Pathogenic α₁-Antitrypsin Polymers Are Formed by Reactive Loop-β-Sheet A Linkage*

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Pasupathy Sivasothy‡§¶, Timothy R. Dafforn¶**†, Peter G. W. Gettins‡‡, and David A Lomas‡§

From the ‡Respiratory Medicine Unit, Department of Medicine and §Department of Haematology, University of Cambridge, The Wellcome Trust Centre for Molecular Mechanisms in Disease, Cambridge Institute for Medical Research, Cambridge, CB2 2XY, United Kingdom and the ¶Department of Biochemistry and Molecular Biology, University of Illinois at Chicago, Chicago, Illinois 60612-4316

α₁-Antitrypsin is the most abundant circulating protease inhibitor and the archetype of the serine protease inhibitor or serpin superfamily. Members of this family may be inactivated by point mutations that favor transition to a polymeric conformation. This polymeric conformation underlies diseases as diverse as α₁-antitrypsin deficiency-related cirrhosis, thrombosis, angioedema, and dementia. The precise structural linkage within a polymer has been the subject of much debate with evidence for reactive loop insertion into β-sheet A or C as strand 7A. We have used site directed cysteine mutants and fluorescence resonance energy transfer (FRET) to measure a number of distances between monomeric units in polymeric α₁-antitrypsin. We have then used a combinatorial approach to compare distances determined from FRET with distances obtained from 2.9 × 10⁶ different possible orientations of the α₁-antitrypsin polymer. The closest matches between experimental FRET measurements and theoretical structures show conclusively that polymers of α₁-antitrypsin form by insertion of the reactive loop into β-sheet A.

α₁-Antitrypsin is synthesized in the liver and secreted into the plasma where it is the most abundant circulating protease inhibitor. It is the archetypal member of the serine protease inhibitor or serpin superfamily (1), and like other members of this family it shares a common molecular structure based on a mobile reactive center loop and a five-stranded β-sheet A (2–5). The reactive loop acts as a peptide "bait" for the cognate protease, and after docking the loop is cleaved and the acyl intermediate is inserted into β-sheet A. This major conformational change results in the translocation of the protease to the end of the molecule distal to the initial docking site (6–8) and its inactivation by distortion of the catalytic triad (9).

Mobility of the reactive loop is essential for inhibitory function but also favors aberrant conformations associated with disease (10). In particular the loop is able to insert into the β-sheet of a second molecule to form well ordered polymers that are the basis of the profound plasma deficiency of the Z (11), Siiyama (12), and Mmalton (13) variants of α₁-antitrypsin. The polymerized protein accumulates in the endoplasmic reticulum of the hepatocyte to form inclusions that are associated with juvenile hepatitis, cirrhosis, and hepatocellular carcinoma (14). The accompanying plasma deficiency predisposes the Z homozygote to early onset emphysema (15). Loop sheet polymers have also been reported with dysfunctional mutants of C1-inhibitor (16), α₁-antichymotrypsin (17), and antithrombin (18) in association with angioedema, emphysema, and thrombosis, respectively, and polymers of neuroserpin underlie a novel inclusion body dementia (19). Moreover, this conformational transition occurs spontaneously in plasminogen activator inhibitor-2 and is likely to be important in the control of intracellular proteolysis (20).

The precise protein-protein linkage that underlies polymer formation remains unclear. Polymerization of Z α₁-antitrypsin can be blocked by peptides that are homologous to the reactive center loop by annealing to β-sheet A (11, 21). It was therefore proposed that polymers were formed by the insertion of the loop of one molecule into the β-sheet A of another. The crystal structure of an antithrombin dimer revealed another mechanism with the loop of one molecule inserting to replace strand 1 of β-sheet C of a second molecule (3, 4). Support for this hypothesis came from the antithrombin variant Rouen VI (18) and the Mmalton variant of α₁-antitrypsin (13), which were predicted to form short chain polymers terminated by a β-sheet C linkage. Epitope mapping of monoclonal antibodies specific to C1-inhibitor also suggested polymer formation via a C-sheet mechanism (22). More recently the crystal structure of plasminogen activator inhibitor-1 has raised the possibility of polymerization in which the reactive loop anneals as strand 7A (5). We have used recombinant α₁-antitrypsin with three cysteine variants to determine the structural mechanism of polymer formation. The data show conclusively that α₁-antitrypsin polymers form by a reactive loop:β-sheet A linkage.

