An antibody raised against a pathogenic serpin variant induces mutant-like behaviour in the wild-type protein

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A monoclonal antibody (mAb) that binds to a transient intermediate may act as a catalyst for the corresponding reaction; here we show this principle can extend on a macro molecular scale to the induction of mutant-like oligomerization in a wild-type protein. Using the common pathogenic E342K (Z) variant of α_1-antitrypsin as antigen – whose native state is susceptible to the formation of a proto-oligomeric intermediate – we have produced a mAb (5E3) that increases the rate of oligomerization of the wild-type (M) variant. Employing ELISA, gel shift, thermal stability and FRET time-course experiments, we show that mAb_{5E3} does not bind to the native state of α_1-antitrypsin, but recognizes a cryptic epitope in the vicinity of the post-helix A loop and strand 4C that is revealed upon transition to the polymerization intermediate, and which persists in the ensuing oligomer. This epitope is not shared by loop-inserted monomeric conformations. We show the increased amenity to polymerization by either the pathogenic E342K mutation or the binding of mAb_{5E3} occurs without affecting the energetic barrier to polymerization. As mAb_{5E3} also does not alter the relative stability of the monomer to intermediate, it acts in a manner similar to the E342K mutant, by facilitating the conformational interchange between these two states.

Key words: aggregation, antibody, α_1-antitrypsin, polymerization, protein stability.

INTRODUCTION

α_1-Antitrypsin (*SERPINA1*) is an acute-phase glycoprotein, secreted by hepatocytes and abundant in the plasma. A member of the serpin superfamily, the primary role of α_1-antitrypsin is the inactivation of neutrophil elastase in the lung. The pathophysiological importance of this protein is underscored by individuals harbouring loss-of-function mutations, who have a pre-disposition to emphysema [1], and those who exhibit a gain-of-function phenotype: the formation of recalcitrant protein deposits in the endoplasmic reticulum (ER) of the cell of synthesis. These inclusions atypically fail to induce the ER unfolded protein response [2,3], but cause distension of the organelle, interfere with protein diffusion [4] and can lead to liver cirrhosis [5]. Currently, the only commonly practised clinical interventions are replacement therapy, which is both costly and controversial as to its efficacy [6], and liver transplantation, where end-stage liver disease has ensued [5].

The most common pathological variant associated with α_1-antitrypsin deficiency is the Z allele (E342K), which is present in $\sim 4\%$ of Northern Europeans. This mutation has been shown to facilitate the transition between the native state and a conformation that is prone to the formation of ordered aggregates, termed polymers [7]. Biophysical approaches have indicated that polymer formation proceeds from monomer through several unimolecular 'activation' steps [8,9]. The pathway culminates in a bimolecular association that, being essentially irreversible, renders the process under kinetic control [10]. Polymers isolated from patients homozygous for the Z allele have an unbranched beads-on-a-string morphology [11,12], and several hypotheses as to the underlying inter-molecular linkage have been advanced based on biophysical [11–14] and crystallographic [15] evidence. Chief among these are the A-sheet model [11], the β-hairpin model [14] and the C-terminal model [15]. Although it remains to be demonstrated which of these polymer states reflects those present in patient populations, these disparate proposals suggest that under certain conditions α_1-antitrypsin can access multiple end-point polymer states. Nevertheless, the inherent stability and size heterogeneity of the final polymer form renders it a non-ideal target for therapeutic interventions to mitigate the intra-hepatic formation or deposition of polymers. Instead, manipulation of the polymerization pathway itself represents a more tractable approach [16]. However, a detailed molecular understanding of this pathway requires tools for probing its intermediates.

Many examples exist of antibodies and related molecules that exhibit conformation-specific recognition of targets such as transthyretin [17], superoxide dismutase [18] and serpins, including plasminogen activator inhibitor-1 [19] and α_1-antitrypsin [20]. In addition, some antibodies are known to alter the behaviour of their cognate antigen, such as stabilizing against [21] and inducing [22] conformational change. From a screen developed to identify monoclonal antibodies (mAbs) that alter the rate of polymerization of α_1-antitrypsin, we identified one, mAb_{5E3}, that demonstrated both conformational specificity and antigen-altering activity. This molecule was found to recognize a cryptic epitope revealed in the transition to a polymerization

intermediate, and that persists in the ensuing polymer. Both the pathogenic E342K variant purified from patient plasma, and the wild-type M variant in the presence of the mAb, showed a similar polymerization activation energy to wild-type protein alone. Thus, this mAb increases conformational flux between native and activated forms without affecting their relative thermodynamic stabilities and without interfering with subsequent oligomerization. These data show that an antibody raised against an unstable mutant can act as a molecular template to induce aberrant behaviour in a wild-type protein.

EXPERIMENTAL

Preparation of α_1-antitrypsin

M (AT_{WT}) and Z (AT_{E342K}) α_1-antitrypsin were isolated from plasma of PI*MM and PI*ZZ α_1-antitrypsin homozygotes as detailed previously [12]. Control AT_{WT} or AT_{E342K} heat-induced polymers were prepared by heating the monomeric protein (0.2 mg·ml^{-1}) at $60\,^{\circ}$C in phosphate-buffered saline (PBS) (pH 7.4) for 3 h, and confirmed by non-denaturing PAGE. Denaturant-induced polymers were generated using 3M guanidinium chloride in PBS for 48 h at room temperature. Variants of recombinant α_1-antitrypsin were generated by site-directed mutagenesis, expressed in *Escherichia coli* XL-1 Blue, and purified to homogeneity by affinity chromatography using nickel-nitrilotriacetate (NTA)–S sepharose (Qiagen) and Q Sepharose (GE Healthcare Life Sciences) as detailed previously [9,10]. Fab fragments were generated using a commercial papain-based kit according to the manufacturers' instructions (Thermo Scientific).

