Serpins are metastable proteinase inhibitors. Serpin metastability drives both a large conformational change that is utilized during proteinase inhibition and confers an inherent structural flexibility that renders serpins susceptible to aggregation under certain conditions. These include point mutations (the basis of a number of important human genetic diseases), small changes in pH, and an increase in temperature. Many studies of serpins from mesophilic organisms have highlighted an inverse relationship: mutations that confer a marked increase in serpin stability compromise inhibitory activity. Here we present the first biophysical characterization of a metastable serpin from a hyperthermophilic organism. Aeropin, from the archaeon Pyrobaculum aerophilum, is both highly stable and an efficient proteinase inhibitor. We also demonstrate that because of high kinetic barriers, aeropin does not readily form the partially unfolded precursor to serpin aggregation. We conclude that stability and activity are not mutually exclusive properties in the context of the serpin fold, and propose that the increased stability of aeropin is caused by an unfolding pathway that minimizes the formation of an aggregation-prone intermediate ensemble, thereby enabling aeropin to bypass the misfolding fate observed with other serpins.

Members of the serine proteinase inhibitor (serpin) superfamily are predominantly proteinase inhibitors whose native conformation is metastable (1–3). They, therefore, represent an exception to the Anfinsen rule that all proteins fold to their native state. They are trapped in a highly stable, covalent serpin-enzyme complex when the serpin native state is perturbed by mutation or small changes in pH. Serpins thus have relevance to important human pathologies. Increased (16). Physiologically, this is manifested in a range of loss-of-function diseases such as emphysema, liver cirrhosis, thrombosis, and dementia (17).

An understanding of the determinants of metastability in serpins thus has relevance to important human pathologies. Extensive biochemical and biophysical studies have demonstrated that residues important to maintaining and controlling metastability are distributed throughout the molecule (18, 19). The introduction of mutations that destabilize the native state of a serpin increase its propensity to polymerize (20, 21). Stabilizing mutations produce more diverse effects; some only moderately enhance stability and retain serpin function (19, 21), while others yield increased stability at the expense of inhibitory specificity (Fig. 1). During proteinase inhibition, the RCL is cleaved by the proteinase and becomes incorporated as a middle strand of the A-sheet, a process referred to as the stressed → relaxed (S to R) transition. As a result, the proteinase is translocated to the opposite pole of the serpin, and the two are trapped in a highly stable, covalent serpin-enzyme complex (7). The serpin thus surrenders its metastability in favor of adopting a more stable conformation that complements proteinase inhibition.

The energetic basis of this inhibitory mechanism is that incorporation of the RCL as a strand into the A-sheet is thermodynamically favorable (8). However, serpins can also adopt another thermodynamically favorable state by inserting their RCL into an adjacent serpin molecule. Propagation of the resulting loop-sheet linkages results in the formation of long-chain A-sheet polymers (9–12). Loop A-sheet polymers occur when the serpin native state is perturbed by mutation or small changes in pH and temperature. Such perturbation results in the partial unfolding of the serpin and the formation of a non-native ensemble of structures, which then readily self-associate (13–15). It has been shown that by increasing the stability of this intermediate ensemble or the rate of its formation, the likelihood of polymerization occurring is also significantly increased (16). Physiologically, this is manifested in a range of loss-function diseases such as emphysema, liver cirrhosis, thrombosis, and dementia (17).

An understanding of the determinants of metastability in serpins thus has relevance to important human pathologies. Extensive biochemical and biophysical studies have demonstrated that residues important to maintaining and controlling metastability are distributed throughout the molecule (18, 19). The introduction of mutations that destabilize the native state of a serpin increase its propensity to polymerize (20, 21). Stabilizing mutations produce more diverse effects; some only moderately enhance stability and retain serpin function (19, 21), while others yield increased stability at the expense of inhibi-
tion, presumably because the flexibility that is required for inhibition is no longer present (22). These studies, which have been restricted to serpins from mesophilic organisms, highlight a serpin metastability paradigm that reflects an exquisite balance between structural stability and flexibility to achieve biological function. This structure/function compromise seemingly precludes the possibility of an active serpin with a greatly increased native state stability. However, this position is at odds with the presence of serpin sequences in the genomes of several moderate to highly thermophilic bacteria and archaea (23–26).

