A Kinetic Mechanism for the Polymerization of α₁-Antitrypsin*

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The mutation in the Z deficiency variant of α₁-antitrypsin perturbs the structure of the protein to allow a unique intermolecular linkage. These loop-sheet polymers are retained within the endoplasmic reticulum of hepatocytes to form inclusions that are associated with neonatal hepatitis, juvenile cirrhosis, and hepatocellular carcinoma. The process of polymer formation has been investigated here by intrinsic tryptophan fluorescence, fluorescence polarization, circular dichroic spectra and extrinsic fluorescence with 8-anilino-1-naphthalenesulfonic acid and tetramethylrhodamine-5-io daacetamide. These biophysical techniques have demonstrated that α₁-antitrypsin polymerization is a two-stage process and have allowed the calculation of rates for both of these steps. The initial fast phase is unimolecular and likely to represent temperature-induced protein unfolding, while the slow phase is bimolecular and associated with loop-sheet interaction and polymer formation. The naturally occurring Z, S, and I variants and recombinant site-directed reactive loop and shutter domain mutants of α₁-antitrypsin were used to demonstrate the close association between protein stability and rate of α₁-antitrypsin polymerization. Taken together, these data allow us to propose a kinetic mechanism for α₁-antitrypsin polymer formation that involves the generation of an unstable intermediate, which can form polymers or generate latent protein.

α₁-Antitrypsin is a member of the serpin protein superfamily that encompasses a wide range of serine proteinase inhibitors involved in coagulation, inflammation, fibrinolysis, and the complement cascade (1–3). The members of the superfamily have a common tertiary structure based on a central β-sheet (sheet A), surrounded by two other sheets (B and C) and a mobile, inhibitory reactive center loop (Fig. 1). The mechanism of action of these proteins as proteinase inhibitors is most unusual in comparison to other serine proteinases, as cleavage of the P₁–P₃ bond by non-target enzymes causes a large rearrangement of the serpin with the loop incorporated into the A β-sheet and the P₁ and P₃ residues separated by over 60 Å (4–6). The loop may be stably incorporated into the A β-sheet in the absence of cleavage by formation of the latent species (7–10). This is typically prepared by heating serpins for prolonged periods in the presence of stabilizing concentrations of sodium citrate (8, 10, 11). The description of this conformational change from x-ray crystal structures of the native (7, 8, 12–15), latent (7–9), and cleaved (4–6, 16) forms of the serpins, combined with the fact that the cleaved and latent forms have a much higher stability compared with the native species (10, 17–20), has led to the suggestion that the serpin fold is metastable (21, 22).

In 1992, we showed that severe deficiency of the Z variant of α₁-antitrypsin (Glu-342→Lys) resulted from a conformational transition and a unique linkage between the reactive center loop of one molecule and a β-sheet of a second (23). This process of polymer formation was temperature- and concentration-dependent (23, 24), and the polymers that formed had the appearance of “beads on a string” when visualized by electron microscopy (25). Since this initial report, investigations have been concerned with the characterization and classification of clinically relevant mutations. These studies have shown that polymerization can occur with a variety of deficiency mutants of α₁-antitrypsin (25–27) and in variants of antithrombin (28, 29) and Cl-inhibitor (30, 31) in association with thrombosis and angioedema, respectively. The structural mechanism by which serpin self-assembly occurs has not yet been determined, but biophysical data show that the polymeric form has an enhanced stability like that of the cleaved form of α₁-antitrypsin (10, 26, 32). Peptides with homology to the reactive center loop can insert into the A β-sheet of the native molecule to block polymerization in vitro (23, 24, 32), and these data suggest that polymerization occurs by sequential insertion of the reactive loop from one molecule into the A-sheet of another. X-ray crystal structures of a dimer of antithrombin have revealed an alternative intermolecular linkage between the reactive center loop of one molecule and the C β-sheet of a second (7, 8, 33). The precise mechanism associated with disease remains uncertain, and although both A- and C-sheet linkages can form in conditions that are dependent on the buffer (10, 34), the recent structures of native α₁-antitrypsin (13, 15) give support to a reactive loop: A β-sheet interaction in vivo.

