Crystallographic and Cellular Characterisation of Two Mechanisms Stabilising the Native Fold of $\alpha_1$-Antitrypsin: Implications for Disease and Drug Design

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Received 16 January 2009; accepted 31 January 2009
Available online 14 February 2009

Keywords: serpin; $\alpha_1$-antitrypsin; polymerisation; rational drug design; conformational disease

Introduction

The plasma protein $\alpha_1$-antitrypsin is principally synthesised by hepatocytes and protects lung tissue from degradation by inhibiting neutrophil elastase.$^1$ It is the archetypal member of the serpin (serine protease inhibitor) superfamily of proteins.$^2$ In common with other inhibitory serpins, the native fold of $\alpha_1$-antitrypsin is metastable and this property is utilised in the characteristic serpin mechanism of action.$^3$ The reactive loop of the molecule is oriented into solution as an ideal substrate for its target protease. Following docking, elastase cleaves the P1–P1′ bond of $\alpha_1$-antitrypsin, allowing the P1–P14 residues of the reactive loop to insert as an additional strand within $\beta$-sheet A. The enzyme remains bound to the P1 residue in a catalytic intermediate state and is translocated 70 Å from one pole of the molecule to the other.$^3$ The energy released by the formation of the new $\beta$-strand disables the catalytic site and stabilises the
covalently bound complex, which is then rapidly cleared from the circulation. This dynamic mechanism renders α₁-antitrypsin vulnerable to point mutations, predisposing it to alternative, pathogenic, conformational transitions—in particular self-association into polymer chains. These accumulate within the endoplasmic reticulum of hepatocytes rather than enter the circulation. The most clinically significant of these is the Z mutation (Glu342Lys) carried by 1 in 27 of North European
populations. Homozygotes for the Z allele have deficiency of circulating α1-antitrypsin with levels that are 10–15% of those found in homozygotes for M (wild type) α1-antitrypsin. Disease ensues from both the toxic effects of polymers aggregated within hepatocytes (neonatal hepatitis, cirrhosis and hepatocellular carcinoma) and the loss of antiprotease activity within the lung (emphysema).

Over the last 15 years, biochemical, biophysical and structural studies have defined the general scheme of α1-antitrypsin polymerisation.7-11 This proceeds via an initial monomeric intermediate termed M*. In a conformation that is activated for the formation of polymers. The existence of this initiating species is indicated by fluorescence studies of α1-antitrypsin polymerisation kinetics and by the structure of the thermodynamically stabilised yet highly polymerogenic α conformer of the closely related protein, α1-antichymotrypsin.8-12 These studies support the hypothesis that the native-to-M* transition results from dysregulated opening of β-sheet A and allows the reactive site loop of a neighbouring molecule to insert aberrantly as an extra β-strand. Successive intermolecular loop–sheet interactions lengthen the polymer chain. Recently, an alternative model has been proposed, based upon the structure of a self-terminating dimer of the serpin antithrombin,13 emphasising polymerisation as an alternative endpoint on the folding pathway. The repeating subunit of the proposed polymer is considerably unfolded compared to the native structure with intermolecular linkage occurring via a β-hairpin formed from the reactive loop and a contiguous β-strand.

Both proposed mechanisms of polymerisation share key characteristics that can be exploited for the purposes of rational drug design. In both cases, increasing stability of the native fold will reduce the population of the unstable intermediate. Moreover, in both cases, polymerisation requires expansion of β-sheet A and the formation of a six-stranded sheet. For α1-antitrypsin, this involves the sealing of a solvent-accessible cavity flanking β-sheet A.14 This, therefore, represents an attractive target for rational drug design as small molecules binding here will impede expansion of β-sheet A and, thus, prevent polymerisation by an allosteric mechanism. Evidence for this comes from the effects of mutating either of two residues bordering the cavity—Thr114 and Gly117—to phenylalanine. Both mutations reduce polymerisation of α1-antitrypsin by an order of magnitude in vitro whilst leaving inhibitory activity unaffected.14 Moreover, both mutations partially rescue the defect in the total levels of secreted Z α1-antitrypsin in a eukaryotic cell model of disease. The importance of this cavity is
highlighted by in silico screens that have identified lead compounds that could fill the cavity and, hence, reduce polymerisation. However, unlike the mutations, all compounds that have proven effective in vitro have also abolished inhibitory activity.