MATERIALS AND METHODS

Wild type Pittsburgh α₁-antitrypsin (M358R) and three cysteine variants (S121C, D159C, and 1360C) were prepared and cloned into the pET16b plasmid as detailed previously (6). The S121C, D159C, and 1360C variants also carried the C232S mutation, ensuring that all mutants contained only one free cysteine residue. The α₁-antitrypsin sequence of each plasmid was confirmed by dideoxynucleotide sequencing.

Expression, Refolding, and Purification of Recombinant α₁-Antitrypsin—Each plasmid containing the mutated α₁-antitrypsin was transformed into BL21(DE3) cells (Novagen). The recombinant proteins were expressed and purified from inclusion bodies as detailed previously (6). Purity was confirmed by SDS and nondenaturing polacrylamide gel electrophoresis, and the proteins were dialyzed into 50 mM Tris, 50 mM KCl, 1 mM dithiothreitol, pH 7.4 and stored at −80 °C until required.

Characterization of Recombinant α₁-Antitrypsin—SDS and nondenaturing polyacrylamide gel electrophoresis, inhibitory activity, and
measurement of the rate of polymerization were performed as detailed previously (23). The proteins were labeled with 5-iodoacetamidofluorescein (5-IAF) and tetramethylrhodamine-5-iodoacetamide (5-TMRIA) according to the manufacturer's instructions (Molecular Probes Inc.). The labeled protein was separated from unreacted label with a NAP-10 (Amersham Pharmacia Biotech) gel filtration column. The 5-IAF labeling of each variant was adjusted to 50% by addition of the corresponding unlabeled mutant.

**Measurements of Fluorescence Resonance Energy Transfer (FRET)—**

All fluorescence measurements were made using a PerkinElmer Life Sciences LS-50B spectrofluorimeter. Spectra were measured at 25 °C using slit widths of 2.5 nm for the excitation beam and 4.0 nm for the emission beam. The 5-IAF label was excited at 495 nm, and emitted light was measured between 500 and 600 nm. Energy transfer between 5-IAF and 5-TMRIA was measured by recording the decrease in intensity of light emitted from 5-IAF. This method was used instead of measuring changes in acceptor signal, because the signal obtained after subtraction of the spectrum of a polymerized sample containing labeled acceptor and unlabeled donor.

**FRET Calculation—**

The change in fluorescence of the 5-IAF was measured by recording the decrease in intensity of light emitted from 5-IAF (24). This method was used instead of determining fluorescent intensity from the ratio of donor to acceptor fluorescence because its polar quality restricts quenching to fluorophore moieties on the surface of the protein (27–29). Unlike the charged quenchers such as iodide, acrylamide shows little sensitivity to the electrostatic environment around the fluorophore. The fluorescence changes were then corrected to take into account any error because of dilution, and the resulting quenched data were plotted. These data were analyzed with respect to the following Stern-Volmer equation (27).

\[
I/Q = 1 + k_{sv}[Q]
\]  
(Eq. 5)

where \(I_0\) is the initial fluorescence, \(I\) is the fluorescence intensity at concentration \(Q\) of quenching agent, and \(k_{sv}\) is the Stern-Volmer constant.

**Conformational Analysis—**

The reactive center loop between the P3 and P8 residues was modeled into a 0.5 Å grid with rotational grid spacings of 30°. This allowed exploration of all possible relative orientations at each grid point. Implementation of this method using translational and rotational (three translational and three rotational) freedom that define the relative positions of monomers within a polymer using the following method: 1) A cube was defined with 120 Å long sides positioned on the center of mass of an α1-antitrypsin molecule. 2) The cube was split up into a grid along all three axes with a spacing of 10 Å. 3) A second α1-antitrypsin molecule was translated to a point on the grid. 4) The second α1-antitrypsin was rotated about its center exploring all three axes of rotational space at 30° intervals. 5) For each rotation a new grid was defined with the second α1-antitrypsin at its center. 6) A third α1-antitrypsin molecule was placed using the same translation and rotations that were used to place the second molecule. 7) Steps 5 and 6 were repeated until a 7-mer polymer was created. 8) Theoretical transfer efficiencies \(E\) were calculated between the fourth monomer in the polymer and all other monomers using Equation 3 and the methods of Miki and Iio (31, 9). The total \(E\) value was calculated, and the structure was saved if it matched the experimental values. 10) The procedure was repeated allowing exploration of all possible relative orientations at each grid point.