We report here the molecular dynamics of α₁-antitrypsin polymer formation. Such a study is important, as it is now becoming apparent that a deeper understanding of the mechanism of polymerization is required to allow the production of mimetics to control diseases caused by serpin misassembly.

EXPERIMENTAL PROCEDURES

Purification of α₁-Antitrypsin—α₁-Antitrypsin was purified from human plasma by 50% and 75% ammonium sulfate fractionation, followed by thiol exchange and Q-Sepharose chromatography as detailed previously (24). α₁-Antitrypsin was also expressed in Escherichia coli using the vector pWombAT (35) and was purified from the crude E. coli cell extract by 8% and 28% w/v PEG1 8000 fractionation. The

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1 The abbreviations used are: PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis; 5-TMRIA, tetramethylrhodamine-5-iodoacetamide; ANSA, 8-anilino-1-naphthalenesulfonic acid.
against bovine by non-denaturing PAGE and a complete loss of inhibitory activity.

5,5

150 mM NaCl, pH 8.5) and eluting with a 0–75 mM glycine gradient.

D and labeled the reactive loop is centered on Glu-342

s5A and loop insertion, is shown as a
trols opening of the gap between s3A and

Determination, the protein being eluted off the column by a 0–0.4 M NaCl

resulting pellet was resuspended in 20 mM phosphate buffer, pH 6.8, containing 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% (v/v) β-mercaptoethanol and subjected to PAE300 chromatographic separation, the protein being eluted off the column by a 0–4 mM NaCl gradient. Fractions containing α1-antitrypsin were then further purified using zine-chelating Sepharose (equilibrated in 50 mM Tris-HCl, 150 mM NaCl, pH 8.5) and eluting with a 0–75 mM glycine gradient.

Finally, the fractions containing α1-antitrypsin were loaded onto a 5.5'-dithiobis(2-nitrobenzoic acid)-charged glutathione-Sepharose column (equilibrated in 100 mM Tris, 5 mM EDTA, pH 8.0), and eluted with 15 ml of 5.5'-dithiobis(2-nitrobenzoic acid)/dithiothreitol (24).

The proteins were stored in 50 mM Tris, 50 mM KCl, pH 7.4, and their concentration quantified by measurements of UV absorbance at 280 nm with an extinction coefficient (1 mg/ml) of 0.53. Purity was confirmed by 7.5–15% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE) and 7.5–15% (w/v) non-denaturing PAGE.

Preparation of Conformations of α1-Antitrypsin—M α1-antitrypsin polymers were prepared by heating plasma M α1-antitrypsin (0.25 mg/ml) at 60 °C for 3 h as described previously (24) and were confirmed by non-denaturing PAGE and a complete loss of inhibitory activity against bovine α-chymotrypsin (10). Cleaved α1-antitrypsin was prepared by incubation with Staphylococcus aureus V8 proteinase (24), which cleaved the P4–P5 bond of the reactive loop, and full cleavage was confirmed by a 4-kDa band shift on SDS-PAGE. Latent α1-antitrypsin was prepared by heating at 67 °C in 0.7 mM citrate for 12 h as detailed previously (10).

Fluorescence Measurements—Fluorescence measurements were made using a Perkin Elmer LS 50B spectrofluorimeter. Intrinsic tryptophan fluorescence of α1-antitrypsin was measured in 20 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, and 0.1% (w/v) PEG 8000, pH 7.4, using an excitation wavelength of 295 nm and detecting photons emitted at 90° to the excitation beam. Wherever possible, the slits controlling the intensity of the excitation light source were kept at the minimum machine-permissible limit of 2.5 nm; any other values for these slit widths were as detailed in the text. Emission slit widths were varied between 2.5 and 15 nm, dependent on the experimental conditions in order to give an optimal emission signal.

The majority of experiments used a 0.5-ml cuvette with a path length of 1 cm on the excitation axis and 0.2 cm on the emission axis. Throughout all experiments the sample temperature was maintained by a heated water jacket within the cuvette holder, the temperatures quoted within the text being those within the cuvette.