To understand the structural mechanisms by which the effects of the Thr114Phe and Gly117Phe mutations are conferred, we have crystallised both α1-antitrypsin variants and solved their structures to 2.2 and 3.2 Å, resolution, respectively. The structure of Thr114Phe α1-antitrypsin allows us to define a novel target for pharmacophores to block polymer formation whilst preserving inhibitory activity. The structure of the Gly117Phe mutant shows that this mutation exerts its effects by changes in both local packing and the position of the F helix. We have gone on to demonstrate that the two mutations ameliorate the defect in folding of Z α1-antitrypsin, which has been previously characterised. Apart from the cavity flanking the reactive loop,56–58 interstrand spacing in β-sheet A,56 strand 1 of β-sheet C,56 the F helix,56,12,27,28 and the shutter region56 (Fig. 1a). These are essentially unchanged in the mutant structure compared to that of wild-type α1-antitrypsin.

### Results and Discussion

#### Effects of the Thr114Phe mutation arise from partial cavity blockade

The 2.2 Å crystal structure of Thr114Phe α1-antitrypsin adopts the classic structure of a serpin fold in the native conformation (Fig. 1a and Table 1). The polypeptide chain spanning residues 24–393 is highly ordered with the exception of residues 344–351. These comprise the P15–P8 section of the reactive loop, a section that is disordered or associated with high B-factors in other crystal structures of metastable serpins.9,16,20–22 The key P1 methionine residue that defines the inhibitory specificity of a serpin adopts a canonical conformation.

Comparison of the structure of Thr114Phe α1-antitrypsin with the 2.0-Å structure of wild-type α1-antitrypsin that is also in the native conformation [Protein Data Bank (PDB) code: 1QLP]16 shows a high degree of overall similarity between the two proteins (Fig. 1b). The root-mean-square deviation (r.m.s.d.) of the mutant and wild-type structures, based on Cα positions, is 0.4 Å. A number of critical regulators of conformational change in α1-antitrypsin have previously been characterised. Apart from the cavity flanking β-sheet A, these are the proximal hinge of the reactive loop,23 the arginine pocket stabilising the loop at the P5 position,24 interstrand spacing in β-sheet A,25 strand 1 of β-sheet C,26 the F helix,56,12,27,28 and the shutter region26 (Fig. 1a). These are essentially unchanged in the mutant structure compared to that of wild-type α1-antitrypsin.

#### Table 1. X-ray data collection and refinement statistics for structures of Thr114Phe α1-antitrypsin and Gly117Phe α1-antitrypsin

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Thr114Phe α1-antitrypsin</th>
<th>Gly117Phe α1-antitrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell dimensions</td>
<td>a (Å) 113.8</td>
<td>58.8</td>
</tr>
<tr>
<td>Molecules in asymmetric unit</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Space group</td>
<td>C2</td>
<td>P2₁</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>87.0–2.2</td>
<td>77.1–3.2</td>
</tr>
<tr>
<td>R cryst (%)</td>
<td>10.6 (49.3)</td>
<td>13.8 (58.8)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>94.2 (90.2)</td>
<td>99.7 (99.7)</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>15.3 (5.5)</td>
<td>11.5 (2.5)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>6.8 (6.8)</td>
<td>4.7 (4.8)</td>
</tr>
</tbody>
</table>

Values in parentheses refer to the highest-resolution shell.

The lack of perturbation elsewhere in the overall structure of Thr114Phe α1-antitrypsin implies that the mutation must be mediating its effect on the fold stability of native α1-antitrypsin (Fig. 1c) from within the cavity flanking β-sheet A. The cavity is well defined in this high-resolution structure, permitting interpretation of the effect of the mutation on cavity filling at the side-chain level. The precise location of the aromatic side chain of Phe114 was strongly indicated by the initial density seen within the cavity adjacent to residue 114 when this was modelled as an alanine (to minimise model bias) prior to any restrained refinement (Fig. 1d). Phe114 makes no interactions with nearby residues, and therefore, its effects are entirely due to partial filling of the cavity and changes in its packing environment during unfolding and polymerisation. The aromatic group of Phe114 closes the upper part of the cavity (Fig. 1d) with its side chain just 3.7 Å from Leu103 and 4.0 Å from Asn104 at the top of helix D, distances that permit favourable van der Waals contacts. These data support the importance of β-sheet A compaction in stabilising the native conformer against initial unfolding.
Proof-of-principle *in silico* fragment screening based upon the Thr114Phe mutation

