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REVIEW

Lisa D. Cabrita · Stephen P. Bottomley

How do proteins avoid becoming too stable? Biophysical studies into metastable proteins

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Abstract The vast majority of theoretical and experimental folding studies have shown that as a protein folds, it attempts to adopt a conformation that occurs at its lowest free energy minimum. However, studies on a small number of proteins have now shown that this is a generality. In this review we discuss recent data on how two proteins, α-lytic protease and α_1-antitrypsin, successfully fold to their metastable native states, whilst avoiding more stable but inactive conformations.

Introduction

The classic protein folding experiments performed by Anfinsen (1973) demonstrated that ribonuclease A could reversibly unfold and fold, revealing that the amino acid sequence functions as a "blueprint" for folding to the native state. These experiments also revealed that a protein can form its native conformation whilst avoiding the myriad of other possible structures which are accessible to the folding polypeptide chain. These "other possible structures" are often stable, non-native conformations that are of increasing biomedical importance, as in vivo protein misfolding is the molecular basis for a number of diseases (Carrell and Gooptu 1998; Dobson 2001; Horwich 2002). Disorders such as cystic fibrosis, emphysema, liver cirrhosis and many neurodegenerative diseases are characterised by the intracellular aggregation of an aberrantly folded protein. In some cases, it is during the folding process that the protein becomes trapped in a non-native conformation that forces it through an alternative folding pathway (Yu et al. 1995). Conversely, many proteins fold correctly but their native conformations are destabilised such that they readily unfold to a non-native species that subsequently aggregates (Devlin et al. 2002). In both scenarios, however, these processes are accelerated by mutations or changes in solution conditions that alter the stability of structures formed as part of the unfolding/folding pathway.

Numerous experimental and theoretical approaches have delineated many of the steps involved in protein folding (Daggett and Fersht 2003) and have led to the proposal of a number of mechanisms such as the framework, hydrophobic collapse and nucleation–condensation models. Recent ultra-rapid kinetic experiments and theoretical studies have converged to suggest that the nucleation model can describe the folding pathway most successfully (Daggett and Fersht 2003). In this model, the transition state ensemble encompasses molecules that exist in distorted forms of the native state, which are dominated by an extensive network of long-range native interactions that stabilise elements of secondary structure. This experimentally derived folding model is also well supported by funnel landscapes and polymer models theory. In all of these models, folding is driven by the energetic attainment of the native state that is thermodynamically favoured, by being the conformation with the lowest Gibbs free energy. Thus, successful protein folding involves a biased downhill search from the highly disordered denatured state to the well-ordered native state. In so doing, the folding protein avoids energetic "potholes" which can result in misfolding. However, for some proteins adopting a conformation that is not at the thermodynamic minimum is necessary for biological function (Baker and Agard 1994; Cunningham et al.1999) . The formation of a metastable native state has been observed for a number of proteins such as influenza hemagglutinin (Bullough et al. 1994) , gp120 and gp41 from HIV (Chan et al. 1997), protein E from flavivirus tick-borne

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L. D. Cabrita · S. P. Bottomley (✉)
Department of Biochemistry and Molecular Biology,
Monash University,
3800 Clayton,
Victoria, Australia
E-mail: steve.bottomley@med.monash.edu.au
Tel.: +61-3-9905-3703
Fax: +61-3-9905-3726

encephalitis virus (Stiasny et al. 2001) and certain heat shock transcription factors (Orosz et al. 1996).

In all these examples there are other conformational states that are more stable than the native state, which raises the question: how do proteins fold to these states whilst avoiding the more thermodynamically favourable conformations? In this review, we will highlight advances in our understanding of how metastable proteins fold using recent data from two of the most characterised members of this family: α-lytic protease (αLP) and α_1-antitrypsin (α_1-AT). In the case of αLP, its native state is considerably less stable than even its unfolded conformation (Sohl et al. 1998). Moreover, in order to fold, αLP requires a pro-region, for in its absence, the protein remains in a folding purgatory—as a "trapped inactive intermediate". Similarly α_1-AT, the archetypal member of the serine protease inhibitor (serpin) superfamily is metastable, and it has the ability to adopt a number of other more stable but inactive and lethal conformations (Whisstock et al. 1998; Bruch et al. 1988).

