Serpin Polymerization Is Prevented by a Hydrogen Bond Network That Is Centered on His-334 and Stabilized by Glycerol*

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Polymerization of serpins commonly results from mutations in the shutter region underlying the bifurcation of strands 3 and 5 of the A-sheet, with entry beyond this point being barred by a H-bond network centered on His-334. Exposure of this histidine in antithrombin, which has a partially opened sheet, allows polymerization and peptide insertion to occur at pH 6 or less when His-334 will be predictably protonated with disruption of the H-bond network. Similarly, thermal stability of antithrombin is pH-dependent with a single unfolding transition at pH 6, but there is no such transition when His-334 is buried by a fully closed A-sheet in heparin-complexed antithrombin or in α1-antitrypsin. Replacement of His-334 in α1-antitrypsin by a serine or alanine at pH 7.4 results in the same polymerization and loop- peptide acceptance observed with antithrombin at low pH. The critical role of His-334 and the re-formation of its H-bond network by the conserved P8 threonine, on the full insertion of strand 4, are relevant for the design of therapeutic blocking agents. This is highlighted here by the crystallographic demonstration that glycerol, which at high concentrations blocks polymerization, can replace the P8 threonine and re-form the disrupted H-bond network with His-334.

The serpin family of serine protease inhibitors (1) provides a clear example of the way in which dysfunction and disease can result from conformational instability (2, 3). The inhibitory function of the serpins is dependent on a triggered opening of the five-stranded A β-sheet of the molecule to allow the insertion of the cleaved reactive center loop as an additional strand in the center of the sheet (4–6). As a consequence, the serpins are particularly vulnerable to mutations affecting the critical region of the molecule underlying the point of entry of the loop, between strands 3 and 5 of the sheet (7). Mutations in this region (Fig. 1a) allow the aberrant opening of the A-sheet, with the risk of the insertion, into its lower half, of the reactive loop of another molecule to give intermolecular linkage and polymerization of the serpin. Even minor changes in the shutter region of α1-antitrypsin (8) and antichymotrypsin (9) result in their polymerization and intracellular aggregation with consequent lung and liver disease and similarly with C1-inhibitor (10) and antithrombin mutations (11) resulting in angiodysplasia and thrombosis. But the best example of the critical function of this region comes from recent investigations of a novel form of familial neurodegenerative disease due to the aggregation within neurons of a brain-specific serpin, neuroserpin (12). The polymerization and aggregation of neuroserpin results from mutations in its shutter region. Two of these mutations affect residues previously identified in other serpin diseases, Ser-53 and -56 (template numbering (13) is used throughout this paper). The dysfunction accompanying mutations of serines 53 and 56 is readily explicable by the alteration in packing of residues underlying the A-sheet at the point where a sliding movement opens the gap between strands 3 and 5A (2, 7). But a third and novel mutation in neuroserpin (14, 15) has also focused attention on histidine 334 at the point of bifurcation of strands 3 and 5 (Fig. 1, b and c). This conserved histidine is centrally placed in the shutter region and is less directly involved in the critical packing interactions of sheet opening than either serine 53 or 56, but surprisingly the mutation of His-334 results in a much more severe neurodegenerative disease. An explanation for this is likely to be the key H-bond network centered on His-334, which bridges strands 3 and 5 of the A-sheet and notably links His-334 directly to the base of Ser-53 and indirectly to Ser-56 (Fig. 1, b and c).

The potential role of His-334 as a guardian of sheet opening is indicated in the crystallographic structures of two serpins, antithrombin and heparin cofactor II (16, 17). Both of these serpins are exceptional in having, in their native states, a partial opening of the A-sheet with an initial insertion of the reactive loop (to a level of P14, see Fig. 1a). In both, the hydrogen bonding between strands 3 and 5 does not commence until His-334 (at the level of insertion of P8 in cleaved serpins), which appears as the first barrier to further opening of the sheet (Fig. 1b). The consequences of the loss of this barrier and of opening the A-sheet are potentially 2-fold. It can allow the complete insertion of the uncleaved reactive loop of the molecule to give the inactive latent form or it can allow the incorporation of the reactive center loop of another molecule resulting in serpin polymerization. Transition to the latent state takes place as a physiological mechanism in the plasminogen activator inhibitor-1 (18), which exceptionally among the serpins has a glutamine rather than a histidine at 334 (19). Transition to the latent form is also a significant pathological mechanism in the conformationally unstable mutants of antithrombin in which a premature conversion to the latent conformation can result in a catastrophic decrease in antithrombin activity with massive thrombosis (20). More commonly though, with most serpins such as α1-antitrypsin, the opening of the A-sheet predominantly results in the formation of loop-sheet polymers with the insertion of the P7-3 portion of the loop of one molecule into the lower half of the A-sheet of the next molecule (21). Here we show how studies of both types of
transition in antithrombin and α1-antitrypsin confirm the critical function of His-334 as a barrier to premature opening of the A-sheet.