Production and screening of antibodies that alter polymerization of AT_{E342K}

Balb/c mice were immunized with monomeric AT_{E342K}, and spleen cells were harvested and fused with myeloma cells. The supernatants of the resulting hybridoma clones were screened first by antigen-mediated ELISA using purified AT_{E342K} monomer as the antigen; those of selected clones were then heated at $45\,^{\circ}$C for 45 h in the presence of 20 μg·ml^{-1} AT_{E342K}, and the consequent polymers were quantified using an ELISA with the polymer-specific antibody mAb_{2C1} and a non-conformation sensitive antibody, mAb_{9C5} [20]. To verify whether identified antibodies were interfering with the assay itself, a competitive ELISA was performed in which the plate was coated with polymer-specific mAb_{2C1}, loaded with excess polymer antigen, and different dilutions of cell culture supernatants were applied. Detection was by horseradish peroxidase (HRP)-conjugated mAb_{9C5}. The clone showing increased polymerization levels with respect to a media-only control was sub-cloned by limited dilution, expanded as a cell line, and characterized by antigen-mediated, competitive, polyclonal sandwich and monoclonal sandwich ELISA.

Antigen and sandwich ELISA

The protocols used have been described previously [20]. Plates were coated overnight with antigen-purified rabbit polyclonal anti-α_1-antitrypsin antibody at 2 μg·ml^{-1} in PBS for sandwich ELISA, or purified AT_{E342K} in PBS for antigen-mediated ELISA. Following this they were washed with 0.9 % (w/v) sodium chloride and 0.05 % (v/v) Tween 20 and blocked for 2 h with 300 μl per well of blocking buffer (PBS, 0.25 % BSA, 0.05 % Tween 20 and 0.025 % sodium azide). For sandwich ELISA,

samples were diluted in blocking buffer to a final volume of 50 μl, added to the plate and incubated for 2 h. After washing, for both types of ELISA the wells were incubated for 2 h with the primary mAbs (mAb_{5E3} or polymer-specific mAb_{2C1}) at a concentration of 1 μg·ml^{-1} diluted in blocking buffer, the wells were washed again and rabbit anti-mouse HRP-conjugated antibody (Sigma) was added for 1 h. After removing residual secondary antibody, the reaction was developed for 10 min with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (Sigma–Aldrich), stopped with 1 M H_2SO_4, and an endpoint measurement of HRP activity at 450 nm was made using a SpectraMax M2 plate reader (Molecular Devices). All steps were performed at room temperature.

Native thermal stability assays

Experiments, using the SYPRO Orange fluorescent reporter (Life Technologies) were performed as detailed previously [10], and the temperature midpoint was determined by application of an equation describing thermal denaturation to the data [23,24]:

$$F_T = F_N + m_N(T - T_m) + (F_I + m_I(T - T_m))\frac{e^{C(\frac{1}{T_m} - \frac{1}{T})}}{1 + e^{C(\frac{1}{T_m} - \frac{1}{T})}}$$

where F_T is the fluorescence at temperature T, T_m is the transition midpoint temperature, F_N and F_I are the fluorescence of the native and intermediate states, m_N and m_I describe their temperature dependence around temperature T_m, and $C = \Delta H_m/R$, the enthalpy of unfolding at T_m divided by the gas constant. As the reaction is not at equilibrium [10] the optimized value of ΔH_m was not used.

Thiol labelling of plasma α_1-antitrypsin

α_1-Antitrypsin has a single endogenous surface-exposed cysteine residue (Cys^{232}) that is readily conjugated with thiol-reactive probes [8,25]. Variants of α_1-antitrypsin were reduced using 20 mM β-mercaptoethanol, buffer exchanged into PBS, and incubated with a 10-fold molar excess of Alexa Fluor 488 (AF488) maleimide or Alexa Fluor 594 (AF594) maleimide (Life Technologies) overnight at $4\,^{\circ}$C with reaction quenching using 5 mM cysteine. Application to, and elution from, a 1 ml HiTrap Q Sepharose column was used to remove remaining free label.

FRET-based polymerization assays

α_1-Antitrypsin, labelled with fluorophores at residue Cys^{232}, was diluted to 0.1 mg·ml^{-1} in PBS in the presence and absence of 0.45 mg·ml^{-1} antibody and a final volume of 10 μl. Polymerization was reported by an increase over time in FRET between the AF488 donor dye (excitation at 470 nm) and the AF594 acceptor dye (emission recorded at 605 $\pm$ 15 nm) upon heating in a Realplex^4 quantitative PCR instrument (Eppendorf). The Förster radius of this pair is $\sim$60 Å (1 Å = 0.1 nm) [26], which makes them useful at distances predicted by the different models of polymerization to arise between monomers in a repeating polymer [14,15,25,27,28]. The increase in FRET signal was found to be satisfactorily fitted by a single or double sigmoidal curve in Prism (GraphPad):