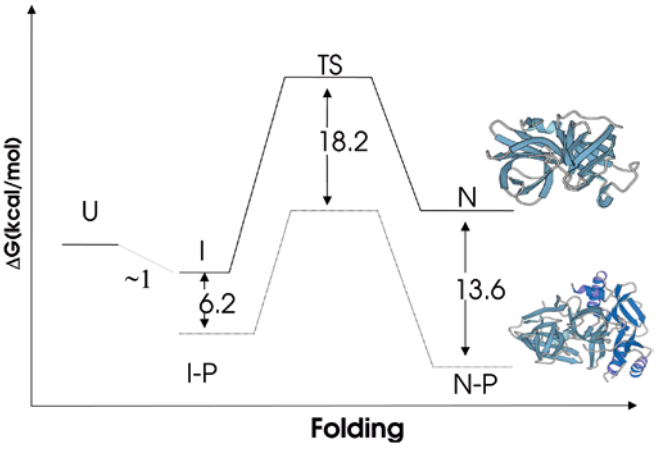

Fig. 1 A free energy diagram of αLP illustrating the relationship between its unfolded (U), intermediate (I) and native (N) states in the absence and presence of the pro (p) peptide. The crystal structures of N (pdb identifier: 2ALP (Fujinaga et al. 1985)) and NP (pdb identifier: 4PRO (Sauter et al. 1998)) are also shown

α-lytic protease

αLP is an extracellular serine protease, originally isolated from the soil bacterium *lysobacter enzymogenes* (Silen et al. 1988). It is synthesised as a 198 amino acid prepropeptide, containing a putative signal sequence directing it to the periplasm. Upon export, the protease self-activates by removing the 32 amino acid pro-region which yields the mature, active protein (Silen et al. 1989).

Evidence for metastability

Native αLP avoids conformational change to its more stable (but inactive) conformations because of high kinetic barriers (Baker et al. 1992). Elegant and extensive work performed by David Agard and colleagues over the past 15 years has shown that when mature αLP is refolded, in the absence of the pro-region, it adopts a molten globule-like intermediate species, termed "I" (Baker et al. 1992). "I" can remain stable for months at physiological pH, and is approximately 1 kcal/mol more stable than the unfolded form of the protein (Sohl et al. 1998). Strikingly, both the unfolded and I forms of αLP are more stable than the native state (N) (Sohl et al. 1998). Detailed kinetic studies determined the rate of folding (I to N; 1.2×10^{-11} s^{-1}) and unfolding (N to U; 1.8×10^{-8} s^{-1}) of the mature protease in the absence of the pro-region. These rates, in which the unfolding rate is faster than the folding rate, show that there is a strong preference towards the I state which is approximately 4 kcal/mol more stable than the native conformation. Cumulatively, these data indicate that the native, active conformation of αLP is not at the minimum of free energy (Fig. 1) (Sohl et al. 1998).

This raises the question of how the active protease conformation is formed if the partially unfolded states are energetically more favourable. The answer to this lies with the pro-region, which plays an important role in the folding of αLP. Addition of the pro-region to I causes rapid formation of the native state, therefore indicating that the pro-region facilitates productive protein folding by reducing the height of the free energy barrier, which blocks folding to the native state (Sohl et al. 1998; Cunningham et al. 2002; Peters et al. 1998). Kinetic measurements showed that the pro-region binds tightly to the folding transition state of the protease and lowers the height of the barrier by over 18 kcal/mol. This increases the rate of αLP folding by a factor of 3×10^9. In addition, the pro-region binds extremely tightly to the native protease and enhances its stability relative to the I–pro complex, therefore favouring folding (Sauter et al. 1998). The native–pro complex is not proteolytically active (Sohl et al. 1997). The pro-region is then removed resulting in formation of the metastable but active protease. Due to the thermodynamics of the system the barrier to unfolding back to I is high enough that the metastable native state is protected from inappropriate conformational change.

Structural basis for metastability

The structural basis for the metastability of αLP has been probed by a number of techniques. Hydrogen–deuterium exchange and proteolysis studies (Jaswal et al. 2002) performed on the native state of αLP demonstrated that it is extremely rigid and that, unlike many proteins, this rigidity is spread throughout the molecule. Titration calorimetry showed that the native state is enthalpically favoured over I by 18 kcal/mol, therefore the stability of I over N must be entropic in origin (Sohl et al. 1998). A possible source for this large entropy difference is thought to arise from the high glycine content of the protein, ≈16% in αLP versus 9% in its close homologue, chymotrypsin, which is not metasta-

ble. A high glycine content increases the number of unfolded conformations available to the protein (due to the conformational freedom conferred by this residue), thereby increasing the entropy of the partially folded states and so their relative stabilities.

αLP is hence an example of a typical metastable protein and the presence of the pro-region allows the folding protein to escape from a stable intermediate conformation. Once the pro-region is removed, the native state is isolated from the more stable partially unfolded forms by high energy barriers.

