challenging as aggregation processes typically involve transient, heterogeneous ensembles of monomeric and oligomeric species.

Nonetheless, a number of studies have focused on the structural characterisation of the partially folded serpin conformation that gives rise to polymerisation. Evidence suggests that the polymerogenic precursor shares structural similarities with the serpin folding intermediate, as both are thought to populate in low concentrations of denaturant (14, 15, 89, 90). In the case of the Z variant of α_1 -antitrypsin, the mutation within the breach region appears to retard the folding of the molecule at the intermediate state (76). Essentially this increases both the concentration and the lifetime of the intermediate species and results in polymerisation, supporting the notion that the folding and polymerogenic intermediates are structurally related.

The binding of the fluorescent dye, 4, 4'-dianilino-1, 1'-binaphthyl-5, 5'-disulfonic acid (bis-ANS), to the intermediate species demonstrates the exposure of hydrophobic pockets within the serpin structure (77, 85),

and this has previously been attributed to the separation of strands three and five of the A β -sheet (77, 85). In support of this notion, destabilisation of the A β -sheet by mutations within the shutter region such as the M_{malton} and Siiyama (Ser⁵³-Phe) variants of α_1 -antitrypsin leads to spontaneous polymerisation and disease (64, 91). Further support for disruption in the A β -sheet of the polymerogenic precursor is provided by elegant work in the laboratory of Robin Carrell. Here, protonation of the conserved His³³⁴ in antithrombin under acidic conditions, or substitution at this position in α_1 -antitrypsin enhances the rates of polymerisation (81). The rationale for this observation has His³³⁴ at the centre of a hydrogen bonding network that maintains the closure of strands 3A and 5A in the shutter region of the serpin.

A β -sheet disruption has also been associated with the partial insertion of the RCL. The strongest evidence to support this notion comes from the delta structure of α_1 -antichymotrypsin. Furthermore, disruption of the breach region of the A β -sheet in the naturally occurring Z deficiency variant of α_1 -antitrypsin has been attributed to the partial insertion of the RCL (92). In the study of Mahadeva *et al.*, an exogenous 6-mer peptide anneals to the lower portion of the A β -sheet in preference to the insertion of a full length 12-mer peptide (92) representing the whole length of s4A. Presumably, 12-mer peptide insertion is blocked by the presence of RCL residues at the top of the A β -sheet. Additional support for partial insertion of the RCL in the polymerogenic intermediate comes from studies illustrating that the proximal hinge residues of the RCL in Z α_1 -antitrypsin have enhanced resistance to proteolytic attack (93). Moreover, the annealing of short peptides to the upper part of the A β -sheet of antithrombin promotes polymerisation (94) and variants of C1 inhibitor that allow for partial insertion of the RCL, spontaneously form multimers (95). These data strongly indicate that disruption of the breach region by partial insertion of the RCL is crucial to the initiation of polymerisation.

Disruption within the top portion of the A β -sheet has also been associated with the movement of the first strand of the C β -sheet. In a study by Chang *et al.*, the annealing of a 6-mer peptide to the top of the A β -sheet of antithrombin initiated a polymerisation process that could be blocked by the addition of a 5-mer peptide with a sequence analogous to s1C (96). Taken together, these data imply that the polymerogenic intermediate has disrupted A and C β -sheets, with partial insertion of the RCL. This structure would be amenable to both loop A- and C-sheet polymerisation mechanisms, with both s2C and the bottom of the A β -sheet available to accept an exogenous RCL.

8. THRIVING UNDER STRESS—THE THERMOPHILIC SERPINS

The recent discovery of serpin homologues in prokaryotes has opened the door to new avenues of research. In particular, the existence of serpins in thermophilic organisms is likely to instigate a revaluation

of our understanding in regards to serpin stability. Serpins have been identified in the bacterium *Thermobifida fusca*, the fresh water bacterium *Thermoanaerobacter tencongensis* and the archaeon *Pyrobaculum aerophilum* which have optimal growth temperatures of 55°C, 75°C and 100°C, respectively (5). Considering that mammalian serpins typically polymerise readily at temperatures over 45°C (61, 81, 86), the thermophilic serpins must have evolved stabilising adaptations to maintain activity in such hot environments. This is antithetical to the notion that inhibitory serpins must maintain a degree of native instability in order to efficiently perform, and creates a paradox in our current rationale.

Research is now focussed on identifying the thermodynamic and structural modifications to accommodate this paradox. The determination of the X-ray structure of the serpin, thermopin, from *T. fusca* highlights two significant abnormalities. The first of these is the deletion of helix-G, and the presence of a four-amino acid C-terminal extension that lies across the top of the A β -sheet (5). Initially it was suspected that this extension may stabilise the serpin by maintaining the A β -sheet in a closed conformation as it makes specific interactions with the breach region. However, deletion of this tail from the structure did little to affect the protein stability, suggesting that it may play a role in folding but not in native stability (5).

9. THE WAY FORWARD

The serpins represent a very intriguing family of proteins from a protein folding perspective. The fact that so many have been identified to be involved in so many essential biochemical pathways, illustrates that they have an evolutionary advantage over other proteinase inhibitors. However, their susceptibility to assuming dysfunctional conformations and their involvement in human disease necessitates a focussed research effort into serpin misfolding and the identification of potent and specific inhibitors of these processes. These problems will likely be addressed by concerted structural studies in order to characterise at high-resolution the precursor or precursors to misfolding. Furthermore, the identification of stabilising structural elements in the thermophilic serpins may provide molecular targets for inhibitor design.

10. ACKNOWLEDGEMENTS

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