The volume partially filling the cavity in Thr114Phe $\alpha_1$-antitrypsin (Fig. 1e) is in a region of high-solvent accessibility in the wild-type protein and also yields the therapeutically desirable combination of effective blockade of polymerisation whilst maintaining inhibitory function. Furthermore, it does not alter the native $\alpha_1$-antitrypsin structure beyond the cavity in any way. It is, therefore, attractive to consider it a refined target or an ideal pharmacophore volume, depicted in Fig. 1e as the difference between surface representations of wild-type and Thr114Phe $\alpha_1$-antitrypsin around residue 114. The estimated size of this ideal pharmacophore is 59 Å$^3$, equivalent to 22% of the total cavity volume in wild-type $\alpha_1$-antitrypsin. A similar reduction in the size of lead compounds compared to those identified by screening against the entire cavity would improve the probability of success in rational drug design. Lipinski’s ‘Rule of 5’ observes that in drug development is highly correlated with the following: $M_r$<500 Da, lipophilic index ($\log P$)<5, ≤5 hydrogen-bond donors, ≤10 hydrogen-bond acceptors. A modified version of this, the ‘Rule of 3′, has been proposed to guide drug design via exploration of a greater diversity of chemical space using still smaller (<300 Da) molecular fragments to identify potential ‘hits’. The mean change in $M_r$ in development from lead compound to drug is +69 Da, and most effective drugs used in clinical practice are 250–320 Da. Currently, the lead compounds designed to block the entire cavity and shown to be effective in vitro are at the upper end of the desirable drug size (310–650 Da).

To consider the region occupied by the Thr114Phe mutation a meaningful pharmacophore target, it must generate hits *in silico* that are plausible in terms of mimicking the mutation’s partial cavity blockade, their predicted binding affinities ($K_d$) and ‘drug-likeness’. The pharmacophore target is centred between the middle of strand 2 of $\beta$-sheet A (s2A) and the top of helix D (Fig. 10). It is close to polar residues (Asn104, Thr114 and His139) and an area favourable for hydrophobic contacts that may aid ligand coordination and binding, respectively. On the other hand, unlike the entire cavity, this region is only bounded by residues in one plane, which may be challenging for some docking strategies. These considerations make it hard to assume *a priori* that plausible docking candidates would emerge from *in silico* screening of fragment libraries.

We have therefore performed a proof-of-concept screen of >70,000 fragments with $M_r$=150–250 Da to assess the potential of the new pharmacophore for identifying small compounds that can be developed in rational drug design. The pharmacophore centroid was set as the midpoint between Thr114 (mutation site, middle of s2A) and Asn104 (top of helix D) for the purposes of our protocol. Screens were performed to select hits on the basis of docking scores and ligand–target centroid proximity. By definition, all hits obey the Rule of 5 and can only deviate from the Rule of 3 a single hydrogen-bond acceptor. The best-scoring 0.1% of ligands were taken forward for induced fit docking, resulting in optimised docking of 65 unique ligands. These had the following mean (±SD) characteristics: $M_r$=207.2 Da (±21.7), estimated $\log P$ value=1.76 (±0.81) and ligand–pharmacophore centroid proximity=5.16 Å (±0.76). The fragments are shown docked ensemble in relation to residues 104, 114 and 139 (Fig. 1f, left). The best docking scores and proximity to the pharmacophore centroid and sizes of all ligands docked by the induced fitting protocol are also shown (Fig. 1f, right). Docking scores calculated by Glide SP correlate with predicted $K_d$ values such that a value $<6.8$ kcal/mol correlates with a predicted micromolar $K_d$ and a value $<8.2$ kcal/mol correlates with submicromolar affinity. Figure 1f illustrates that this screen identified numerous fragment hits achieving scores within this range. Examples of docked ligands ranked highly by docking scores and fragment–target centroid proximity are shown in Fig. 1g.

Optimisation of this strategy is likely to require screening 1–2 orders of magnitude more fragments and ‘growing’ (up to $M_r$~250 Da) of the best hits to optimise specificity and drug-likeness ahead of *in vitro* testing. However, the hits identified by this preliminary screen are good candidates in terms of size, predicted binding affinities and drug-likeness for further development. Moreover, our screen also identified compounds that docked well to sites above the pharmacophore target site or interacted with the front of $\beta$-sheet A (Fig. 1f). Similar hits will aid the ‘growing’ of compounds for future *in vitro* testing.