Fluorescence Polarization—Polarization experiments were carried out in 20 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, and 0.1% (w/v) PEG 8000, pH 7.4, using the same system as that for the fluorescence measurements with the addition of plane polarizing filters between the light source and the sample and between the sample and the detector. Measurements were made on a single sample with five repeat measurements for each time point with the polarizers in the parallel and perpendicular positions. A value for the fluorescence polarization was calculated from two samples using Equation 1 (see Ref. 36).

\[ P = \frac{I_v - G \cdot I_h}{I_v + G \cdot I_h} \]  
(Eq. 1)

P is the polarization of the sample, \( I_v \) and \( I_h \) are the parallel and perpendicular components of the fluorescence signal, and G is the instrument factor for the machine that normalizes the polarization introduced by the optical components of the detection system.

Production of Tetramethylrhodamine-5-iodoacetamide (5-TMRIA)-labeled α1-Antitrypsin—The single cysteine of plasma α1-antitrypsin was labeled with 5-TMRIA according to the manufacturer’s instructions (Molecular Probes, Inc.) This gave a labeling efficiency of 20%. Changes in the fluorescence characteristics of the probe during α1-antitrypsin polymerization were measured in 20 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, and 0.1% (w/v) PEG 8000, pH 7.4, by exciting at 543 nm and measuring light emitted at 567 nm. The results are the average of eight polymerization experiments.

8-Anilino-1-naphthalenesulfonic Acid (ANS) Binding—A saturated solution of ANSA was prepared in 50 mM Tris, 50 mM KCl, pH 7.4, and filtered through a 0.2-μm pore size filter. Fifty μl of this stock solution was added to 500 μl of 20 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, and 0.1% (w/v) PEG 8000, pH 7.4, in a fluorescence cuvette. The experiment was initiated by the addition of α1-antitrypsin (typically 50 μl) to the cuvette to give a final concentration of 0.1 mg/ml. The fluorescence changes of the ANSA during α1-antitrypsin polymerization were then measured by exciting with light at 370 nm and detecting fluorescence at 450 nm.

Circular Dichroism—Circular dichroism (CD) experiments were undertaken using a JASCO J-720 spectropolarimeter. Samples were prepared using 50 mM phosphate buffer (pH 7.4), and CD spectra of samples were collected using a 0.05-cm path length quartz cuvette at an α1-antitrypsin concentration of 0.5 mg/ml. Changes in the secondary structure of α1-antitrypsin with time and temperature were measured by monitoring the CD signal at 222 nm with the protein at 0.5 mg/ml in 50 mM phosphate, pH 7.4. The temperature within the cuvette was maintained by a computer-controlled water bath connected to a water jacket integral to the cuvette holder, and monitored by a sensor directly located in the holder. Thermal unfolding experiments were performed using a heating rate of 60 °C/h and measuring the change in signal at 222 nm. The second derivative of the resulting data was then used to calculate the inflection point of the transition and hence Tm.

Data Fitting—The kinetic data were fitted to single or double exponential functions of the following type:

\[ F = A_0(1 - e^{-k_1t}) \]  
(Eq. 2)

for a single transition (Equation 2) or

\[ F = A_0(1 - e^{-k_2t}) + A_0(1 - e^{-k_{20}t}) \]  
(Eq. 3)

for a double transition (Equation 3), where \( A_0 \) and \( A_0 \) are the amplitudes of the phase, with rates of \( k_1 \) and \( k_{20} \), and \( t \) is time. The choice of the curve used in the fit was determined by the one that gave the
smallest fit was calculated using Grafit (version 3.00, 1992, Erithacus Software Ltd).