MATERIALS AND METHODS

Materials—Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, and oligonucleotides were synthesized by MWG-Biotech. The expression vector pQE31 and Ercherica coli strain SG13009 (pREP4) were from Qiagen. Isopropyl-β-D-thiogalactopyranoside was from Melford Laboratories Ltd. (Suffolk, England). Kanamycin sulfate was from Roche Molecular Biochemicals. Heparin-Sepharose, HiTrap Q-Sepharose, and HiTrap chelating columns were from Amersham Biosciences. Trypsin and ampicillin were from Sigma. The sub-2.1mM glycine for pH 9–10. All buffers were filtered, and samples were centrifuged before the experiment. Thermal unfolding experiments were performed by monitoring the CD signal at 222 nm between 25 and 95 °C using a heating rate of 2 °C/min at a concentration of 0.25 mg/ml for antithrombin variants and 0.5 mg/ml for antitrypsin. Melting points (Tm) were calculated using an expression for a two-state transition as described previously (30). All the results are the average of three experiments. The pH-dependent antithrombin thermal melting points were calculated as a single transition between pH 5 and 8, and the pKa of this transition was fitted by GraFit (version 3.0, Erithacus Software Ltd.).

Equilibrium Unfolding of Antithrombin—Equilibrium unfolding was monitored by spectrophotometry (λ = 280 nm, λ = 350 nm) in the presence of guanidine chloride (GdmCl). α1-Antitrypsin was incubated in a buffer (50 mM phosphate or sodium acetate, 50 mM NaCl, and 1 mM EDTA) containing various concentrations of GdmCl at 25 °C for about 16 h before spectral measurements. The final concentration of protein was 20 μg/ml. Equilibrium unfolding was fitted to a two-state model, and the midpoints [GdmCl]1/2 of unfolding curves at different pH were plotted against pH.

PolymORIZATION of Antitrypsin Variants—Antitrypsin variants were incubated at 1 mg/ml in 20 mM Tris-HCl, 50 mM NaCl, and 1 mM EDTA for 1 h at temperature from 37 to 65 °C. Samples were loaded onto an 8% (w/v) native gel. The proteins were visualized by staining with Coomassie Blue.

Peptide Insertion—Antitrypsin at 0.5 mg/ml or α1-antitrypsin variants at 0.68 mg/ml were incubated at 37 °C in the presence of a 100-fold molar excess of the P14-P3 12-mer peptide (acyetyl-SEAAASTAVVIA) for different time intervals. The results were analyzed by loading the samples onto an 8% (w/v) native gel with 7% urea.

Crystallization of Antithrombin/Peptides Complexes—Native antithrombin (1 mg/ml) was incubated with 2 nM of P14-8 peptide of antithrombin (acyetyl-SEAAAS) and a tri-peptide (formyl-MLF) at 37 °C for 24 h. Samples were then washed several times with 10 mM Tris-HCl, pH 7.4, in a concentrator to remove most of the free peptides, with concentration to 14 mg/ml. Crystallization was performed using hanging drop methods as previously described (31) with the sequence modifications. Antithrombin-peptide complexes were mixed with equal amounts of antithrombin and equilibrated against 10–20% PEG 4000 in 50 mM sodium cacodylate buffer, pH 6.8, and 0.2 mM NH4F, with or without 12% glycerol. Crystals grew to full size in ~1 week.