The Gly117Phe mutation alters aromatic ring packing in the interface of $\beta$-sheet A with the $\beta$-helix interface and induces a half-turn downward shift in $\beta$-helix position

The structure of Gly117Phe $\alpha_1$-antitrypsin was solved to 3.2 Å resolution (Table 1 and Fig. 2a), allowing confident interpretation of the secondary structure of the protein. The asymmetric unit contained three molecules with minimal intermolecular contacts and in an arrangement not suggestive of biologically significant multimerisation. No significant changes are evident in $\beta$-sheet A or B sheets or strand 1 of $\beta$-sheet C in Gly117Phe $\alpha_1$-antitrypsin relative to the wild-type protein. Electron density was not seen for residues 349–356 (copy A), 348–356 (copy B) and 348–358 (copy C) of the hypermobile reactive site loop.

Before incorporation of the Phe mutation into the model or any restrained refinement had been performed, clear density was apparent at the mutation site, indicating that its side chain was oriented away from $\beta$-sheet A towards the $\beta$-helix where significant changes were seen. To investigate
these changes, we omitted the mutation site and the overlying part of the F helix to generate a difference density map of these areas with model bias minimised. This was based upon the initial rigid-body fit of the search model and did not use noncrystallographic symmetry (NCS) restraints, yet it confirmed a half-turn shift of the F-helix along its axis for all three molecules as well as side-chain density for the underlying Phe117 residue (Fig. 2a, boxed). In the refined structure, the F-helix is displaced downwards (towards the bottom of s3A) by half a turn relative to its position in the structure.
of native wild-type $\alpha_1$-antitrypsin (2.7–3.5 Å between equivalent C$^\alpha$ atoms). This compares with an overall C$^\alpha$ r.m.s.d. of 1.2 Å for the rest of the two structures. The F-helix shift is unlikely to be an artefact of crystal packing as different copies of Gly117Phe $\alpha_1$-antitrypsin have different packing environments. Moreover, no F-helix changes are required to permit packing of three molecules of the native wild-type protein in orientations identical with those of the three copies of Gly117Phe $\alpha_1$-antitrypsin in the asymmetric unit (not shown). Conversely, repacking of helix F$\rightarrow$β-sheet A interface residues, in particular Tyr160, is necessary to avoid steric clashes with the adjacent Phe117 side chain and sustain favourable hydrophobic interactions. Figure 2b compares packing of the helix F$\rightarrow$β-sheet A interface in wild-type and Gly117Phe $\alpha_1$-antitrypsin. In the refined structure of Gly117Phe $\alpha_1$-antitrypsin, Phe117 forms the centre of a cluster of aromatic side chains, interacting with Phe119, Phe143 and Tyr187 (all at distances of 3.5–5.5 Å). The side chain of Tyr160 is in a solvent-exposed position in this mutant in contrast to its buried position within the helix F$\rightarrow$β-sheet A interface in wild-type $\alpha_1$-antitrypsin but remains well placed for aromatic interactions with two residues of this cluster, Phe119 and Phe143. These differences therefore account for the changed conformation of helix F in Gly117Phe $\alpha_1$-antitrypsin compared to the wild-type protein. They introduce an extra aromatic ring into the helix F$\rightarrow$β-sheet A interface in the mutant to increase favourable packing interactions between strands 1–3 of the β-sheet. These changes do not obviously increase the degree of hydrophobic interaction between the F helix (or its linker to s3A, both shown in gold) and β-sheet A (blue). However, it is striking that the biochemistry of this mutant resembles that of $\alpha_1$-antitrypsin, carrying what can now be regarded as a complementary mutation, Tyr160Trp. Here, an extra aromatic ring is introduced from the F-helix side into the helix F$\rightarrow$β-sheet A interface to project into a site adjacent to Gly117. The Tyr160Trp mutation is associated with a near-identical increase in thermal stability to that seen with the Gly117Phe mutation. Both mutations are also alike in achieving this without any reduction in inhibitory activity. The increased biochemical stability conferred by the Gly117Phe mutation on the native fold of $\alpha_1$-antitrypsin (Fig. 2c) is therefore related to increased stabilisation of the F helix. The relative contributions of the alterations in packing of the helix F$\rightarrow$β-sheet A interface and its half-turn displacement towards the lower pole of the molecule remain to be determined. Since these effects involve alterations of the final folding of native $\alpha_1$-antitrypsin and are mediated via interactions in a hydrophobic interface, they may be hard to mimic via rational drug design. However, the fact that the position of the F helix is affected in the absence of any other significant changes to the overall fold supports the conclusion that this helix forms relatively late in the folding process and independent from the folding of other structural features.