RESULTS

Intrinsic Tryptophan Fluorescence—The polymerization of α₁-antitrypsin is both concentration- and temperature-dependent (24). Plasma α₁-antitrypsin was incubated over a range of temperatures and concentrations to obtain the optimum conditions for the assessment of rates of polymer formation by intrinsic tryptophan fluorescence. At temperatures over 50 °C, the rate was too fast to dissect out the different phases of polymerization, and at lower temperatures, the rate was too slow to be assessed during a 24-h incubation. At 45 °C and 0.1 mg/ml, there was an increase in intrinsic tryptophan fluorescence (Fig. 2a) and a 2.5-nm blue shift, which was 50% complete by 36,000 s (10 h), 80% complete by 80,000 s (22 h), and 95% complete after 151,200 s (42 h). Mathematical analysis of the change in the fluorescence intensity with time revealed a fast and slow exponential phase. Examination of the protein at various time points throughout polymerization by non-denaturing PAGE showed no high molecular mass species in the first 7200 s (2 h; Fig. 2b) and then a rate of decrease in intensity of the native band, which agreed well with the slower rate calculated from intrinsic tryptophan fluorescence (Fig. 2c). In order to demonstrate the specificity of these signals, the experiment was repeated with α₁-antitrypsin cleaved at the reactive center loop. This form is unable to undergo polymerization (24) and showed no change in tryptophan fluorescence when incubated under the same conditions. The phenomena reported by the tryptophan fluorophores during the fast and slow phase of polymerization were assessed with a variety of other spectroscopic methods (Table I).

Fluorescence Polarization—Measurement of the polarization of the fluorescence from tryptophans within the protein showed a decrease in fluorescence polarization with time (Fig. 3a). This indicates an increase in the speed of motion of the tryptophans and may be explained by either an increase in the rate of motion of this moiety within the protein scaffold or a decrease in tumbling time of the protein overall (36). The rate of decrease in fluorescence polarization over 6000 s was similar to the rate of the fast phase observed with intrinsic tryptophan fluorescence (Table I). The slow phase was not resolved with this technique.

Extrinsic Fluorescence—The changes in properties of α₁-antitrypsin during polymer formation were assessed following labeling of cysteine 232 (Fig. 1) with an extrinsic rhodamine probe, 5-TMRRA. The fluorophore reported an increase in fluorescence and a 2.0-nm blue shift with a rate constant similar to that of the slow phase detailed previously (Fig. 3c). Further analysis showed a fast signal change (Inset) followed by a larger decrease in fluorescence which occurs at a rate close to that of the slow phase. These data show that in the early stages of the experiment α₁-antitrypsin transiently exposes hydrophobic domains before the slow reburial of these exposed residues.

Circular Dichroism—Changes in the far-UV CD spectra were observed between the five-stranded native and the six-stranded reactive loop cleaved and latent forms of α₁-antitrypsin (Fig. 4a). An increase in the magnitude of the signal at 222 nm was observed as α₁-antitrypsin formed polymers when incubated at 0.1 mg/ml and 45 °C (Fig. 4b). These data were the mean of five experiments and allowed the calculation of a fast phase (Table I), which closely matched that observed by intrinsic fluorescence. Spectra were also taken at the beginning of the experiment and after 24 h when the polymerization was 80% complete as assessed by change in fluorescence signal (Fig. 4a). This shows a decrease in intensity at 222 nm (the region associated with α-helix content) of only 0.25 × 10⁻² millidegrees cm² dmol⁻¹, which is compatible with α₁-antitrypsin polymerization. Polymers were also prepared by heating α₁-
antitrypsin at 0.25 mg/ml at 50 °C for 12 h in 50 mM Tris, 50 mM KCl, pH 7.4 (Fig. 4c). The far-UV CD profile of these polymers was significantly more negative than that of native α1-antitrypsin and α1-antitrypsin polymers formed at 45 °C (Fig. 4a). The Concentration Dependence of the Fast and Slow Phase of Polymerization—Previous studies have shown that the overall rate of α1-antitrypsin polymer formation was dependent on protein concentration (24). The fast phase of the fluorescence signal during polymerization was unaffected by protein concentration, confirming that the process was unimolecular (data not shown). The slow rate did show dependence on concentration, which became non-linear at high protein concentrations (Fig. 5). Incubation of α1-antitrypsin at 0.1 mg/ml and 45 °C with stabilizing sodium citrate, which favors the formation of the latent protein, reduced the rate of polymerization by approximately 15-fold (Fig. 5).