Data Collection and Refinement—Diffraction data to 2.8 Å were collected from a single frozen crystal at Daresbury Synchrotron Radiation Source (station 14-2) and processed using Mosflm (32) and Scala (33). Processing statistics are given in the Table I. Since the crystal of the ternary peptide complex was essentially isomorphous with the structure of the previously solved antithrombin/binary-peptide complex (34), the original binary complex coordinates (PDB accession number 1D6B) were used as the starting model. Refinement to 2.8 Å was performed using the crystallographic NMR software (35) using the ~1

Stochiometries and Rates of Inhibition—Stochiometries of inhibi-
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Table I

X-ray data collection and refinement statistics

| Space group | P2₁ | a = 68.81Å, b = 99.91Å, c = 87.29Å, β = 104.44° |
|-------------|-----|--------------------------------------------------|
| Wavelength (Å) | 0.978 | |
| No. of reflections | 91,051 | |
| Rmerge (a) | 0.104 (0.551) | |
| Completeness (%) | 96.7 (81.5) | |
| Redundancy | 3.3 (2.5) | |
| Resolution (Å) in CNS | 2.89 (3.00) | |
| No. of unique reflections | 25,977 | |
| R(free) | 15.6 (20.2) | |
| Weighted r.m.s. deviation from ideality: bond lengths (Å)/bond angles (°) | 0.007/1.30 | |
| Total no. of atoms | 6,763 | |
| No. of water molecules | 40 | |

(a) Rmerge = Σ |Iobs−<I>obs|/ΣIobs.
(b) For highest resolution shell 2.93-2.80Å.
(c) R-factor = Σ|Fo−Fc|/ΣFo.
(d) Root mean square.

Results

Polymers, Latency, and Glycerol—Prolonged heating of antithrombin and α₁-antitrypsin at temperatures above 50 °C results not only in their polymerization (Fig. 2a) but also in a transition of a proportion of molecules to the latent conformation. This transition to the latent form is minor and barely detectable with α₁-antitrypsin. However, latent antithrombin is readily formed, though it may not be apparent due to the immediate formation of a heterodimer (20) with reversible sheet C linkage of the latent antithrombin to a molecule of active native antithrombin (Fig. 2b). With antithrombin, the addition of glycerol to the incubation buffer suppresses polymer formation but allows the latent transition (20), such that incubation at 50 °C of antithrombin in

Fig. 1. Histidine 334 in the shutter region of antithrombin. a, structure of native antithrombin (from PDB 1E04) with reactive center loop in red, A-β-sheet in green, and strand 6 of B-β-sheet and helix B in yellow. A red arrow indicates entry of the reactive loop as strand 4. b, shows the hydrogen bonds (dashed line) between main chains of the A-sheet. Histidine 334 forms the first hydrogen bonds between strands 3 and 5 of the A-sheet. c, detailed interactions in the shutter region (circled in a) showing oxygen atoms as red balls, nitrogen atoms in blue, and the position of Ca atoms in black. Hydrogen bonds are shown as cyan dashed lines. The imidazole ring of His-334 forms side chain to main chain interactions with Ser-53 and Asn-186 and with the side chain of Asn-186. The hydroxyl of Ser-53 forms a hydrogen bond with the main chain amine of Ser-56, while the hydroxyl of Ser-56 interacts with the side chain of Asn-186. d, close-up of the shutter region of antithrombin ternary complex (antithrombin with P14-9 and a tripeptide). The glycerol in green occupies the P8 position and hydrogen bonds to the imidazole of His-334 and the carbonyl group of Phe-333 and carboxyl group of P9 residue of the P14-9 peptide. e, stereo picture as in d showing αA weighted 2Fo−Fc electron density of the inserted peptides: P14-9 above and formyl-P6-4 below. The glycerol is separately buried between them in the position usually occupied by the side chain of the P8 threonine. The key H-bonds, to the imidazole and its bond to the amine of Ser-53, are shown as dotted lines.
The samples were analyzed on an 8% 7 M urea gel to give clear excess of a P14-3 peptide (acetyl-SEAAASTAVVIA) at different pH values. The rapid formation of the complex at pH 5 as compared with antithrombin incubated for 12 h at 37°C for 16 h at different pH values. Antithrombin was incubated at 37°C with 100-fold molar excess of a P14-3 peptide (acetyl-SEAAASTAVVIA) at different pH values. The samples were analyzed on an 8% 7 M urea gel to give clear separation of the peptide-antithrombin binary complex (BC).