Serpin structure and function

Serpins are an ever-expanding superfamily of proteins found ubiquitously in animals, plants, fungi and recently in many forms of bacteria (Irving et al. 2000). Their diversity in origin is matched in the variety of their functions. In man alone, serpins are associated with, or regulate: coagulation, fibrinolysis, amyloid plaque formation, metastasis and angiogenesis (Silverman et al. 2001).

Serpins are large (typically 300–400 amino acids) single-domain proteins, which share a remarkable similarity in their tertiary structure consisting of 3 β sheets (A $\rightarrow$ C) and 8–9 α helices (A $\rightarrow$ I) which surround the β sheet scaffold (Fig. 2) (Whisstock et al. 1998). The most distinct feature is the flexible reactive centre loop (RCL), containing the scissile bond (P1–P1′) which mediates a serpin's inhibitory specificity. Some serpins deviate from this basic architecture, such as thermopin (Irving et al. 2003), which lacks the G helix, or MENT and PAI-2 which contain additional structural motifs.

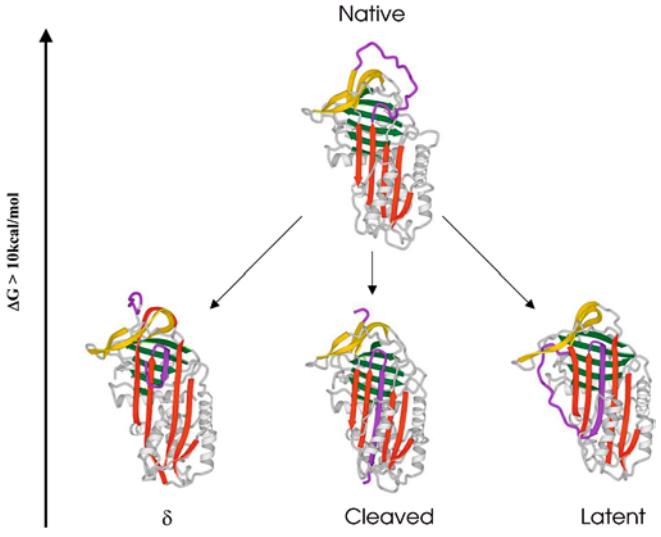

Fig. 2 The different monomeric serpin conformations that are available to the folding polypeptide are shown. Note the insertion of the reactive centre loop (*purple*) into the centre of the A β-sheet (*red*) in the production of alternative, more stable states. Native α_1-AT (pdb identifier: 1QLP (Elliott et al. 1998)), latent structure (pdb identifier: 1ANT (Carrell et al. 1994)), δ structure (pdb identifier: 1QMN (Gooptu et al. 2000))

Unlike the small protease inhibitors such as BPTI, serpins do not merely interact with cognate proteases in a "lock-and-key" fashion, but function via an irreversible "suicide-substrate" mechanism (Fig. 3) (Huntington et al. 2000; Stratikos and Gettins 1999). Upon cleavage by a protease, the serpin undergoes a dramatic conformational change, whereby the RCL is cleaved and inserts into the centre of the A β-sheet, dragging with it the tethered protease. The RCL integrates as an additional strand into the A β-sheet, and the protease is translocated 70 Å to the distal end of the molecule. This structural transition results in a dramatic stabilisation of the serpin and extensive distortion of the protease's tertiary structure, which inactivates its catalytic machinery (Huntington et al. 2000; Tew and Bottomley 2001a; Kaslik et al. 1995).

One primary structure, multiple tertiary conformations

To facilitate the rapid and gross conformational changes required for inhibition, the native serpin structure is metastable. A range of biochemical (Bruch et al. 1988; Mast et al. 1992), biophysical (Powell and Pain 1992; Herve and Ghelis 1990) and structural studies (Loebermann et al. 1984) have shown that upon RCL insertion into the A β-sheet, the molecule becomes significantly more stable. These data have also shown that the RCL can insert either partially, as in the δ structure (Gooptu et al. 2000), or fully, as in the latent (Carrell et al. 1994) or cleaved conformations (Loebermann et al. 1984) (Fig. 2). All these conformations are inactive as protease inhibitors but they are considerably more stable than the active native conformation (Fig. 2). These conformations can be reached from the native state due to small perturbations in solution conditions such as temperature or pH. Alternatively, a number of mutations have been identified, from patients presenting with plasma deficiencies of these serpins, which cause the native protein to rapidly adopt these inactive conformations (Stein and Carrell 1995). In the case of plasminogen activator inhibitor 1, the transition from the native to the latent form does not require changes in environment or the presence of a mutation, as this transition occurs with a half-life of approximately 2 h at 37 °C (Berkenpas et al. 1995). The ability of serpins to readily adopt these RCL inserted forms is important for function, but how the folding polypeptide chain avoids these thermodynamically favourable but inactive conformations is still poorly understood.