pH Dependence of Polymerization—The results obtained during these studies suggest that increasing the conformational flexibility of α1-antitrypsin plays an important role in increasing the proteins’ propensity to polymerize. This was further assessed by changing the solution pH to destabilize the protein and assessing the rate of polymerization with intrinsic fluorescence. The rate of polymerization was measured at pH 5, 6, 7, 8, and 9 using buffers with protonation states that were insensitive to temperature changes. Rates of polymerization were considerably increased at high and low pH (Fig. 6a), and this was associated with reduced melting temperatures as assessed by CD spectra at 222 nm (Fig. 6b).

Effect of Point Mutations on Rates of Polymerization—Our interest in the polymerization of α1-antitrypsin results from its association with plasma deficiency, cirrhosis, and emphysema (38). Thus detailed studies of the effect of medically relevant mutations on the protein’s behavior are important. In this study, we have taken advantage of the availability of such mutants and have studied their propensity for polymerization using the methods detailed above. Six α1-antitrypsin variants were used in this study, which represent both naturally occurring variants purified directly from blood plasma (Z, S, I, and M α1-antitrypsin) and mutations introduced into a recombinant framework (wild type, Phe-51 → Leu, Glu-354 → Gln, and Glu-354 → Ser α1-antitrypsin) and expressed in E. coli. Analysis of these variants showed that an increased rate of polymer formation by the protein correlated with a reduced melting temperature (Table II).

**TABLE I**

| Measured Effect                                      | Rate at 1 mg/ml | Rate at 0.1 mg/ml |
|------------------------------------------------------|-----------------|-------------------|
| Intrinsic fluorescence                               | 6.0 ± 0.13 × 10⁻⁴ | 3.65 ± 0.20 × 10⁻⁵ |
| Fluorescence polarization                             | 2.7 ± 0.9 × 10⁻⁴ | ND                |
| 5-TMRIB fluorescence                                 | ND              | ND                |
| ANSA fluorescence                                    | 6.9 ± 0.16 × 10⁻⁴ | 4.1 ± 0.05 × 10⁻⁵ |
| CD                                                    | 3.9 ± 0.2 × 10⁻⁴ | ND                |

**DISCUSSION**

The primary objective of this study was a detailed analysis of the process of α1-antitrypsin polymer formation. The data from tryptophan fluorescence and the extrinsic probe ANSA showed that there were two processes with 10-fold different rates during polymer formation. The faster of the two processes was independent of protein concentration and was observed as the sole process during the measurement of fluorescence polarization and circular dichroism. This is consistent with a conformational change occurring within the protein induced by a

**Fig. 3.** Measurement of the changes in conformation of plasma α1-antitrypsin during polymerization at 0.1 mg/ml and 45 °C. Panel a, tryptophan fluorescence polarization. Panel b, fluorescence produced by exciting covalently linked rhodamine at 492 nm with a slit width of 2.5 nm and measuring emitted light at 540 nm through an 8.0-nm slit. Inset is the fluorescence profile for the first 1000 s. Panel c, the fluorescence produced by exciting ANSA at 370 nm with a slit width of 2.5 nm and measuring emitted light at 450 nm through an 8.0-nm slit. Inset shows the increase in fluorescence between 0 and 10,000 s, which corresponds to the fast phase of Fig. 2a.
change in temperature. This process did not alter the fluorescent characteristics of the rhodamine probe bound to Cys-232 in the C-sheet, implying that the conformational change occurs elsewhere in the protein. The change in tryptophan fluorescence must result from the perturbation of tryptophan residues at either 194 or 238 (Fig. 1). Trp-238 is on the surface of α_{1}-antitrypsin, 6.5 Å from Cys-232, with no stable hydrogen bonds visible in the native structure (13, 15). It seems improbable that a conformational change within the protein could increase the fluorescence of Trp-238, and indeed site-directed mutagenesis has shown that this residue makes no contribution to tryptophan fluorescence on refolding of α_{1}-antitrypsin (39). Thus, increasing temperature perturbs the environment of Trp-194 in α_{1}-antitrypsin, reflecting disorganization of the strands at the top of the Aβ-sheet.