This article reports the first characterization of a serpin from a hyperthermophilic organism: the crenarchaeon, *Pyrococcus aerophilum*, which thrives in deep thermal vents at temperatures exceeding 100 °C (23, 27, 28). We demonstrate that this serpin, aeropin, possesses a remarkable resistance to chemical and thermal denaturation with respect to all other characterized serpins, while paradoxically remaining an effective proteinase inhibitor. More intriguingly, the data reveal a potential mechanism by which aeropin and other hyperthermophilic proteins are able to avoid aggregation.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification**—Genomic DNA was extracted from *P. aerophilum* (DSMZ). The following sense and antisense oligonucleotides were used to amplify the aeropin gene (GenBankTM ID 1464742): (5′–3′) ctaattggatccttgcgcttacgggcttgaa and (5′–3′) gcccgggtcgacttaagtcggcgtaaagcc. The PCR fragment was cloned into pRSETC (Invitrogen).

Mutations, created using the QuickChange mutagenesis kit (Stratagene), were introduced to remove the disulfide bonds. The following oligonucleotide sequences were used to introduce the mutations, the mutation is underlined.

- C102S (forward): gcggagaacccccgctcccggcg and C102S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc; C136S (forward): aataagttgagaagcatcggggccgag and C136S (reverse): cgcggggagcacgctggggtcttcgcc.

For expression and purification, the expression vectors containing aeropin and the disulfide mutants were transformed into Rosetta-Gami plysS *Escherichia coli* (Novagen). Expression of aeropin and the disulfide mutants was induced at an *A*$_{600}$ nm of −0.6 and continued for 5 h at 37 °C. The protein was purified using both Ni-NTA affinity and size exclusion (Superdex 200 16/60) chromatography. It was found that the concentration of NaCl had to be maintained at 200 mM to ensure the protein remained soluble, a characteristic that has been seen for other proteins derived from *P. aerophilum* (29, 30). The most pure fractions as determined by SDS-PAGE were concentrated and stored at −80 °C. Cleaved aeropin was prepared as previously described except following digestion, the protein was incubated at 100 °C for 60 min to remove residual uncleaved material (24).

**Enzyme Kinetics**—All kinetic measurements were performed in 50 mm Tris, 200 mm NaCl, 10 mm CaCl$_2$, 0.1% (w/v) PEG 8000, pH 7.8 as previously described (31).

**Spectroscopic Methods**—Stock solutions of guanidine hydrochloride (GdnHCl) were prepared in 20 mm NaPO$_4$, 200 mm NaCl, pH 7.8, and the concentration was confirmed using refractive index measurements (32). Equilibrium unfolding and refolding curves were performed as previously described (33).

**Heat-induced Polymerization**—Polymerization was followed using non-denaturing PAGE analysis (13). The protein was at a concentration of 0.5 mg/ml in 20 mm NaPO$_4$, 200 mm NaCl pH 7.8 for 15 min and analyzed between 20 and 100 °C.

**Size-exclusion Chromatography Analysis**—Samples of aeropin at a concentration of 0.2 mg/ml were incubated in 0–6 m GdnHCl overnight. The samples were then separated using a Superose 12 10/30 column in 20 mm NaPO$_4$, 200 mm NaCl, pH 7.8.

**RESULTS**

**Aeropin Is a Serine Proteinase Inhibitor**—The aeropin (GenBankTM ID 1464742) gene was cloned into an *E. coli* expression system, and the protein expressed and purified to homogeneity with its integrity confirmed by mass spectrometry (data not shown). Sequence alignment of aeropin with other serpins (Fig. 1B) demonstrates that it possesses most of the highly conserved residues of the serpin superfamily (34), suggesting that it conforms to the common serpin structure, as shown in the molecular model in Fig. 1A. Aeropin most likely lacks the D-helix, an element also absent from the structure of crmA (35), based on a significantly truncated region containing two proline residues. The sequence alignment also indicates that aeropin is likely to be a proteinase inhibitor due to the presence of an inhibitory hinge motif (23) (Fig. 1B). The primary determinant of the inhibitory specificity of a serpin is typically a dipeptide sequence around 14 residues C-terminal to the hinge region motif (36), known as the P$_1$–P$_1^{′}$ site (37). However, the cognate proteinase inhibited by aeropin is not known, and the aeropin P1–P1$^{′}$ residues (V–C) did not immediately propose a model target proteinase.