**Effect of Thr114Phe and Gly117Phe mutations on secretion of Z $\alpha_1$-antitrypsin from COS-7 cells**

To relate our findings to drug design for $\alpha_1$-antitrypsin deficiency, we attempted to characterise the stability and folding of the Thr114Phe/Z and Gly117Phe/Z double mutants of $\alpha_1$-antitrypsin. These were produced as recombinant proteins using the same overexpression system as the crystallised proteins and purified by the same method. The yield of both double mutants was between 1 and 2 orders of magnitude lower than that of wild-type and single-Phe mutant $\alpha_1$-antitrypsin. The products were conformationally heterogeneous with a mixture of native, latent [the major product on non-denaturing and urea polyacrylamide gel electrophoresis (PAGE) and polymeric conformers. The effects of the Thr114Phe and Gly117Phe mutations on transient expression and secretion of $\alpha_1$-antitrypsin containing the Z mutation in a more physiological, eukaryotic cell system were then assessed. Previous work has shown that the Thr114Phe and Gly117Phe mutations partially rescue the profound defect in the overall secretion of $\alpha_1$-antitrypsin caused by the Z mutation in a *Xenopus* oocyte system. These effects are modest in proportion to the overall defect but are of considerable clinical interest since disease severity correlates closely with rates of polymerisation and ( inversely) with circulating levels of $\alpha_1$-antitrypsin. However, this study did not characterise the conformational state of the secreted material. We therefore transformed and transiently expressed M, Z, Thr114Phe/Z and Gly117Phe/Z $\alpha_1$-antitrypsin in COS-7 cells. Analysis of cell lysates and culture media by Western blot analysis of sodium dodecyl sulfate (SDS)-PAGE showed increased secretion of $\alpha_1$-antitrypsin in the double mutants compared with Z $\alpha_1$-antitrypsin (Fig. 3a and b). These data were consistent with those quantified by the more sensitive pulse-chase system in *Xenopus* oocytes. This increase in total $\alpha_1$-antitrypsin secretion was related to a clear increase in secretion of native, monomeric $\alpha_1$-antitrypsin as assessed by Western blot analysis of non-denaturing PAGE (Fig. 3c, arrow). The absolute concentrations of $\alpha_1$-antitrypsin in the culture media were very low relative to those conventionally used to measure the inhibitory activity of $\alpha_1$-antitrypsin. However, we were able to detect inhibitory activity using an ELISA technique in which bovine α-chymotrypsin was used to capture functional $\alpha_1$-antitrypsin from concentrated culture media. This indicated that more functional material was secreted in the presence of the double mutations (1.35 μg/ml for Thr114Phe/Z and 0.35 μg/ml for Gly117Phe/Z $\alpha_1$-antitrypsin) than with the Z mutation alone (<0.16 μg/ml of active protein; below linear range of detection by ELISA). These effects were modest compared with the amount of functional M $\alpha_1$-antitrypsin detected in the culture media (>5 μg/ml; exceeding the maximum assesseable in...
The Thr114Phe and Gly117Phe mutations act by different mechanisms to increase the global stability of native α1-antitrypsin. Expansion of β-sheet A is a common feature in both proposed mechanisms of polymerisation. In order for α1-antitrypsin to adopt either M* conformation, residue 114 must move laterally by 4.6 Å in the plane of β-sheet A. This is limited by local steric clashes in Thr114Phe α1-antitrypsin. In the case of the Gly117Phe mutation, the structural mechanism whereby the changes in the vicinity of the F helix may impede formation of a β-hairpin donor/acceptor M* species is less intuitive. There is no apparent increase in stabilising interactions between the helix F–s3A linker and s5A, and the changes in packing in the interface between the F helix and β-sheet A would not of themselves be expected to impede opening of the β-sheet. Nevertheless, our data are not inconsistent with this model since they may simply reflect overall cooperativity of the final steps on the folding pathway (and initial steps on the unfolding pathway) for α1-antitrypsin. However, our findings can be mechanistically integrated with other biochemical data for the native-to-M* transition posited for the single-strand linkage model in which opening of the s4A site is associated with partial intramolecular loop insertion. Various data support the following features of this model: upper s4A opening precedes lower s4A opening and is associated with partial insertion of the reactive site loop. Subsequent lower s4A opening around the site of P8 residue insertion is associated with breaking of a network of interactions between s4A, s5A and shutter region residues.