Serpin folding and metastability

A limited number of studies have been carried out on the serpin folding mechanism (Powell and Pain 1992; Herve and Ghelis 1990; Cabrita et al. 2002; Pearce et al. 2000; Tew and Bottomley 2001b; James et al. 1999), in part due to the difficulty in studying a pathway which has so

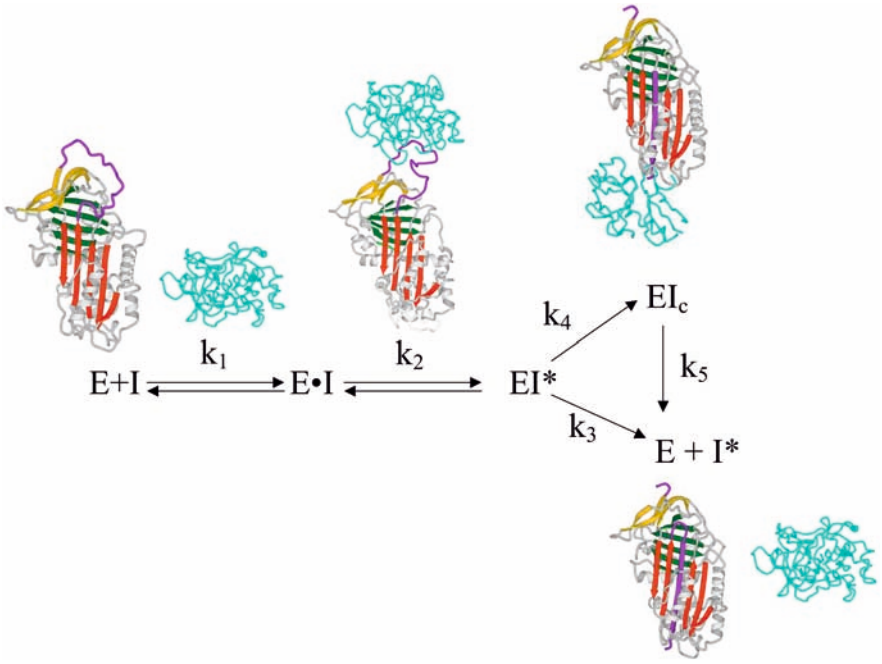

Fig. 3 The kinetic scheme for the serpin–protease interaction. The serpin (I) reacts with a serine protease (E), forming the Michaelis complex (EI) (pdb identifier: (Ye et al. 2001)). EI then undergoes a conformational change through various intermediate species to form the stable serpin enzyme complex (EI_c) (pdb identifier: 1EZX (Huntington et al. 2000)). EI_c eventually breaks down to form E + I* (cleaved serpin). Alternatively, the protease can cleave the serpin and not form the covalent complex; this yields the products E + I*

many competing (and thermodynamically attractive) side reactions. These studies, however, all reveal that the folding and unfolding reaction of many serpins (α_1-AT (James et al. 1999), antichymotrypsin (ACT) (Pearce et al. 2000), maspin (Liu et al. 1999), thermopin (Irving et al. 2003), antithrombin (Fish et al. 1985), PAI-1 (Wang et al. 1996)) involves at least one intermediate ensemble. This intermediate species has considerable secondary structure consisting of a well-formed B β-sheet along with partially formed A and C β-sheets. Detailed information into its structure is limited; however, investigations into the denatured state show residual structure around the B/C barrel (which refers to the region formed between the B and C β-sheets) in both α_1-AT and ACT (Tew and Bottomley 2001b). In addition, it was recently shown that if the interactions in the B/C barrel are disrupted, both the intermediate ensemble and the native state become destabilised, such that α_1-AT becomes kinetically unstable and converts to the more stable latent form (Im et al. 2002). Random mutagenesis has identified other residues that are important for maintaining the serpin in a metastable state (Im et al. 2000, 1999; Kwon et al. 1994; Lee C et al. 2000, Lee KN et al.1996). Not surprisingly, there appears to be no one single region or regulatory element responsible, although many of the residues identified lie on the path of RCL insertion, in particular side chains that link the F helix to the A sheet (Fig. 2) (Im and Yu 2000).