Subsequently, aeropin was found to be a potent chymotrypsin inhibitor with a stoichiometry of inhibition of 1.2 molecules of inhibitor required to inhibit 1 molecule of proteinase at 37 °C (Fig. 2 and Table 1). SDS-PAGE analysis under reducing conditions confirmed that the proteinase and inhibitor were locked in a covalent inhibitory complex (Fig. 2B). Furthermore, the association rate constant (k$_{ass}$) at 37 °C was found to be 1.8 × 10$^5$ M$^{-1}$ s$^{-1}$ (Table 1). Cumulatively, these data indicate that aeropin is a highly efficient proteinase inhibitor, that employs the serpin mechanism of inhibition, and hence aeropin is capable of undergoing the $S \rightarrow R$ transition.

**Aeropin Is Thermostable**—The thermal stability of aeropin was measured by observing changes in secondary structure using far-UV circular dichroism (CD), during an increase in temperature. Aeropin, in non-denaturing buffer, showed no appreciable change in signal over the entire temperature range (Fig. 3A), which suggested that its melting temperature ($T_m$) exceeds 95 °C. The thermal melt was repeated in the presence of 3.5 m GdnHCl, which was shown in preliminary experiments to preserve the native conformation of aeropin. The presence of denaturant resulted in an observable thermal unfolding transition from the baseline, with a $T_m$ of 65 °C (Fig. 3A and Table 1).
It is interesting to note that under the same denaturing conditions the archetypal serpin antitrypsin (\( \alpha_1 \)-AT), used as a benchmark for comparison throughout this study, is >80% unfolded (33). These data demonstrate that aeropin possesses a markedly enhanced thermostability with respect to all other inhibitory serpins studied to date. A hallmark of serpin metastability is the transition to a more stable state upon proteolytic cleavage of the RCL. To determine whether aeropin was indeed metastable, the thermal stability of the chymotrypsin-cleaved form of aeropin was analyzed. As can be seen in Fig. 3A, in the presence of 3.5 M GdnHCl cleaved aeropin does not unfold even at temperatures in excess of 90 °C, indicating that RCL cleavage leads to an increase in stability.

The thermostability profile of aeropin was also monitored using non-denaturing PAGE analysis (Fig. 3B). Consistent with the thermal melt data, aeropin remained monomeric until >90 °C after which there was a disappearance of aeropin protein from the running gel. Such a disappearance presumably

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**FIGURE 1. Ribbon diagram of aeropin.** A, molecular model of aeropin, constructed using MODELLER (62) based upon ten native structures (1by7, 1imv, 1ova, 1sek, 1t1f, 1dvm, 1jmj, 1qlp, 1sng, and 1yxa); the RCL is based on 1dvm, whose conformation was found to be most compatible with the presence of the RCL disulfide. Major secondary structure elements are labeled, and the position of disulfides shown. The figure was prepared using PyMOL. B, structure-based sequence alignment of the serpins listed above, and aeropin, was performed using MUSTANG (63) and ClustalW (64); a subset of these is shown with consensus secondary structure and antitrypsin numbering. Shading indicates residues that are nonpolar (light), polar (medium), and identical to highly conserved positions of the serpin family (white-on-black).
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reflects aggregation and not polymerization as no laddering consistent with serpin polymerization was observed. This characteristic has been seen previously with other serpins (38).

Aeropin Is Resistant to Changes in pH—We next determined whether the high stability of aeropin was maintained over a broad pH range. Thermal denaturation, monitored by far-UV CD, was undertaken at different pH values were carried out, and the midpoint of denaturation determined for both aeropin and α1-AT (Table 2). Aeropin proved to be remarkably stable to changes in pH and did not display an unfolding transition until the pH was lowered to around 4. In contrast α1-AT displayed a decreased $T_m$ value below pH 6 and was completely unfolded at pH 3.5 (39).