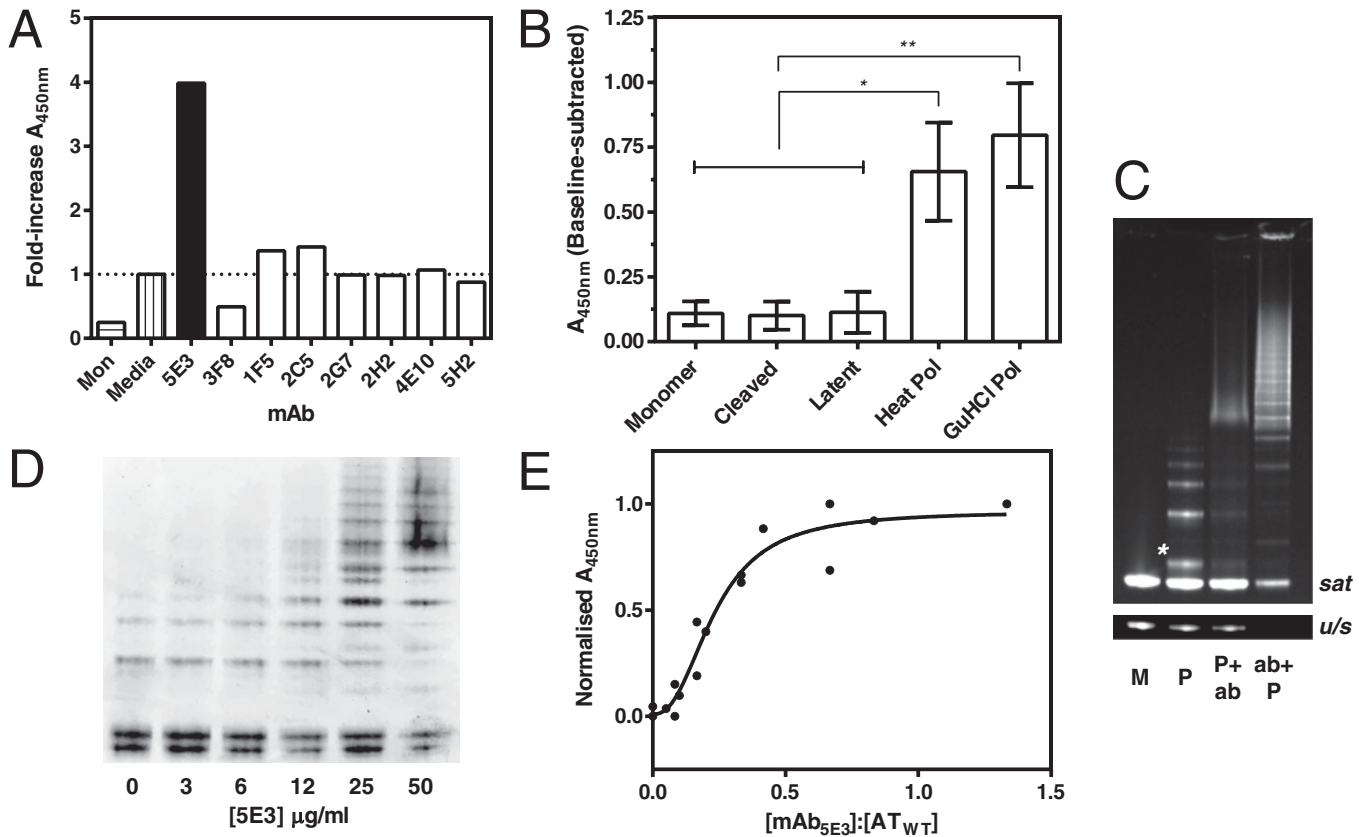$$F_t = H \times t^2 \times \left(\frac{w}{(t_{0.5,1}^2 + t^2)} + \frac{(1-w)}{(t_{0.5,2}^2 + t^2)}\right) + L$$

Figure 1   Screens for mAbs that alter polymerization, and the specificity of mAb$_{5E3}$

(**A**) Cell culture supernatants of hybridoma clones including 5E3 (black bar) were incubated at 45°C for 45 h with AT$_{E342K}$ at 20 μg·ml^{-1}, and polymer levels were quantified by sandwich ELISA using plates coated with the polymer-specific mAb$_{2C1}$ and detection by mAb$_{9C5}$ conjugated to HRP. Values shown are fold-increases in polymer levels with respect to a media-only control (shaded bar). (**B**) A sandwich ELISA performed using plates coated with anti-α_1-antitrypsin polyclonal antibody, bound for 2 h with serially diluted conformers of AT$_{WT}$, and detection by mAb$_{5E3}$ at 1 μg·ml^{-1} and an HRP-conjugated secondary antibody. After baseline subtraction the data at different dilutions were fitted to a four-parameter logistic function and the values at a total antigen load of 2.5 ng recorded (mean$\pm$S.E.M. for four independent experiments). A one-way ANOVA using Bonferroni's correction indicated a significant difference between polymers and monomers (*$P < 0.05$; **$P < 0.01$). (**C**) Gel shift experiment using AF488/AF594-labelled AT$_{WT}$ protein separated by 3–12% (w/v) non-denaturing PAGE: 'M', unheated monomer; 'P', monomer heated at 53°C for 12 h; 'P + ab', the same heated monomer mixed with a 1.5-fold molar excess of mAb$_{5E3}$ 5 min prior to electrophoresis; and 'ab + P', monomer incubated for 5 min with mAb$_{5E3}$ prior to heating at 53°C for 12 h. Detection was by fluorescence using a UV transilluminator. The upper panel has deliberately been overexposed ('sat') to highlight the oligomeric region of the gel; the lower panel shows the affected monomer region at a lower intensity ('u/s'). The asterisk indicates a species with a migration intermediate between monomer and dimer that appears to represent a polymerization intermediate [33]. (**D**) Purified AT$_{E342K}$ was incubated at 20 μg·ml^{-1} for 45 h at 45°C in the presence of 0–50 μg·ml^{-1} mAb$_{5E3}$ and the resulting polymers were quantified by silver-stained non-denaturing PAGE. (**E**) Combined data from mAb$_{2C1}$ ELISA following incubation of 20 μg·ml^{-1} or 100 μg·ml^{-1} AT$_{E342K}$ for 45 h at 45°C with a 0–1.33-fold molar ratio of mAb$_{5E3}$. The resulting absorbance readings from each of three experiments were normalized to lie between 0 and 1, and the abscissa shows the molar ratio of mAb$_{5E3}$ to α_1-antitrypsin concentration. The curve reflects a four-parameter logistic equation; a half-maximal effect was observed at a molar stoichiometry of 0.23 $\pm$ 0.03.

where F_t is the fluorescence at time t, H is the dynamic range of the curve, L is the baseline, w is the fraction contribution to the signal by the curve with a half-time of $t_{0.5,1}$ and $(1 - w)$ is the fraction contribution to the signal by the curve with a half-time of $t_{0.5,2}$. This equation permitted numerical calculation by the software of the overall time to half-maximal fluorescence intensity.