**Table I**

| Measurement                  | S121C | D159C | Pittsburgh wild type | I660C | Wildtype antitrypsin |
|------------------------------|-------|-------|-----------------------|-------|----------------------|
| Unlabeled intrinsic fluorescence | 2.62 ± 0.34 × 10⁻⁵ | 2.37 ± 0.41 × 10⁻⁵ | 1.43 ± 0.33 × 10⁻⁵ | 3.65 ± 0.55 × 10⁻⁵ | 3.65 ± 0.20 × 10⁻⁵ |
| 5-TMRIA fluorescence         | 0.51 ± 0.25 × 10⁻⁵ | 2.10 ± 0.57 × 10⁻⁵ | 3.17 ± 0.58 × 10⁻⁵ | 2.85 ± 0.34 × 10⁻⁵ | 2.70 ± 0.03 × 10⁻⁵ |

The abbreviations used are: 5-IAF, 5-iodoacetamidofluorescein; 5-TMRIA, tetramethylrhodamine-5-iodoacetamide; FRET, fluorescence resonance energy transfer; RCL, reactive center loop.
RESULTS AND DISCUSSION

Polymerization of the Labeled Variants—Wild type Pittsburgh \( \alpha_1 \)-antitrypsin (M358R) and the S121C, D159C, and I360C variants were purified to homogeneity and migrated as a single band on SDS-polyacrylamide gel electrophoresis. They were 45, 71, 70, and 77% active, respectively, as inhibitors when assessed against bovine \( \alpha \)-chymotrypsin. The polymerization rates of the cysteine variants of \( \alpha_1 \)-antitrypsin in both native and derivatized form (with covalently bound 5-TMRIA) were measured by monitoring changes in both intrinsic tryptophan fluorescence and fluorescence of the bound probe (Table I) and were confirmed by nondenaturing polyacrylamide gel electrophoresis. The values were similar to those of wild type recombinant \( \alpha_1 \)-antitrypsin (358M), but polymerization of the four variants corresponded to an overall biphasic decrease in fluorescence. This is the reverse of that observed in our previous study using plasma derived protein and other recombinant mutants (23). The difference results from the redox state of Cys-323, which greatly affects the fluorescence signal from polymerization but does not affect the polymerization process itself (unpublished observations). The close proximity of Cys-323 to one of the two tryptophans, Trp-238 (6.5 Å), and the substitution of Cys-323 for a serine in the S121C, D159C, and I360C variants are likely to change the signal. Measurements of the changes in 5-TMRIA fluorescence during polymerization of each variant at 45 °C show that in three of the four variants the derivatization process does not alter the rate of polymerization. However the S121C mutation reduced the polymerization rate by 80%, which suggests that the region around residue 121 is important in the polymerization process.

Measurement of FRET between Polymerizing Molecules—We used the optical phenomenon of fluorescence resonance energy transfer to investigate the orientation of monomeric units within the \( \alpha_1 \)-antitrypsin polymer. \( \alpha_1 \)-Antitrypsin was labeled with fluorophores via cysteines at four locations throughout the protein. These residues were located 1) on the reactive center loop at residue 360, 2) on the C-sheet at residue 232, 3) at the bottom of \( \alpha \)A at residue 121, and 4) on the top of helix F at residue 159 (Fig. 1A). Mixtures were then prepared using protein labeled at 360 and 159 with 5-TMRIA to act as FRET acceptors and protein labeled at 121, 159, 232, and 360 with 5-IAF to act as FRET donors. These mixtures were then polymerized by incubation at 45 °C for 48 h. To ensure that no monomeric material remained in the sample, FRET efficiencies were also measured following incubation at 55 °C for 48 h. The results from this experiment were identical to those carried out at 45 °C. The efficiency of FRET was measured by comparing emission spectra derived from exciting at the FRET donor wavelength of polymerized material with and without an acceptor (Fig. 1B). Fluorescence anisotropy measurements were also made for each labeled protein to assess whether the labels showed an isotropic distribution and hence that the assumption of a value of 0.65 for \( \kappa^2 \) was valid. The values obtained ranged from 0.08 for the label on residue 232 to 0.26 for the label on residue 121. Assessment of the effect of the lack of isotropy on the value of \( R_0 \) was carried out using the methods of Dale et al. (34) and showed that this would affect the accuracy of \( R_0 \) by 1.1 Å at most. These data were then used in combination with the respective \( R_0 \) to calculate the distances between the labels within the polymer (Table II). The FRET efficiencies for polymerized \( \alpha_1 \)-antitrypsin were well within the region that allowed accurate values for \( R \) to be determined for all FRET pairs except 121–159. The measurement of FRET between proteins labeled at 159 and 360 in both directions (159 donor to 360 acceptor and 360 donor to 159 acceptor) provided an internal check of the consistency of FRET meas-

![Fig. 1.](image)
urements. The FRET efficiencies calculated from these 2 pairings were in very close agreement (159 → 360, \( E = 0.53 \); 360 → 159, \( E = 0.47 \)), providing confidence in the accuracy of the measurements.