The slower of the two processes was also observed as changes in tryptophan and ANSA fluorescence and the fluorescence of rhodamine linked to Cys-232. This process is dependent on protein concentration and was associated with the formation of high molecular mass loop-sheet polymers on non-denaturing PAGE. The fact that the fluorescence of a moiety attached to the C-sheet is affected during polymer formation is consistent with the proximity of the residue to the reactive loop, which provides the “linker” segment in models of loop-sheet polymers (13, 40). Although the slow phase of polymer formation was dependent on protein concentration, at high concentrations the process was saturated (Fig. 5). This suggests that, at high concentrations, a second process, such as nonspecific aggregation competes with polymer formation. On the basis of these results, we propose the following kinetic mechanism for the polymerization of a serpin.

\[
\begin{align*}
    & \text{Step 1} & \text{Step 2} \\
    & M + M & \rightarrow M^* + M^* \\
    & k_1 & \rightarrow P \\
    & k_2 & L \\
    & k_3 & \\
\end{align*}
\]

**Scheme I**

Step 1 represents the conformational change of the serpin to a polymorphic monomeric form (M*), step 2 represents the formation of polymers (P), and step 3 represents a side pathway that leads to the formation of the latent conformation (L).

If polymer formation is a two-step process, then we would expect the rate of conversion from M to P ($k_2$) to be dependent on the concentration of M* and hence the rate of M $\rightarrow$ M* ($k_1$).

Our data show that the difference in rates between the two processes is on the order of 10-fold (Table I). Thus, following the initiation of the reaction, there should be a short lag in the production of P as the concentration of M* rises. Two of our experiments measure only the production of P: non-denaturing
PAGE and rhodamine fluorescence. Rhodamine fluorescence indeed shows a small lag in the increase in signal (Fig. 3b, inset) in the early stages of the experiment, which would agree well with a build-up of M*. As further proof, comparisons of simulations of the production of P with time (using numerical solutions to differential equations describing the above mechanism), with the data from rhodamine fluorescence show good agreement. Parameters extracted from these simulations also give a value for $k_1$ ($5.2 \times 10^{-4}$ s$^{-1}$) that is very similar to those shown in Table I. Further to this, non-denaturing gels shows no higher molecular weight species forming in the first 7200 s, again suggesting a lag in the formation of polymers. Taken together, these data support the proposal that polymerization is a sequential two-step process: an initial fast conformational change within the protein producing a polymogenic intermediate, which then undergoes loop-sheet linkage.

FIG. 6. Panel a, rates of $\alpha_1$-antitrypsin polymer formation over a range of pH. The rates were determined by measuring the change in intrinsic tryptophan fluorescence of a 0.1 mg/ml solution of plasma $\alpha_1$-antitrypsin at 45 °C, exciting at 295 nm (2.5-nm slit) and measuring light emitted at 340 nm (6.0-nm slit). The resulting data were fitted to Equation 3 and $k_{app}$ obtained as the polymerization rate. Panel b, the melting points of $\alpha_1$-antitrypsin at different pH values were measured by monitoring the CD signal at 222 nm while increasing the sample temperature at 60 °C/h. Inset is a plot of the melting point obtained versus the solution pH.
latent $\alpha_1$-antitrypsin (17) disrupted fluorescence measurements due to solution turbidity. A lower concentration of citrate was therefore used (0.5 mM), which produced insufficient latent $\alpha_1$-antitrypsin to be visualized by non-denaturing PAGE. Despite this, the citrate reduced the rate of polymerization by an average of 15-fold (Fig. 5). This suggests that the action of citrate (even when present at a suboptimal concentration) was to reduce the value of $k_{app}$ and favor the formation of latent protein.