Gel-based experiments

Bis-Tris non-denaturing PAGE (3–12% (w/v) acrylamide) (Life Technologies) was used to determine the oligomeric state of α_1-antitrypsin and perform gel shift experiments, with 2–4 μg of protein per lane for staining with Coomassie Blue and 0.05–2 μg for fluorescence detection using a UV transilluminator. This gel system was also used for mobility shift experiments; antibodies cause a decreased rate of α_1-antitrypsin migration due both to the increased size of the antibody–antigen complex and an elevated overall isoelectric point. Densitometry was performed using ImageJ [29].

RESULTS

Identification of a monoclonal antibody that increases the rate of polymerization

We set out to identify, from a panel of mAbs raised against an oligomerization-prone variant of α_1-antitrypsin, one that could accelerate polymerization, and determine whether it could induce comparable behaviour in the wild-type protein. Standard protocols were followed to identify hybridomas expressing mouse monoclonal antibodies against purified Z α_1-antitrypsin (AT$_{E342K}$) [20,30]. Aliquots of cell culture supernatant from these hybridomas were mixed with AT$_{E342K}$, heated at 45°C for 45 h, and polymer levels were quantified by a sandwich ELISA using plates coated with the polymer-specific mAb$_{2C1}$ and detection of bound polymers with conformation-insensitive mAb$_{9C5}$–HRP [20]. One clone, 5E3, increased the level of polymer ∼4-fold with respect to a media-only control (Figure 1A), and the effect was confirmed using purified antibody in a sandwich ELISA (Supplementary Figure S1A) and interference assay (Supplementary
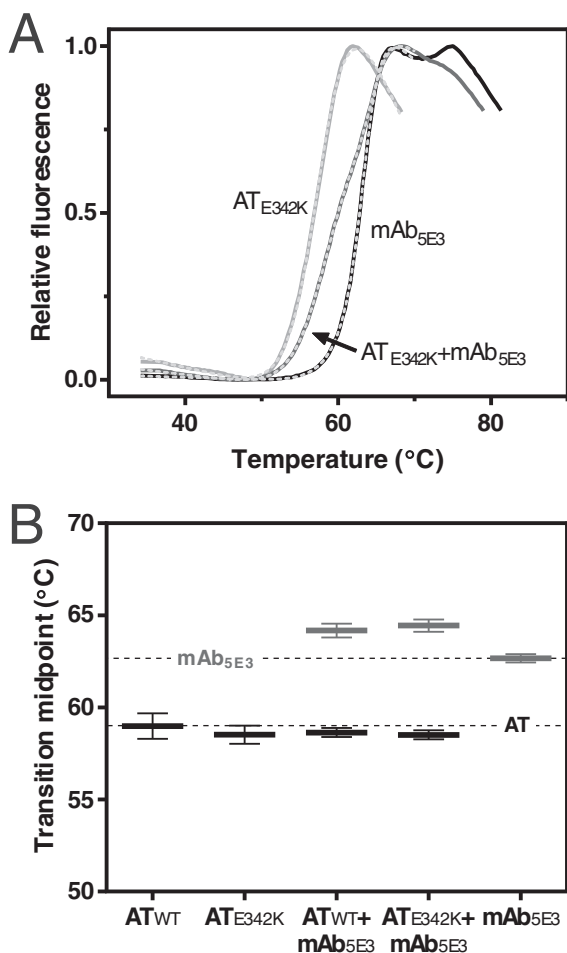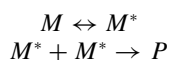
concentration and the activation energy barrier to oligomer formation. Consequently, an increased polymerization rate could be achieved through changes in thermodynamic stability of different components of the pathway or a reduction in energy required to transition from one state to the next [7]. Thus, any conformational selectivity could play a role in the apparent polymerization-enhancing activity of mAb$_{5E3}$.

Experiments were performed to characterize any preferential binding of mAb$_{5E3}$ to different conformations of α_1-antitrypsin. A Western blot of heated AT$_{E342K}$ samples indicated that mAb$_{5E3}$ is able to recognize heat-induced polymer (Supplementary Figure S1C). However, it is unlikely that binding to the terminal polymer form would increase the rate of polymerization, as this form is itself extremely stable [11,31] with at best marginal dissociation reported [32]. Recognition of different monomer conformations and heat- and guanidium chloride-induced polymers were compared by ELISA (Figure 1B). The three main proposed models for the structure of the pathological polymer countenance a monomeric unit that is in a loop-inserted conformation, consistent to varying degrees with the cleaved and latent states of the protein. However, these results revealed a distinct preference for polymer over monomer, and in contrast with mAb$_{2C1}$ [20], no clear selectivity between denaturant- and heat-induced polymer forms.

Monoclonal antibodies migrate more slowly in non-denaturing gels than α_1-antitrypsin due to their larger size and higher isoelectric point. We exploited this in performing a gel shift experiment using AT$_{WT}$ that had been fluorescently labelled at Cys^{232} with AF488 and AF594 dyes (Figure 1C). M α_1-antitrypsin (AT$_{WT}$) was polymerized at 53°C for 12 h, either in the presence of a 1.5-fold molar excess of mAb$_{5E3}$ (lane 4) or alone (lane 2) and pre-made polymers were also mixed with the same proportion of antibody 5 min prior to electrophoresis by non-denaturing PAGE (lane 3). A comparison of lanes 2 and 3 of the gel revealed sequestration of the oligomeric material by the antibody into a slower-migrating species, without a reduction in intensity of the monomer band. Interestingly, a band interposed between monomer and dimer was also bound by the antibody; such a species has been identified previously as a hallmark of the polymerization intermediate [33]. AT$_{WT}$ polymerized in the presence of mAb$_{5E3}$ demonstrated an increased quantity of higher-order material and a corresponding decrease in the amount of monomer left at the end of the incubation, providing a secondary confirmation of the polymerization-enhancing activity.