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**Conclusion**

The data presented here demonstrate in crystallographic detail two mechanisms that greatly stabilise the native fold in α1-antitrypsin without abolishing its inhibitory function. These mechanisms are likely to
underlies the partial rescue of secretion of native α₁-antitrypsin when the Thr114Phe or Gly117Phe mutations occur in the context of the Z mutation in eukaryotic cells although the double mutants remain highly polymerogenic. The high-resolution structural characterisation of the Thr114Phe mutation presents a more precise pharmacophore target for screening of small fragments capable of mimicking its effects. These data are generally consistent with either the single-strand or β-hairpin linkage models of polymerisation. However, they suggest a specific mechanism of propagated conformational change in the case of the single-strand model and, therefore, open up new approaches for further studies to test its applicability in this important pathological pathway.

Materials and Methods

Site-directed mutagenesis, protein expression, purification and characterisation

QuickChange mutagenesis (Stratagene, California, USA) was used to introduce the Thr114Phe, Gly117Phe and/or Z (Glu342Lys) mutations into His-tagged α₁-antitrypsin in the pQE31 vector to create single- or double-mutant constructs. The full-length sequences of the mutated α₁-antitrypsin genes were confirmed, and plasmids were transformed into XL-1 Blue Escherichia coli cells. The wild-type and mutant proteins were expressed and purified as described previously. The final products were stored in 10 mM Tris/HCl, pH 7.4, 10 mM NaCl and 1 mM ethylenediaminetetraacetic acid. Product purities were confirmed by non-denaturing PAGE, SDS-PAGE and transverse urea gradient (TUG)-PAGE. Further characterisation of urea stability by circular dichroism (CD) was performed as described previously.

The Z, Thr114Phe/Z and Gly117Phe/Z α₁-antitrypsin constructs for transient transfection of COS-7 cells were prepared by a combination of cloning, subcloning and site-directed mutagenesis in the pcDNA3.1 vector. All constructs were confirmed by sequencing of the whole gene. COS-7 cells were transfected and cultured as described previously. The Z, Thr114Phe/Z and Gly117Phe/Z α₁-antitrypsin constructs for transient transfection of COS-7 cells were prepared by a combination of cloning, subcloning and site-directed mutagenesis in the pcDNA3.1 vector. All constructs were confirmed by sequencing of the whole gene. COS-7 cells were transfected and cultured as described previously.
bovine α-chymotrypsin. The plate was then washed before incubating for 15 min at 37 °C with 100 μl of culture medium concentrated 7.5 times (this did not affect relative proportions of polymeric to total α1-antitrypsin).

After further washing to remove unbound material, the amount of functional material captured was assessed by incubation with 100 μl of purified polyclonal antibody against α1-antitrypsin used at 1 μg/ml for 2 h at 4 °C in the presence of 0.25% (w/v) bovine serum albumin as a blocking agent, followed by washing and incubation with 100 μl of horseradish-peroxidase-conjugated anti-rabbit secondary antibody (Sigma-Aldrich, Dorset, UK). The plate was washed and the enzymatic activity was calculated and quantified using SURFNET. As described previously, as described previously, grid separations of 0.8 Å and probe spheres of 2.0–4.0 Å radii were used. The extra volume occupied within the cavity by the Thr114Phe mutation was calculated by subtracting the volume of the cavity in the mutant from that in the wild-type protein. Similarly, an ideal pharmacophore capable of mimicking the partial cavity blockade caused by the mutation was calculated by subtracting the volume of the surface map of residue 114 in wild-type α1-antitrypsin from that of Thr114Phe α1-antitrypsin using SURFNET.