Equilibrium Unfolding—Unfolding studies of aeropin were conducted using both far-UV CD and intrinsic tryptophan fluorescence. Refolding was also attempted; however, in common with many other serpins aeropin did not refold to 100% active material. As a result, a full thermodynamic analysis could not be performed on the data; hence, transition midpoints on the unfolding curve were used as the measure of protein stability. No significant change in the secondary structure occurred below 3.5 M GdnHCl, at which point a single transition occurred with a midpoint of 4.3 M GdnHCl, and complete unfolding was observed in 6.5 M GdnHCl (Fig. 4A). The unfolding failed to reveal the plateau region, associated with a highly populated intermediate ensemble, that is characteristic of all other serpins studied to date (24, 33, 40–44). Intrinsic tryptophan fluorescence was also used to monitor unfolding. As observed with far-UV CD, the unfolding of aeropin showed a native baseline to 3.5 M GdnHCl (Fig. 4B) after which there was a single highly co-operative transition to the unfolded state centered around a midpoint of 4.0 M GdnHCl. While individually the fluorescence and CD unfolding curves suggest a two-state unfolding mechanism, the observation that they are not superimposable indicates that aeropin follows a three-state model with an unfolding intermediate. The presence of an unfolding intermediate has been previously characterized in several serpins by their ability to bind the hydrophobic dye, bis-ANS (33, 42). To test whether the intermediate ensemble adopted by aeropin possessed such a property, we incubated denatured aeropin in the presence of bis-ANS, and recorded the resulting spectra. However, there was no detectable change in the fluorescence signal (data not shown), indicating that the intermediate ensemble possesses different characteristics to intermediates previously studied.

Equilibrium Unfolding Monitored by Size-exclusion Chromatography—It has been previously reported that incubation at high protein concentration and low denaturant concentrations causes α1-AT to polymerize (45). To assess whether aeropin was subject to aggregation during the unfolding process, aeropin (0.2 mg/ml) was incubated in increasing GdnHCl

TABLE 1
Inhibitory and stability characteristics of aeropin and its variants

| SI | $k_{app}$ | $k_{on}$ | $T_m$ (in 3.5 M GdnHCl) | °C |
|----|----------|----------|-------------------------|----|
| Aeropin | 1.2 ± 0.1 | $1.5 \times 10^3$ ± 0.2 | $1.8 \times 10^5$ | 65 ± 0.5 |
| ΔCysRCL | >200 | ND* | — | — |
| ΔCysAE | 1.2 ± 0.1 | $1.0 \times 10^3$ ± 0.2 | $1.2 \times 10^5$ | 56 ± 0.5 |
| ΔCys | >200 | ND | — | 55 ± 0.5 |

* ND, not determined.

FIGURE 3. Thermal stability of aeropin. A, thermostability of aeropin (solid line) and cleaved aeropin (dashed line) were measured by following the change in CD at 222 nm in the presence of 3.5 M GdnHCl. The inset shows the unfolding of aeropin in the absence of denaturant. B, thermostability of aeropin, cleaved aeropin, and α1-AT assessed using non-denaturing PAGE.

TABLE 2
The effect of pH upon aeropin stability

| pH | Aeropin $T_m$ | Antitrypsin $T_m$ |
|----|--------------|------------------|
| 3.5 | 81 | Unfolded |
| 4.0 | 93 | 39 |
| 5.0 | >95 | 57 |
| 6.0 | >95 | 64 |
| 7.0 | >95 | 62 |
| 8.0 | >95 | 59 |
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FIGURE 4. Equilibrium unfolding monitored by far-UV CD, intrinsic fluorescence and size-exclusion chromatography. Equilibrium unfolding of aeropin was followed by far-UV CD (A) and intrinsic tryptophan fluorescence (B). All data in A and B were normalized to unfolded fraction. C, elution profile of aeropin (0.2 mg/ml) in increasing concentrations of GdnHCl from a Superose 12 10/30 column.

centration for the two rates k₁ (N → I, solid line) and k₂ (I → U, dashed line). Inset, representative trace of aeropin unfolded in 6 M GdnHCl.