mAb$_{5E3}$ increases polymerization in a dose-dependent manner

The experiments in Figures 1(B) and 1(C) indicate that mAb$_{5E3}$ is able to form a stable complex with polymer and possibly an intermediate species as well. If a stable interaction is the basis of its activity, then the antibody would be expected to act in a dose-dependent fashion at a comparable range of concentrations to α_1-antitrypsin. A contrasting scenario would be one in which the antibody acts as a catalyst by transiently binding and stabilizing a transition state on the polymerization pathway, which could occur at markedly lower molar ratios. Aliquots of AT$_{E342K}$ were incubated with different concentrations of mAb$_{5E3}$ at 45°C for 45 h, and the presence of polymers was identified by non-denaturing PAGE (Figure 1D) and quantified by ELISA using mAb$_{2C1}$ (Figure 1E). Both techniques were found to report a dose-dependent increase in polymers at higher mAb$_{5E3}$ concentrations with respect to the control, with the latter data fitted to a four-parameter logistic equation yielding an EC$_{50}$ value of 0.23 ± 0.03. As IgG are divalent, this is equivalent to a binding-site ratio of
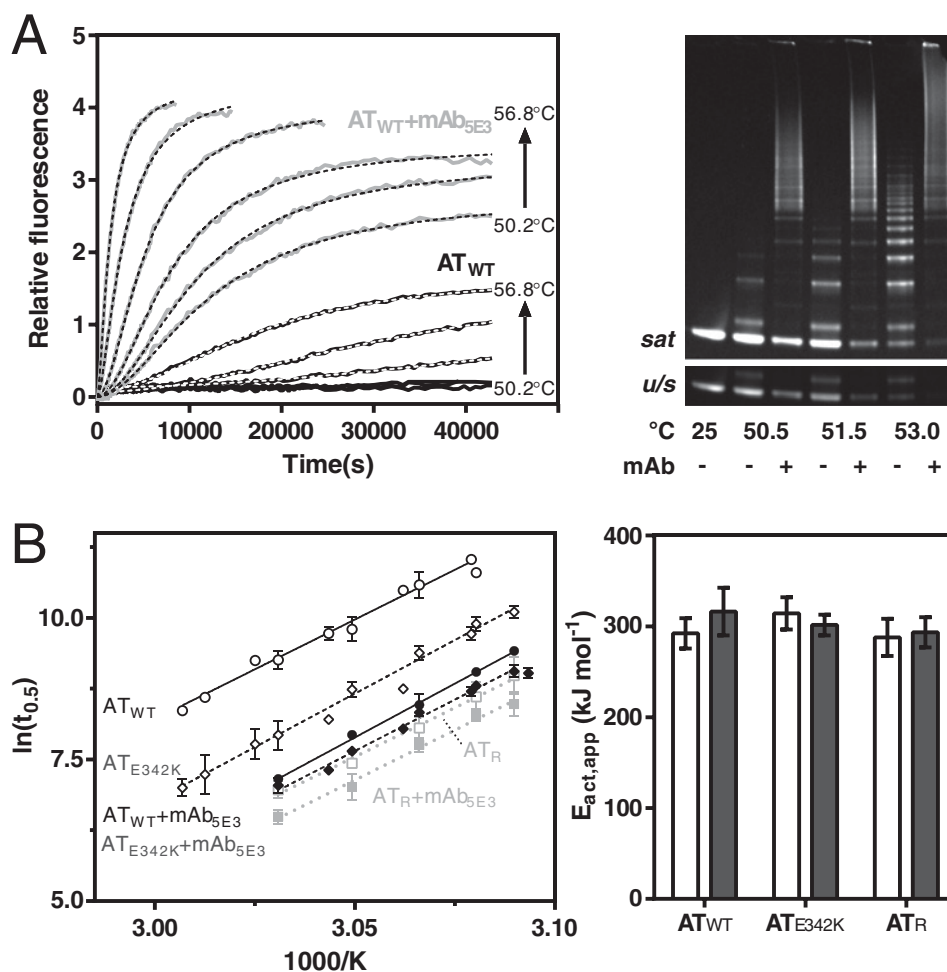
Figure 2   Effect of mAb$_{5E3}$ on the transition to a polymerization intermediate

(**A**) A representative SYPRO Orange thermal shift experiment using 0.1 mg · ml^{-1} AT$_{E342K}$ in the presence (medium grey) and absence (light grey) of a 1.5-fold molar excess of mAb$_{5E3}$ (black). Samples were heated from 25°C to 95°C at a rate of 1°C · min^{-1}. The fluorescence of SYPRO Orange with α_1-antitrypsin reports transition to a polymerization intermediate [10]. Transition midpoints were inferred for each protein by non-linear regression, shown as a white overlaid dashed line, as described in the text. (**B**) Thermal midpoints of denaturation were calculated for 0.1 mg · ml^{-1} AT$_{WT}$, AT$_{E342K}$ and a 1.5-fold molar concentration of mAb$_{5E3}$ alone or in combination. Data were fitted by a single or double two-state unfolding equation, used to de-convolute the contribution from mAb$_{5E3}$ (grey) and α_1-antitrypsin (black) components. The temperature gradient was 1°C · min^{-1}. Error bars reflect $\pm$SEM of at least four individual experiments.

Figure S1B). Subsequent experiments made use of purified mAb$_{5E3}$.

mAb$_{5E3}$ shows specificity for heat- and denaturant-induced polymers

Polymerization is a kinetically controlled process, and comprises at least one unimolecular activation step and a bimolecular association step [9]:

$$M \leftrightarrow M^*$$
$$M^* + M^* \rightarrow P$$

The consequence of this scheme is that the reaction does not reach equilibrium; there is a positive flux of monomer incorporated into oligomers in a manner influenced by protein