40% glycerol results in the quantitative preparation of latent antithrombin (25), free of polymers (Fig. 2b).

pH Dependence of Transitions—The incubation of antithrombin at 50°C for 16 h over a range of pH values (Fig. 2c) shows the ready formation of polymers up to pH 5.5 but insignificantly so at pH values greater than 6. Above pH 6 there is a much slower conformational transition, with the formation of latent antithrombin (apparent as the heterodimer) and much less polymer formation until pH 10. The effect of pH on the opening of the A-sheet of serpins can also be assessed by the readiness with which they complex with synthetic P14-3 peptides. This is clearly seen in Fig. 2d which shows the formation of the binary complex (seen as a 7 M urea stable component) in antithrombin incubated for 12 h at 37°C with the P14-3 peptide. The rapid formation of the complex at pH 5 as compared with pH 6 and 7 indicates that the low pH favors sheet opening and hence peptide annealing.

pH and Thermal Stability—Serpins typically have a well-defined thermal transition with antithrombin having a melting point (Tm) of 57.6°C at pH 7.4. Antithrombin differs, however, from the archetypal serpin α1-antitrypsin in that its Tm undergoes an atypical change with pH (Fig. 3a) with a transition at apparent pH of 6.0 ± 0.1 (Fig. 3b). A transition at pH 6 was similarly observed with guanidine chloride-induced antithrombin unfolding (data not shown). The inflection point at pH 6 suggested that the thermal stability of antithrombin was dependent on the protonation of an individual histidine residue, with the likely candidate being His-334. This histidine is uniquely exposed in antithrombin, which has a partially opened A-sheet, with His-334 forming the first interlinking H-bonds between s3A and s5A (Fig. 1, b and c). By comparison, in α1-antitrypsin, which is more thermally stable with Tm 63.4°C, His-334 is protected by the full closure of strands 3 and 5 in the A-sheet. To confirm that this difference in pH dependence is related to the exposure of His-334, the change in Tm was determined with a closed A-sheet conformation of antithrombin. Closure of the A-sheet of antithrombin was induced by addition of the core heparin pentasaccharide, which has been shown to result in full closure of the A-sheet with burial of His-334 to give a conformation of antithrombin superimposable with that of α1-antitrypsin (31). As shown in Fig. 3c, addition of the heparin pentasaccharide to antithrombin results in an increased thermal stability with conversion to a pH dependence curve similar to that of α1-antitrypsin and without the inflection at pH 6.

Recombinant Replacement of His-334—To assess the contribution of His-334 to the stability of α1-antitrypsin, variants were expressed recombinantly with substitutions of His-334 by serine and alanine and with the variants named here as Wt for the wild type and H334S and H334A, respectively, for the variants. Both α1-antitrypsin variants retain their inhibitory activity and form stable complexes with trypsin identical to those of Wt (Fig. 4a), and rates and stoichiometries of trypsin inhibition were unchanged (data not shown). Incubation of H334A for 1 h at a range of temperatures showed substantial polymerization at 45°C, some 5–10°C prior to equivalent polymerization of the recombinant WT α1-antitrypsin (Fig. 4b). The H334S variant had a smaller increase in polymerization, intermediate between the Wt and H334A forms. The more ready opening of the A-sheet of H334A versus WT α1-antitrypsin is shown by the increased rate of peptide (P7-2) insertion with the H334A variant (Fig. 4c). The inherent change in stability of the three forms is reflected in their thermal stability, with a decrease from a Tm of 63.4°C in WT α1-antitrypsin to 59.6°C in H334S and to 58.2°C in H334A. The H334A mutant has a similar pH-dependent Tm curve to those of antitrypsin and antithrombin-heparin pentasaccharide complexes (data not shown). This indicates that the transitions between pH 4 and 5.5 (Fig. 3, a and b) do not represent the protonation of His-334, which is consistent with the burial of His-334 both in antitrypsin and heparin-pentasaccharide-complexed antithrombin.