Recently, we proposed that productive non-native interactions play a role during the folding of the molecule (Cabrita et al. 2002; Tew and Bottomley 2001b). Such interactions are generally proposed to slow down, and therefore be detrimental to, the folding reaction. In the case of α_1-AT, we suggested that these interactions would be protective, in that they restrict the conformational space available to the folding polypeptide chain and prevent adoption of these more stable structures. Evidence for 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 (Cabrita et al. 2002; Tew and Bottomley 2001b). These two areas are intimately linked and involved in RCL insertion. Our data in conjunction with the δ-structure (Gooptu et al. 2000) suggest that the folding intermediate of α_1-AT has a partially inserted RCL, which is prevented from full insertion due to contacts between the F-helix and the underlying A β-sheet. Further studies are underway to test the validity of this model.

Inappropriate conformational change

Whilst the metastability of α_1-AT and other inhibitory serpins is essential to their biological function, it renders the molecule susceptible to inappropriate conformational change. For example, the mutation Glu342 to Lys (also known as the Z mutation) is present in approximately 1 in 2,000 people of northern European descent. Homozygotes for this mutation are predisposed to both liver disease and emphysema (Lomas et al. 1992). As a result of the Z mutation, the protein does not fold to its native conformation, but forms a highly stable polymeric structure, which has a tendency to aggregate within its site of synthesis, the hepatocyte. These polymers are formed by a mechanism known as "loop–sheet polymerisation" (Sivasothy et al. 2000). It involves the RCL of a donor molecule inserting into the A β sheet of an acceptor, forming a long chain, reminiscent of beads on a string when viewed using electron microscopy (Lomas et al. 1992). The Z mutation results in the loss of a crucial salt bridge, which leads to inappropriate opening of the

A sheet and hence allows the polymerisation event to take place. A small proportion (15%) of native monomeric Z α_1-AT does get secreted into the circulation. This material is active, however the mutation destabilises the structure such that it is in a non-native conformation (Mahadeva et al. 2002; Lomas et al. 1993) and polymerises readily at physiological temperatures.

Mechanism of serpin polymerisation

Serpin polymerisation occurs through a three-step mechanism. The first step is the accumulation of an intermediate species, termed M*, that is highly polymerogenic (Devlin et al. 2002; Dafforn et al. 1999). Recent data have provided insight into the conformation of M*. Compared to the native state (M), M* displays several distinct characteristics: it is able to bind specific peptides (Mahadeva et al. 2002) and hydrophobic fluorescent dyes (Dafforn et al. 1999; James and Bottomley 1998), is intrinsically hyperfluorescent (Pearce et al. 2000), and has a disrupted F-helix (Cabrita et al. 2002). These data all indicate that the A β-sheet is expanded (peptide/dye binding), the top of the A sheet is occupied by some RCL residues (hyperfluorescence) and the F-helix which lies across the face of the A sheet is significantly distorted (biophysical data). This conformation is therefore poised to accept the RCL residues of another molecule.

Sedimentation velocity experiments have now shown that from M*, a dimer species accumulates in a reversible reaction (Devlin et al. 2002). This species, which we termed the protodimer, is spectroscopically indistinguishable from M*. The protodimers then self-associate to form the long-chain polymers. These data therefore indicate that polymerisation occurs not from the addition of monomers to the end of a growing chain, but through addition of dimers and higher-order oligomers.

A serpin that attempts to break the rules

Inappropriate conformational changes in serpins can be initiated through exposure to partially denaturing conditions such as elevated temperatures. The recent discovery of serpins in thermophilic organisms represents a new paradox in the field—how does a serpin at high temperatures maintain its structure without undergoing a conformational change? From an evolutionary perspective, these serpins appear to share many highly conserved residues present in other serpins (Irving et al. 2002). The first thermophilic serpin to be characterised was named ''thermopin'' (Irving et al. 2003). It showed enhanced thermostability and was considerably more resistant to polymerisation than its mesophilic counterpart (α_1-AT). It is intriguing that in spite of its enhanced stability, it is able to retain inhibitory activity.

The X-ray crystal structure of the cleaved variant of thermopin revealed the presence of shorter loops and a more compact molecule. However, the most likely source of enhanced stability comes from a unique C-terminal ''tail''. Residues in this tail form extensive interactions with Glu342 (Z residue) effectively ''hyperstabilising'' this area which is critical for serpin stability. It appears, therefore, that through natural protein engineering a serpin has found a way of increasing its thermostability and maintaining its inhibitory activity.

Conclusion

The adoption of metastable conformations is invariably linked to biological function; evolution has carefully chosen these precarious structures in order to fulfil a range of biological phenomena. It appears, therefore, that unlike αLP which relies on an additional piece of structure to bypass the ''kinetic trap'' of folding, serpins make use of many conserved interactions which are distributed around the molecule to stay in the kinetic trap until a protease comes along and initiates further thermodynamically favourable conformational changes.

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