Figure 3   mAb$_{5E3}$ increases the rate of α_1-antitrypsin polymerization

(**A**) Left panel: an equimolar population of AT$_{WT}$ labelled either with a donor (AF488) fluorophore or an acceptor (AF594) fluorophore was incubated at 0.1 mg · ml^{-1}, and polymerization was monitored as an increase in FRET. Curves representing samples incubated at different temperatures in the presence (grey lines) and absence (black lines) of 0.45 mg · ml^{-1} mAb$_{5E3}$ are shown. Overlaid dashed lines (black and white, respectively) show the curves of best fit. Right panel: 0.1 mg · ml^{-1} AT$_{WT}$ labelled with AF488 and AF594 maleimide heated at various temperatures for 12 h in the absence and presence of a 1.5-fold molar ratio of mAb$_{5E3}$, separated by non-denaturing PAGE and visualized using a UV transilluminator. The upper panel has been overexposed ('*sat*') and the lower panel shows the affected monomer region at a lower intensity ('*u/s*'). (**B**) Left panel: an Arrhenius analysis of polymerization half-times as determined from FRET-based progress curve recorded at temperatures of 50–60°C for 0.1 mg · ml^{-1} AT$_{WT}$ (black, unbroken lines, circles), AT$_{E342K}$ (medium grey, dashed lines, diamonds) and AT$_R$ (light grey, dotted lines, squares) in the presence (shaded symbols) or absence (open symbols) of a 1.5-fold molar concentration of mAb$_{5E3}$. The data arise from at least four independent experiments and are means ± S.D. of two or more observations at a given temperature. Right panel: the apparent activation energy (mean±S.E.M.) for the polymerization of α_1-antitrypsin was calculated from the slopes of linear regressions. There was no significant difference between the different forms of α_1-antitrypsin in the presence and absence of mAb$_{5E3}$, with values of ~300 kJ · mol^{-1}.

0.46:1 required to achieve a half-maximal effect. Thus, mAb$_{5E3}$ most likely operates by binding in a near-stoichiometric fashion to a component of the polymerization pathway following activation of the monomer species.

mAb$_{5E3}$ has no effect on the equilibrium between native and intermediate forms

Thermal shift experiments using SYPRO Orange report the transition from the α_1-antitrypsin native state to a pre-polymer intermediate [10], and thus should indicate whether the antibody plays an active role in this process. From normalized thermograms (Figure 2A), it was possible to obtain thermal midpoint temperatures (T_m) of the individual components alone or in combination by non-linear regression of a double two-state unfolding equation (Figure 2B). It was observed that the presence of the antibody had a negligible effect on the transition midpoint

of α_1-antitrypsin, but a slight stabilization (by 1.7 ± 0.3°C) of the antibody. As mAb$_{5E3}$ unfolding occurs at a higher temperature than the conversion of α_1-antitrypsin to intermediate, this is probably to be the result of stabilization due to complex formation either with the intermediate or a later-stage species such as polymer.

Progress curves report a mAb$_{5E3}$-induced increased rate of polymerization

AT$_{WT}$ was labelled via the endogenous cysteine residue at position 232 with AF488 maleimide and AF594 maleimide dyes. Despite stark differences, the various polymer models broadly agree that fluorophores of adjacent monomers would be brought within a distance of less than 80 Å in the polymer chain [8,14,15,27,28]. Thus, temperature-induced polymerization could be monitored based on the increase in FRET between this pair of probes – whose Förster radius is 60 Å [26] – with excitation of AF488

Sample	$t_{0.5}$ at 53°C (s)	E_{act} (kJ · mol^{-1})
AT$_{WT}$	37.8(±2.3) × 10^3	292 ± 17
AT$_{WT}$ + mAb$_{5E3}$	4.9(±0.3) × 10^3	316 ± 26
AT$_{E342K}$	10.7(±0.7) × 10^3	314 ± 17
AT$_{E342K}$ + mAb$_{5E3}$	3.8(±0.1) × 10^3	301 ± 11
AT$_R$	3.3(±0.2) × 10^3	287 ± 20
AT$_R$ + mAb$_{5E3}$	2.2(±0.1) × 10^3	293 ± 17

at 470 nm and emission recorded from AF594 at 605 nm (±15 nm). Figure 3(A, left panel) shows progress curves recorded for AT$_{WT}$ at temperatures between 50.2°C and 56.8°C. A clear stimulatory effect was seen in the presence of the antibody, even at temperatures otherwise with no detectable polymerization, highlighted by a non-denaturing gel (Figure 3A, right panel), which showed at the conclusion of the experiment a depleted monomer and substantial amount of sequestered polymer in the presence of mAb$_{5E3}$ with respect to antibody-free controls. This acceleration was not due simply to a template mechanism by the antibody, as in dose–response experiments characteristics of the FRET curves were found to be monophasic with respect to concentration, rather than bell-shaped (Supplementary Figures S1D–S1E).

mAb$_{5E3}$ does not affect the polymerization activation energy

In the typical range of temperatures used to induce polymerization, the rate at which α_1-antitrypsin polymers form shows an Arrhenius-type temperature dependence [9,10]. AT$_{E342K}$ and recombinant wild-type (AT$_R$) were fluorescently labelled in the same manner as AT$_{WT}$. Both have an inherently increased rate of polymerization with respect to the wild-type protein: AT$_{E342K}$ has a decreased kinetic stability against conversion to the polymerization intermediate [7], whereas, due to expression in *E. coli*, AT$_R$ lacks the three glycan chains present in plasma-derived material (on aparagine residues at positions 46, 83 and 247), which are associated with maintaining a compact monomeric state [34]. Half-times of polymerization were derived using an empirical sigmoidal, or double-sigmoidal, function from FRET progress curves of AT$_{WT}$, AT$_{E342K}$ and AT$_R$ in the presence and absence of mAb$_{5E3}$ at temperatures between 50°C and 60°C (Figure 3B, left panel). Consistent with its well-characterized tendency to form polymers, polymerization half-times for AT$_{E342K}$ alone were lower than for AT$_{WT}$. The rate of polymerization of both variants was substantially increased in the presence of mAb$_{5E3}$, such that the two became almost equivalent in behaviour, as seen in the strikingly similar regression lines. Thus, despite the intrinsic kinetic instability of AT$_{E342K}$ [7], mAb$_{5E3}$ caused a convergence of the response of both variants to thermal challenge. Notably, the rates for the glycosylated plasma proteins in the presence of antibody are comparable to AT$_R$ (expressed in *E. coli* and therefore non-glycosylated) without antibody. The presence of mAb$_{5E3}$ caused a detectable, but less pronounced, acceleration of AT$_R$ polymerization.