Measurement of Stern-Volmer Constants—Stern-Volmer constants were derived from the linear slopes of fluorescence plotted against increasing acrylamide concentration. In each case the linear fit to the experimental data was achieved with a correlation coefficient (\( r \)) of greater than 0.98, thus indicating the presence of only collision quenching (27–29). Stern-Volmer constants were measured for each labeled mutant before and after polymerization (Table III and Fig. 2). These measurements allowed the change in solvent accessibility of the probe to be assessed during polymer formation. It might be expected that the transition of a protein from monomer to polymer must obscure at least some of the protein surface from solvent. Our measurements of Stern-Volmer constants for each labeled protein showed that in none of the four cases did \( k_{sv} \) decrease substantially upon transition to the polymeric form. A decrease in \( k_{sv} \) might have been predicted if the probe had become less solvent accessible because of the binding of a neighboring monomer in the polymer. In fact in two cases, following labeling at residues 121 and 360, the \( k_{sv} \) increased, which is indicative of the probe becoming more accessible to solvent.

Interpretation of FRET Distances and Stern-Volmer Values with Respect to Existing Models of Polymers—Three models were used in the initial assignment of FRET distances (Fig. 3A). These models represented the proposed A- and C-sheet linkages (2, 32) and the \( s7A \) linkage (5). Measurements were made between fluorophores in all possible \( \alpha_1 \)-antitrypsin molecules inhabiting three positions within the polymer either side of the monomer containing the donor fluorophore. This allowed for the possibility that helical arrangements of the polymers may bring nonconsecutive units into close contact (like the 1–4 interaction observed between amino acids in an \( \alpha \)-helix). Comparison of the values for \( E \) determined by FRET and those calculated from the three existing models show distinct differences in each case. For the A-sheet model, the values for the 121–360 and 159–360 pair show significant differences, although there is good agreement with all the values in which 159 acted as acceptor. In contrast the C-sheet model has discrepancies with the transfer efficiency values for all pairings apart from 232–159 and 159–159.

The Use of FRET Data to Build \( \alpha_1 \)-Antitrypsin Polymers—Conventional modeling techniques that have been employed in studies of the serpin-protease complex using FRET are not applicable to polymers. The FRET signal produced by a polymeric structure is complicated by the large number of possible interactions observed between amino acids in a \( \alpha \)-helix). Comparison of the values for \( E \) determined by FRET and those calculated from the three existing models show distinct differences in each case. For the A-sheet model, the values for the 121–360 and 159–360 pair show significant differences, although there is good agreement with all the values in which 159 acted as acceptor. In contrast the C-sheet model has discrepancies with the transfer efficiency values for all pairings apart from 232–159 and 159–159.

TABLE II

Comparison of FRET efficiencies with those calculated from modeled structures

| FRET pair        | Experimental FRET signal | Original A-sheet polymer (2) | C-sheet polymer (32) | Strand \( s7A \) A-sheet polymer (5) | 1         | 2         |
|------------------|--------------------------|-----------------------------|----------------------|------------------------------------|-----------|-----------|
| 121–360          | 0.55                     | 0                            | 0.10                 | 0.78                               | 0.55      | 0.57      |
| 159–360          | 0.53                     | >0.90                        | 0.30                 | >0.90                              | 0.83      | 0.90      |
| 232–360          | 0.14                     | 0.15                         | >0.90                | 0.47                               | 0.18      | 0.18      |
| 360–360          | 0.17                     | 0.08                         | >0.90                | 0.79                               | 0.12      | 0.21      |
| 121–159          | 0                        | 0.10                         | 0.08                 | 0.08                               | 0.01      | 0.03      |
| 159–159          | 0.14                     | 0.34                         | 0.12                 | 0.12                               | 0.03      | 0.06      |
| 232–159          | 0.90                     | >0.90                        | 0.12                 | 0.64                               | 0.85      | 0.95      |
| 360–159          | 0.47                     |                               |                      |                                    |           |           |