Kinetics of Aeropin Unfolding—As partial unfolding of the native serpin state is the first step in polymerization (13, 46), we determined the unfolding rate of aeropin using tryptophan fluorescence. Fig. 5 is a representation of the unfolding of aeropin in 6 M GdnHCl. It is clear that aeropin unfolds over a very long timescale (order of minutes) and a kinetic analysis indicates the presence of two kinetic phases. A kinetic unfolding profile for aeropin over a range of denaturant concentrations was determined and the two rates of unfolding (in water) were calculated by extrapolation as previously described (32). The N → I rate was found to be 2.41 × 10⁻⁷ s⁻¹, while the I → U transition was ~200-fold faster at 4.31 × 10⁻⁷ s⁻¹. These rates were found to be concentration-independent, indicating that aggregation was not occurring during the unfolding reaction.

Investigating the Role of the Disulfides in the High Stability of Aeropin—Our current data have shown that aeropin has both high stability and inhibitory activity. To identify a possible structural basis for this property, an initial homology model of aeropin was constructed based on the structure with the highest sequence identity (31%) antithrombin (23). As noted previously the model suggested the possible presence of two disulfide bonds that could play a significant role in both its activity and stability (Fig. 1A) (23). Using antitrypsin numbering, these cysteines are Cys¹⁰², Cys¹³⁶, Cys²²⁴, and Cys³⁵⁹. We predicted that the Cys¹⁰²–Cys¹³⁶ pair would form a disulfide between the E-helix and the region preceding s2A, while Cys²²⁴–Cys³⁵⁹ would form a disulfide between the C sheet and the P1′ of the RCL.

When aeropin is analyzed by SDS-PAGE in the absence and presence of dithiothreitol, there is a significant band shift indicating the presence of disulfide bonds which restrict the SDS-induced unfolding of aeropin (Fig. 6). The presence of two bonds was confirmed first by sulphydryl titration using the Ellman assay (47) in which aeropin was reacted with DTNB, and the release of the NTB anion was followed spectrophotometrically. In a non-reducing environment, there was no appreciable change in absorbance, indicating an absence of free thiols. When aeropin was incubated under reducing conditions, a concentration of 4 thiols per aeropin molecule was determined, indicating that there are 2 disulfide bonds present in the native molecule.

Three mutants were constructed in which the cysteines were replaced with serines to probe the role of the disulfides in aeropin stability and inhibitory properties. ΔCysRCL represents the variant with substitutions at positions Cys²²⁴ and Cys³⁵⁹, ΔCysAE has substitutions at Cys¹⁰² and Cys¹³⁶, and ΔCys has all the cysteines replaced by serine (Fig. 6). The absence of free thiols in the double mutants determined using the tiration assay detailed above, confirmed the disulfide bond pattern predicted from the homology model. The inhibitory activity of the variants was tested against chymotrypsin. As shown in Table 1, the inhibitory activity of both ΔCysRCL and ΔCys was significantly compromised with SI values in excess of 200, whereas ΔCysAE, had an SI identical to the wild-type protein (SI = 1.2).

The thermostability of the disulfide variants was assessed using far-UV CD (in the presence of 3.5 M GdnHCl). ΔCys and ΔCysAE were found to have the greatest difference in stability relative to wild-type, with Tₘ values of 55 °C and 56 °C, respectively (Table 1), while ΔCysRCL had a Tₘ of 61 °C, which is
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**DISCUSSION**

This report presents the first biophysical characterization of a serpin from an extremophile. Aeropin was found to be metastable and an active, efficient proteinase inhibitor, consistent with the serpin mechanism of function. Moreover, it possesses a remarkable tolerance to both chemical and thermal denaturation, and did not polymerize in either low denaturant concentrations, with heating or changes in pH as observed with other serpins (13, 33, 49). Partial unfolding of α₁-AT and other serpins has been shown to be a critical first step toward aggregation (13, 33, 39). All of the serpins studied to date unfold via a three-state mechanism, which involves the adoption of an unfolded intermediate species and subsequent polymerization at physiological temperatures (16).