Crystallographic Detail of His-334 Interaction with Glycerol—A crystallographic finding, reported here (PDB accession number 1LK6), provides an unexpected insight with particular relevance to His-334. The finding was incidental to the determination of the structure of a ternary complex of antithrombin as part of a larger2 series of serpin-peptide complex structures. The ternary complex was formed by incubation of antithrombin with P14-9 and P6-4 synthetic loop peptides in the presence of glycerol. The 2.8 Å structure shows a glycerol molecule sited in the position occupied in six-stranded antithrombin by the side chain of threonine P8 (Fig. 1, d and e). Critically, a hydroxyl of the glycerol forms a hydrogen bond with the bond length of 2.9 Å with the bond length of 2.9 Å being identical to that formed by the hydroxyl of the P8 threonine. Moreover in each case the His-334 imidazole is oriented such that its e nitrogen is maintained at an optimal hydrogen bond distance of 3.1 Å from the main chain amine of Ser-53. But as well as this specific linkage to His-334, a series of hydrogen bonds are also formed with surrounding structures including the main chain oxygen of Phe-333 and the carboxyl group of the P9 residue. Subsequently, we have also shown crystallographically that glycerol insertion, as in Fig. 1, d and e, takes place even upon rapid exposure of formed crystals to glycerol just prior to diffraction.

2 A. Zhou, P. E. Stein, J. A. Huntington, and R. W. Carrell, unpublished data.
DISCUSSION

The findings here open a clearer understanding of the mechanisms leading to aberrant conformational changes in the serpins. In particular the pH dependence of these changes strongly supports structural evidence as to the critical role of His-334 in maintaining the metastable inhibitory conformation. In antithrombin, His-334 in strand 5A is clearly seen as a barrier to further insertion of the reactive loop, due to the bridging linkage of its imidazole side chain to Asn-186 in strand 3A and to Ser-53 in the underlying B-sheet (Fig. 1, b and c). The significance of these interactions is highlighted by the identification of mutations in the shutter region causing familial dementias: at His-334, Ser-53, and also at Ser-56, which bonds to Asn-186 (14, 15). The mutations predictably result in a laxity in sheet opening at 37 °C similar to that induced by incubation of the normal protein at 50 °C. The consequence of such incubation (Fig. 2, a–c) is the formation of either intermolecular loop-sheet linkages to give polymers or the monomeric transition to the latent conformation with a full insertion of the reactive loop into the A-sheet. The demonstration here that both polymerization and peptide annealing of antithrombin occurred at pH below 6 (Fig. 2, c and d) together with the structurally known exposure of His-334 in antithrombin, indicates that sheet opening is facilitated by the protonation of His-334. This protonation of the ε nitrogen of the imidazole will break the hydrogen bond that anchors His-334 to Ser-53 in the underlying B-sheet (Fig. 3 d). The accompanying acquisition of a positive charge will disfavor the burying of the imidazole with the combined effects giving disruption of the hydrogen bonds that link strands 3 and 5 of the A-sheet.

In keeping with the conclusion that sheet opening is influenced by the protonation of His-334, pentasaccharide complexes were also fitted, but these transitions do not represent the protonation of His-334 as a similar transition was observed with the H334A mutant. D, diagram showing how protonation disrupts the hydrogen bonding of His-334. The δ nitrogen of His-334 imidazole ring forms hydrogen bonds with carbonyl groups of Asn-186 and, prior to protonation, the ε nitrogen interacts with the main chain amine of Ser-53, with an optimal N–N hydrogen bonding distance of 3.1 Å.
ence by the protonation of His-334, the thermal stability of antithrombin was shown to be pH-dependent, with a characteristic transition at pH 6 (Fig. 3b). In comparison to this, there is no such pH transition in stability in α1-antitrypsin in which the histidine is buried in a tightly closed A-sheet. Moreover, when antithrombin is converted to a form with a similarly closed A-sheet, by complexing with heparin, its $T_m$ curve loses the inflection at pH 6 and reverts to the typical serpin response as seen with α1-antitrypsin (Fig. 3c). Although there are four histidines in antithrombin, only His-334 undergoes a radical change in environment, due to buring, on heparin activation. Evidence that His-334 has the same protective function against aberrant sheet opening in α1-antitrypsin is provided by recombinant substitutions at 334 with alanine (H334A) and serine (H334S). These substituted variants of α1-antitrypsin have properties similar to that of antithrombin when its pH value is decreased to below 6. The H334A and H334S variants of α1-antitrypsin, as compared with the wild type recombinant, have a large decrease in $T_m$ and more readily form polymers and accept synthetic loop-peptides (Fig. 4). The shutting control of sheet opening is a delicately tuned mechanism, and even slight perturbations may have disastrous functional consequences.

For example, two relevant natural antithrombin variants with minor shutter mutations and a decrease in $T_m$ of just over 1 °C result in severe episodic thrombosis (24, 42).