Apparent activation energy ($E_{act,app}$) values were calculated from the linear regressions (Table 1). At ∼300 kJ · mol^{-1} – consistent with previous calculations for wild-type recombinant α_1-antitrypsin [10] – no significant difference was observed
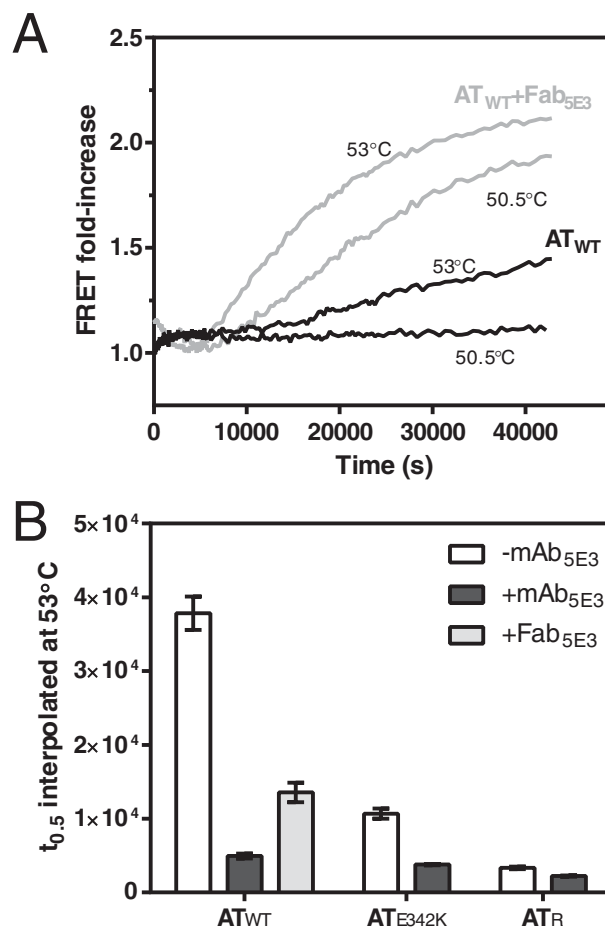
Figure 4   Increased rate of α_1-antitrypsin polymerization in the presence of Fab$_{5E3}$

(**A**) Representative FRET-based progress curves showing the polymerization of AT$_{WT}$ in the presence (grey lines) and absence (black lines) of a 1.5-fold concentration of Fab$_{5E3}$. (**B**) Calculated polymerization half-times (means ± S.E.M.) at a reference temperature, 53°C, from at least nine independent data points.

between AT$_{E342K}$, AT$_{WT}$ or AT$_R$ either in the presence or in the absence of antibody (Figure 3B, right panel). This analysis shows that neither the Z mutation, or the absence of glycan chains from asparagine residues at positions 46, 83 and 247, nor the presence of mAb$_{5E3}$ affects the overall energetic barrier to polymerization.

A monovalent Fab fragment increases the rate of polymerization of AT$_{WT}$

To determine the extent to which divalent binding by mAb$_{5E3}$ contributes to the observed polymerization rates, Fab$_{5E3}$ fragments were generated by digestion with papain and tested on AT$_{WT}$ in FRET-based time-course experiments. As with the full antibody, the induction of polymerization at an otherwise non-favoured temperature was observed (Figure 4A). To quantify this effect, a comparison was made between the half-times of polymerization at a reference temperature (Figure 4B). It was found that the presence of Fab$_{5E3}$ induced a similar rate of polymerization in AT$_{WT}$ to that of AT$_{E342K}$, with a half-time of $13.5(\pm0.4)\times10^3$ s at 53°C. Thus, binding of the mAb$_{5E3}$ epitope by a monovalent ligand resulted in behaviour comparable to that of the antigen against which the antibody had been raised.
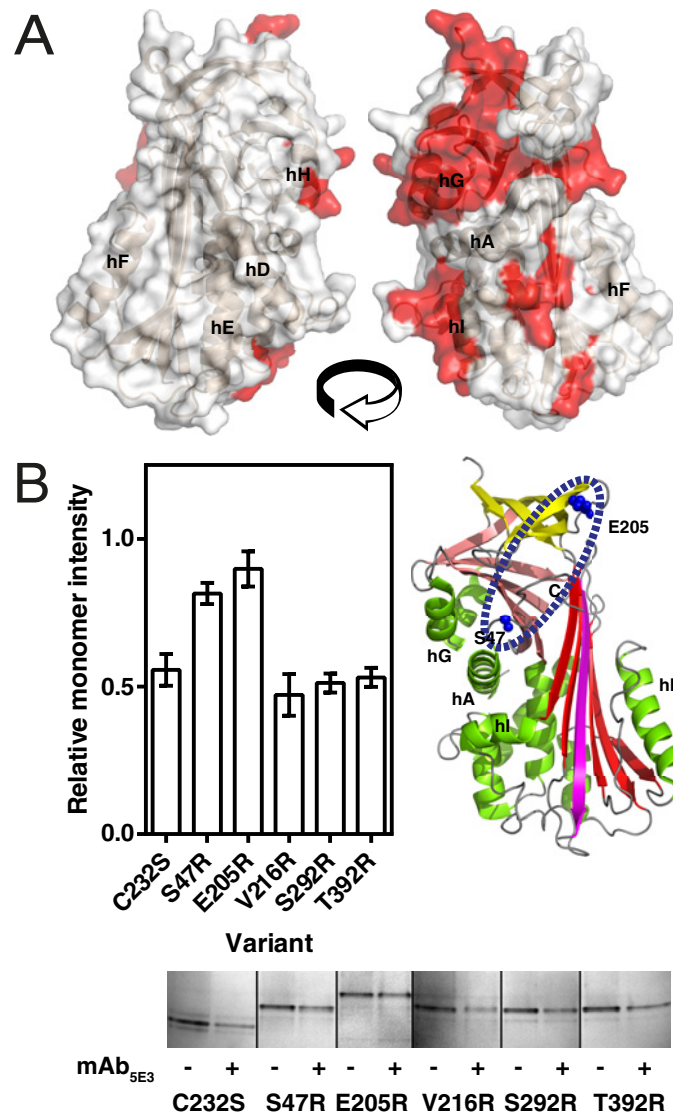
Figure 5   Site of the mAb$_{5E3}$ epitope