TABLE III

Comparison of Stern-Volmer values calculated from acrylamide quenching experiments

| Protein                       | Stern-Volmer constants |
|-------------------------------|------------------------|
|                               | Monomeric | Polymeric |
|                               | \( M^{-1} \) | \( M^{-1} \) |
| S121C                         | 0.35      | 0.56      |
| D159C                         | 0.41      | 0.48      |
| Pittsburgh wild type (232C)   | 0.54      | 0.59      |
| I360C                         | 0.26      | 0.50      |

FIG. 2. Plots showing the dependence of the fluorescence intensity of fluorescein on the concentration of acrylamide for four labeled proteins. The plots are shown for S121C, D159C, I360C, and wild type (232C) Pittsburgh \( \alpha_1 \)-antitrypsin, each with their single cysteine labeled with fluorescein. The open circles represent plots for protein in the monomeric form, and closed circles represent protein preincubated at 45 °C for 48 h.

Positions that donors and acceptors can occupy. This problem can only be solved if the polymer forms a regular extended three-dimensional structure in which FRET only occurs between near neighbors in the polymer. Electron micrographs of serpin polymers show this to be the case (11, 12). This greatly simplifies the situation allowing a solution to be determined by computation. For example, an acceptor molecule on monomer \( n \)
in an extended polymer may participate in FRET with a donor on the $n + 1$ monomer as well as $n + 2$, $n + 3$, etc., and $n - 1$, $n - 2$, etc. We have developed a computational method that automates this analysis. The program CharmM was used to develop an algorithm that performs an exhaustive search of possible conformations of $\alpha_1$-antitrypsin polymers. Calculations of theoretical efficiencies for transfers from $n - 3$ to $n + 3$ were then made for each new conformation, and a total efficiency for the conformation was calculated. This allowed us to test almost 3 million conformations. A heptameric model of the serpin polymer used as initial modeling studies showed that even in the most compact polymer, the FRET from monomers more distant than $n + 3$ and $n - 3$ was insignificant. Results from this search show that the parameters imposed by the experimental FRET results were only matched by 11 polymer structures. Closer examination of these structures showed that three could be discarded because they were represented by a polymer of monomers that were spaced too far apart to allow intermolecular linkage. Two structures were discarded as the monomeric units showed extensive overlap. The six remaining structures are shown in Fig. 3B. In all cases the monomers are arranged such that the RCL of one monomer is in close proximity to the A-sheet of a second monomer. Closer examination of the structures shows that these six candidates can be further classified into two groups: Group 1 contains five structures in which the orientation of the monomers places the RCL of one monomer close to the s7A-s4A position, and Group 2 contains one structure in which the orientation of the monomers places the RCL of one monomer close to the s2A-s3A position.

To simplify the analysis of these structures, a representative of each group was analyzed in more detail in Table II. These structures have five of seven FRET efficiency values within 0.05 of those determined by experiment. The other two anomalous FRET efficiency values were measured using experimental values determined using fluorescein attached to residue 159. This may suggest that the position of 159 in the crystal structure of the monomer used to build the polymers is different to the position of 159 in the polymer.

Stern-Volmer quenching constants were used to discriminate between s4A and s7A linkage. Perhaps unexpectedly there was no decrease in the solvent accessibility of any of the probes. However, closer examination of the positions of the labeled residues showed that none of them became buried in any of the polymer models. Measurement of the Stern-Volmer constant for protein labeled at position 121 showed an increase in solvent accessibility in the polymeric form. Examination of the positions of this residue in structures of $\alpha_1$-antitrypsin with and without insertions into the s4A position showed that upon insertion into s4A residue 121 becomes 25% more exposed. This demonstrates that the polymer must result from s4A linkage.

In summary the measurement of FRET using probes at different points within $\alpha_1$-antitrypsin has allowed an assessment of the viability of all possible structures of the $\alpha_1$-antitrypsin polymer. The results of this search demonstrate that candidate structures cluster into two groups. The use of Stern-Volmer quenching experiments have allowed us to propose that the linkage is by an insertion of the reactive loop of one molecule into s4A of a second. Understanding this linkage provides a firm basis for rational drug design to block reactive center loop-$\beta$-sheet A interactions and so ameliorate the associated disease.

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