This kinetic scheme also allows the interpretation of the effects of destabilization of the protein by extremes of pH and mutations on the rate of conformational change (Fig. 6 and Table II). Any factor that decreases protein stability (as assessed by a reduced melting temperature) will result in an increase in the equilibrium concentration of M$^*$ and so will increase the formation of both polymers and the latent conformation. Naturally occurring deficiency mutants of the serpins cluster in the shudder domain (Siiyama and Mmalton $\alpha_1$-antitrypsin) and the proximal (Z $\alpha_1$-antitrypsin) and distal hinge regions (38). These have been predicted to open the A $\beta$-sheet of the protein, thereby increasing M$^*$ and favoring the formation of polymeric and latent protein at lower activation temperatures (21, 38). Mutants that increase the stability of the serpins (such as Phe-51 → Leu $\alpha_1$-antitrypsin) will reduce M$^*$ and so reduce conversion to the latent or polymerized conformation (22, 41). Thus, it is possible to explain the observation that mutant serpins with reduced thermal stabilities more readily undergo polymerization (Table II).

Circular dichroism may be used to assess conformational transitions of proteins and major differences are apparent in the far UV spectra between the native (five-stranded) and cleaved (six-stranded) forms of the inhibitory serpins (18). This technique was therefore used to assess a second six-stranded species, latent $\alpha_1$-antitrypsin, with the anticipation that it would be similar to the cleaved form (Fig. 4a). Surprisingly, the CD profile of latent $\alpha_1$-antitrypsin was very different from cleaved $\alpha_1$-antitrypsin, which suggests that the reactive loop may not be fully inserted into the A $\beta$-sheet in the latent species or that the position of s1C is important in the far-UV CD profile, as it will be fixed in the C-sheet in the cleaved structure (4) but must be displaced in latent $\alpha_1$-antitrypsin (8). The change in CD signal at 222 nm was also used to assess the conformational change that was associated with polymerization to give a fast rate that was very similar to that obtained by intrinsic fluorescence (Fig. 4b and Table I). The far-UV CD profile of $\alpha_1$-antitrypsin polymerized at 45 °C for 24 h was markedly different from that obtained after polymerizing $\alpha_1$-antitrypsin at 50 °C and 0.25 mg/ml for 12 h (Fig. 4a). The polymers formed at 50 °C are longer (10), and the tendency to aggregate makes it difficult to accurately determine protein concentration and thereby the position of the CD profile in relation to native $\alpha_1$-antitrypsin.

The phenomena of proteins self-assembling to form a polymeric structure may be mechanistically classified into two subtypes (42). The first type represents those most commonly encountered in which self-assembly is mediated by surface interactions between folded proteins. This sort of interaction causes sickle cell hemoglobin to polymerize and proteins to crystallize. The second type of self-assembly has been studied with great interest over the past few years, as it is central to the mechanisms of the transmissible spongiform encephalopathies and Alzheimer’s disease. The self-assembly event of $\alpha_1$-antitrypsin fits well with this form of oligomerization (43), as $\alpha_1$-antitrypsin polymerization, like the amyloid protein transthyretin (44–46), is enhanced by destabilizing the structure of the protein with extremes of pH and point mutations. It also seems reasonable to think of native $\alpha_1$-antitrypsin as a folding intermediate (21, 22), albeit a very stable one with the end product of the folding pathway being the six-stranded latent form of the protein.

In summary, we present here a detailed analysis of the processes underlying the polymerization of $\alpha_1$-antitrypsin, linking a variety of spectroscopic signals to changes that occur within the protein. We have also investigated the effect that mutations which interfere with these processes have on polymerization, and have reinforced the link between serpin polymerization and amyloid-like self-assembly.