In silico screens of compound libraries

The fragment-like subset of the ZINC database was selected for initial prescreening to identify drug- and lead-like compounds capable of significant modification to optimise specificity without unfavourably increasing size. The subset labelled ‘usual’, containing all fragment-like molecules protonated in the pH range 5.75 to 8.25, was downloaded. Programs from the Schrödinger Suite 2007 (Schrödinger LLP, New York, NY, USA) were used for in silico docking. Fragments were preprocessed using LigPrep version 2.1. The original ionisation state was retained and ‘desalt’ and ‘tautomer generation’ flag settings were used. Chiralities were determined from the original ZINC database structures, and one low-energy ring conformation was allowed. This procedure resulted in a total of 70,925 small-molecule structures for docking. The protein was prepared using Maestro version 8.0 (Protein Preparation Wizard). Hydrogens were added, and all waters were deleted from the structure of wild-type α1-antitrypsin. Hydrogen bonds were optimised, and the structure was minimised within 0.3 Å r.m.s.d. from the initial structure. The potential energy grid was pre-calculated using a box centred on the centroid of residues Asn104 and Thr114 to prepare the binding site for docking. The box was 39 Å in each dimension, but the ligand centre was constrained to lie within 14 Å of the box centre. No additional constraints were used.

Initial screening of the 70,925 ligands was performed using Glide version 4.5, to generate a range of putative ligand–receptor interactions (poses). The best pose for each ligand was calculated using Emodel and retained. Ranking between ligands was then performed using the Glide SP score. All Glide parameters were left to their default values. The top 70 (>0.1%) ligands resulting from this Glide SP run were then submitted to the Induced Fit Docking protocol, a three-step docking process. Preliminary docking by Glide SP used a scaling factor for van der Waals radii of 0.5 for both the ligand and the receptor. For each ligand, the best 20 poses were retained. Prime version 1.6 was used to optimise receptor side chains within 5.0 Å of each posed ligand. Finally, ligands were re-docked into the optimised structures. The resulting poses were ranked using a composite of the Glide SP (binding site energy) and the Prime (molecular mechanics + solvation energy function for the protein–ligand complex) scores as previously described.

The molecular weight and other descriptors for the set of docked ligands were calculated using the MOE suite (MOE 2006.08, Chemical Computing Group, Montreal, QC, Canada). Prior to descriptor calculation, the partial charges on all atoms were recalculated using the default MOE force field (MMFF94s), and adjustment of hydrogens and lone pairs was allowed.

Modelling α1-antitrypsin in the M conformation

To model wild-type and mutant α1-antitrypsin in the partially loop-inserted M conformations, we used the 2.2-
A crystal structure of latent α1-antitrypsin (IQLP)\(^5\) as the initial template. In the latent conformation, the reactive loop is fully inserted into β-sheet A as its fourth strand (s4A). This structure was chosen as it is the highest-resolution structure of α1-antitrypsin with an expanded β-sheet A. Mutations were restored back to the wild-type sequence. The residues of the reactive site loop were threaded into the conformation adopted in the structure of α-Leu55Pro α1-antichymotrypsin (IOMN)\(^6\) using SWISSMODEL.\(^7\) As the reactive loop of α1-antitrypsin is four residues shorter than that of α1-antichymotrypsin, this required stripping out of s1C to connect it directly with s4B. The model was regularised in Coot before undergoing energy minimisation, simulated annealing and final energy minimisation using SANDER, with the protein parameterised using the AMBER99SB force field.\(^8\) The energy minimisation using SANDER, with the protein going energy minimisation, simulated annealing and final required stripping out of s1C to connect it directly with the rest of the molecule.

**Accession numbers**

Coordinates and structure factors are deposited in the PDB with accession number 3DRM for the structure of Thr114Phe α1-antitrypsin and 3DRU for the structure of Gly117Phe α1-antitrypsin.

**Acknowledgements**

This work was supported by a Wellcome Trust Intermediate Clinical Fellowship to B.G., an Alpah1-antitrypsin Laurell Training Award award to M.M., a Wellcome Trust summer studentship to S.C.L.B., a Wellcome Trust Clinical Training Fellowship to R.L. P., the Medical Research Council (UK), the British Pharmaceutical Society and Papworth National Health Service Trust. C.S. is a Wellcome Trust Research Training Fellow. We thank Dr Nora Cronin (School of Crystallography, Birkbeck College, University of London) for her assistance in collecting crystallographic data and Ms Heike Kröger (Cambridge Institute for Medical Research) for her assistance with the cell biological experiments.

**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/jmb.2009.01.069

**References**