How does aeropin avoid the unfolding trap that leads to polymerization and aggregation? The data presented here suggest two reasons: (1) the intermediate ensemble is different in character to the polymerogenic form observed with mesophilic serpins; and (2) aeropin has evolved a kinetic mechanism to minimize the population of its unfolding intermediate.

We have shown that aeropin follows a three-state unfolding profile, however, unlike the intermediate of α₁-AT and other serpins (13, 33, 42, 52, 53), the aeropin intermediate ensemble was unable to bind bis-ANS and did not show a 20% loss in secondary structure, suggesting that it is in a different conformation.

In addition to the altered intermediate conformation aeropin displays a high kinetic stability with a transition half-life (N → I) of ~80,000 h, that separates the aeropin native state and its intermediate ensemble. The equivalent N → I phase for α₁-AT is difficult to measure because of its speed; however, the presence of 0.4 m sodium sulfate increases its half-life to 0.92 s. A stabilized native state significantly decreases the likelihood of partial unfolding and therefore aggregation. This is consistent with experimental studies on the effects of the stabilizing F51L mutation on the Z variant of α₁-AT, which rescued the folding defect from Z α₁-AT by decreasing the rate of unfolding (54).

Other proteins achieve a modified intermediate ensemble through modification of the stability of the native state. For example, in thymidylate synthase, a single point mutation stabilized the native structure and resulted in the accumulation of the intermediate over a narrower denaturant concentration range than the wild-type protein. The implication was that the protein was less likely to aggregate (55). Similarly, stabilization of the native structure of ricin resulted in the loss of its aggregation-prone intermediate (56). Moreover, suppressor mutations have also been demonstrated to destabilize the aggregation-prone intermediate in a tail spike protein (57, 58).

While fine details underlying the stability of the aeropin native state await an experimental structure, the biophysical confirmation of the presence of two disulfide bonds suggested these as likely stabilizing factors against unfolding and polymerization. Indeed, this was found to be the case for the covalent linkage between helix E and the region preceding strand 2A. However, removal of this linking yielded a mutant that remained relatively stable. Remarkably, removal of the disulfide between the C-sheet and the RCL did not significantly affect

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**FIGURE 6.** The role of aeropin disulfides. A, SDS-PAGE analysis of aeropin in the absence and presence of reducing agent. B, thermostability of aeropin and the disulfide variants was assessed using non-denaturing PAGE.

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1. L. D. Cabrita and S. P. Bottomley, unpublished observations.

**References**

1. Cabrita, L. D. and Bottomley, S. P. (unpublished observations).
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thermal stability. Hence, other stabilizing factors must be present. A sequence comparison with ten mesophilic serpins (listed in Fig. 1B) revealed that aeropin has a lower proportion of uncharged polar residues, and hence a higher ratio of charged to uncharged polar side chains (1.43), than the mesophilic counterparts (1.07 ± 0.12). This has been noted for other thermophilic proteins (59), and may enhance stability through the formation of salt bridges (60, 61). Aeropin also shows a significantly higher number of glycine, alanine, and valine side chains (p < 0.01). Interestingly, aeropin also contains significantly more tyrosine residues than the mesophilic serpins, many of which are predicted from the model to be predominantly surface-inaccessible; the ability of these residues to simultaneously form hydrogen bonds, hydrophobic and cation-pi interactions may further stabilize the protein.

The metastability paradigm describes a delicate balance between structural flexibility and inhibitory activity, where the maintenance of the former property is vital for effective inhibition. Aeropin highlights that it is possible for a serpin to adopt a high native state stability and an atypical intermediate. As a result, conformational changes required for biological activity are retained while unfolding events that may promote misfolding are less likely to occur. Presumably, the degree of native state stability consistent with serpin inhibitory activity stability depends on temperature, and in the case of an organism existing in extreme heated environments, compensating changes to the polymorphic intermediate have also been necessary. This may thus explain the intolerance that many mesophilic serpins have shown to an increase in stability. Moreover, as aeropin retains an unfolding profile that in character, if not degree, is seen with other serpins, it has reinforced the importance of the intermediate ensemble and the relationship between unfolding, stability, and activity.

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