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REFERENCES
1. Travis, J., and Salvesen, G. S. (1983) Annu. Rev. Biochem. 52, 655–709
2. Gettins, P., Patston, P. A., and Schapira, M. (1993) BioEssays 15, 461–467
3. Potempa, J., Korzus, E., and Travis, J. (1994) J. Biol. Chem. 269, 15957–15960
4. Loebermann, H., Tokunaga, R., Deisenhofer, J., and Huber, R. (1984) J. Mol. Biol. 177, 531–556
5. Baumann, U., Huber, R., Bode, W., Grosse, D., Lesjak, M., and Laurell, C.-B. (1991) J. Mol. Biol. 218, 595–606
6. Baumann, U., Bode, W., Huber, R., and Potempa, J. (1992) J. Mol. Biol. 226, 1207–1218
7. Schreuder, H. A., de Boer, B., Dijkema, R., Mulders, J., and Hol, W. G. J. (1994) Eur. J. Biochem. 226, 273–276
8. Carrell, R. W., Stein, P. E., Fermi, G., and Wardell, M. R. (1994) Structure 2, 257–270
9. Mattonen, J., Strand, A., Symskry, J., Sweet, R. M., Danley, D. E., Geoghegan, K. F., Gerard, R. D., and Goldsmith, E. J. (1992) Nature 355, 270–273
10. Lomas, D. A., Elliott, P. R., Chang, W.-s. W., and Carrell, R. W. (1995) J. Biol. Chem. 270, 5282–5288
11. Lomas, D. A., Elliott, P. R., and Carrell, R. W. (1997) Eur. Respir. J. 10, 672–675
12. Wei, A., Rubin, H., Cooperman, B. S., Schechter, N., and Christianson, D. W. (1992) J. Mol. Biol. 236, 273–276
13. Elliott, P. R., Lomas, D. A., Carrell, R. W., and Abrahams, J.-P. (1996) Nat. Struct. Biol. 3, 676–681
14. Ryu, S.-E., Choi, H.-J., Kwon, K.-S., Lee, K. N., and Yu, M.-h. (1996) Structure 4, 1181–1192

### Table II

The polymerization rates for naturally occurring and recombinant mutants of $\alpha_1$-antitrypsin at 45 °C and 0.1 mg/ml measured by the change in intrinsic tryptophan fluorescence with time

| $\alpha_1$-Antitrypsin variant | Environment of mutation | $T_m$ (°C) | $k_{app}$ ($s^{-1} \times 10^{-5}$) |
|-----------------------------|------------------------|---------|-----------------|
| Phe-51 → Leu (recombinant)  | Shuttle                | 63.6    | 3.9 ± 0.1       |
| WT (plasma)                 | Shuttle                | 61.4    | 4.7 ± 0.1       |
| S Glu-264 → Val (plasma)    | Shuttle                | 60.9    | 9.7 ± 0.4       |
| I Arg-39 → Cys (plasma)     | Shuttle                | 60.6    | 4.9 ± 0.0       |
| WT (recombinant)            |                        | 59.6    | 10.1 ± 0.4      |
| Glu-354 → Ser (recombinant) | Reactive center loop   | 58.8    | 32.7 ± 1.3      |
| Glu-354 → Gln (recombinant) | Reactive center loop   | 58.8    | 20.7 ± 0.3      |
| Z Glu-342 → Lys (plasma)    | Proximal hinge         | 53.4    | 70.4 ± 4.1      |

The phenomena of proteins self-assembling to form a polymeric structure may be mechanistically classified into two subtypes (42). The first type represents those most commonly encountered in which self-assembly is mediated by surface interactions between folded proteins. This sort of interaction causes sickle cell hemoglobin to polymerize and proteins to crystallize. The second type of self-assembly has been studied with great interest over the past few years, as it is central to the mechanisms of the transmissible spongiform encephalopathies and Alzheimer’s disease. The self-assembly event of $\alpha_1$-antitrypsin fits well with this form of oligomerization (43), as $\alpha_1$-antitrypsin polymerization, like the amyloid protein transthyretin (44–46), is enhanced by destabilizing the structure of the protein with extremes of pH and point mutations. It also seems reasonable to think of native $\alpha_1$-antitrypsin as a folding intermediate (21, 22), albeit a very stable one with the end product of the folding pathway being the six-stranded latent form of the protein.

In summary, we present here a detailed analysis of the processes underlying the polymerization of $\alpha_1$-antitrypsin, linking a variety of spectroscopic signals to changes that occur within the protein. We have also investigated the effect that mutations which interfere with these processes have on polymerization, and have reinforced the link between serpin polymerization and amyloid-like self-assembly.

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