An understanding of the mechanism controlling sheet opening is needed for the design of therapeutic agents to prevent the pathological polymerization of serpins. Although the backbone position of His-334 is at the level of P8 of a fully inserted reactive center loop, the imidazole side chain of the histidine, by its H-bonding to Asn-186, effectively blocks entry to the sheet beyond the level of P12 (Fig. 1, b and c). So entry of the reactive loop or its homologue peptides, to level P10 and beyond, will disrupt the linkage between s2A and s5A and allow full opening of the sheet. Thus insertion of P14-9 or P14-8 peptides will enable the entry of the P7-3 sequence of the reactive loop of another molecule into the lower half of the opened sheet, with the resultant formation of polymers. But normally there will be preferential entry of the molecule’s own reactive loop, facilitated by the ability of the side chain of its P8 threonine to re-form the H-bond network with His-334 of s5A and Asn-186 in s3A. The threonine at P8 is conserved in almost all serpins (13) with a notable exception being in human α1-antitrypsin where there is a methionine (this explains why the P14-3 homologue peptide of antithrombin more readily inserts into α1-antitrypsin than does its own P14-3 peptide (43)). The clear preference for a threonine at P8 is also shown in another particularly relevant structure, that of a mutant shutter (L55P) of α1-antichymotrypsin (44). This pathological mutant is seen in a frozen transitional form, with the reactive loop inserted to P12 but with further entry blocked by the insertion into the P8 position of a threonine from the adjacent F helix. Intriguingly this threonine at position 165 at end of the F helix is invariantly conserved in the inhibitory serpins.

A demonstration of the way insertion of the reactive loop to P9 disrupts the His-334-Asn-186 bond between s3 and s5A is seen in our crystallographic structure of a complex of antithrombin with a P14-9 peptide (Fig. 1, d and e). In this structure, stabilized by a further insertion of a P6-4 tripeptide, a gap has opened between His-334 and the side chain of Asn-186 that is normally bridged by hydrogen bonding to the side chain of the P8 threonine. However, the unexpected finding in the structure is the presence in the P8 position of a glycerol molecule, oriented similarly to that of the side chain of the threonine normally at P8. The glycerol, as with the threonine, is hydrogen-bonded to His-334 with the precise bond distances that ensure the anchoring of the imidazole ring to the main chain of Ser-53. Thus the glycerol has in effect re-formed the bonding network that stabilizes His-334. Although this new network does not link to Asn-186 in s3A, the presence of the glycerol will impede the insertion of external peptides in the P8-P7 position. This, together with the potential for glycerol to also insert into other vacant strand positions, provides an additional explanation for the efficiency of glycerol in the protection of antithrombin against polymerization (Fig. 2e).

The protective effect of high concentrations of glycerol had been assumed to be due to its decreasing the rate of diffusion and hence of the collisions between individual molecules required for polymerization. But whereas polymerization will result from random intermolecular collisions, the monomeric transformation to the latent form is due to the ordered entry of the molecule’s own reactive loop into the sheet. This entry of the optimally oriented side chains of the reactive loop, including the P8 threonine, should readily displace any in situ glycerol molecules. Thus the latent transition is less affected by the presence of glycerol (Fig. 2b). Nor is it as dependent as polymerization on the opening of the A-sheet as the latent transition continues to occur at higher pH values when the His-334 network is intact and the sheet is predictably closed (Fig. 2c). The likely limiting factor in the latent transition is not so much the opening of the A-sheet as the release of the intact loop by thermal dissociation at its distal hinge, s1C (25, 45). This is consistent with previous observation that replacement of glutamine with histidine at 334 in plasminogen activator inhibitor-1 does not decrease latent transition (46).

The prevention of polymerization by peptides that insert into the opened A-sheet has been demonstrated in vitro and shown to be selectively achievable (34, 47–50). The therapeutic challenge is to convert these peptides into pharmacologically effective in vivo agents. The identification here of a focal point for sheet opening gives encouragement as to the feasibility of designing smaller and more effective peptide blockers of polymerization to prevent the pathological polymerization of serpins.

Specifically, the demonstration that glycerol can readily act as a surrogate for the critical side chain of the P8 threonine, opens the prospect of achieving an ultimate aim in the field, the development of non-peptide blocking agents.

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