(**A**) A molecular surface representation of loop-inserted α_1-antitrypsin (PDB accession 1EZX, chain A), used to approximate the parsimonious conformation of the monomer proposed by the three main models for the terminal polymer [10], with β-sheet A oriented along the Z plane. Peptide fragments associated with transition to a heat- or denaturant-induced polymerization intermediate were identified by reconciling three previous studies [35–37] as described in the text. (**B**) Left panel: several surface-exposed residues that were situated on the face of α_1-antitrypsin primarily implicated in intermediate formation were mutated to arginine to explore possible involvement in the mAb$_{5E3}$ cryptic epitope. Recombinant protein was heated at 54°C for 30 min in the presence and absence of mAb$_{5E3}$ and residual monomer was quantified using densitometry of non-denaturing PAGE gels (a representative gel is shown in the bottom panel). Results are mean±S.E.M. proportions of residual monomer for each variant in the presence of mAb$_{5E3}$ relative to its absence, calculated from four independent experiments. Right panel: mutations that reduced the effect of mAb$_{5E3}$ on polymerization are shown on a schematic representation of loop-inserted α_1-antitrypsin. Structure figures were prepared using PyMOL (http://www.pymol.org).

The mAb5E3 epitope is in the vicinity of residues Ser[47] and Glu[205]

Although polymers induced by heat share a conformational epitope with *ex vivo* material that denaturant-induced polymers do not [33], mAb$_{5E3}$ is able to recognize both forms (Figure 1B). On this basis, an attempt was made to reconcile regions of α_1-antitrypsin implicated by three previous studies in the transition to intermediate, but that do not change in the transition from intermediate to polymer, based on observations in the literature. Peptide fragments were shortlisted that (a) were among nine exhibiting the greatest difference in hydrogen/deuterium (H/D) exchange between native and intermediate states [35]; (b) were also among nine fragments having the least difference in H/D exchange between intermediate and polymer [35]; and (c) also

spanned at least one residue identified by NMR [36] or surface accessibility experiments [37]. The resulting surface map of α_1-antitrypsin revealed a starkly polarized molecule (Figure 5A).

From this analysis, several initial sites were selected for arginine scanning mutagenesis. Six mutants were expressed in *E. coli* (C232S, S47R, E205R, V216R, S292R and T392R) and purified to homogeneity. Gel-based quantification of recombinant polymer is usually inferred from the loss of monomer following non-denaturing PAGE [38]. These mutants were subjected to a 30-min incubation at 54°C, expected from the AT$_R$ polymerization data in Figure 3(B) to yield roughly a 50% depletion of monomer in the presence of mAb$_{5E3}$. The relative amount of residual monomer in the absence and presence of mAb$_{5E3}$ was quantified by non-denaturing PAGE and densitometry in four experiments
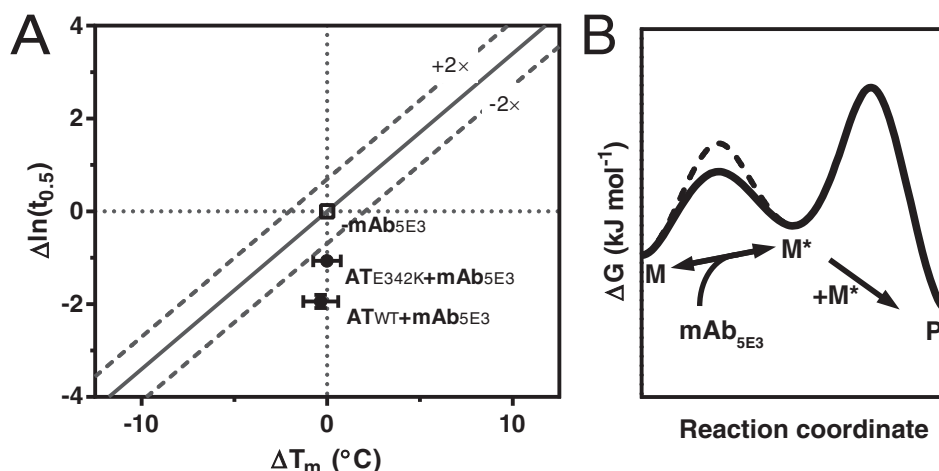
Figure 6   Proposed mechanism for the action of mAb$_{5E3}$

(**A**) A stability-polymerization analysis [10] which compares the average difference in T_m to the change in the natural logarithm of polymerization half-time. The α_1-antitrypsin consensus curve determined in that paper is shown as an unbroken line, flanked by dashed lines which represent a 2-fold greater or lesser rate of polymerization than predicted from effects on native state stability alone. Upon binding to mAb$_{5E3}$, the rate of polymerization increases in a manner that is not accounted for by changes in native state stability for AT$_{WT}$ or AT$_{E342K}$ (circles). (**B**) Notional energy landscape in which the presence of mAb$_{5E3}$ facilitates interchange via a stabilized transition state between the native (M) and intermediate (M*) forms, without affecting the stability of either the intermediate or the oligomerization transition state that yields polymer (P).

(Figure 5B, left and bottom panels). Both the S47R or E205R mutants, on the post-helix A loop and strand 4C, respectively, showed relative insensitivity to the presence of mAb$_{5E3}$ under these conditions, suggesting that they are components of the epitope (Figure 5B, right panel).

DISCUSSION

Effector molecules can be used as tools to probe conformational transitions and thereby provide a means to understand molecular mechanism [21]. In the present study, we have characterized a mAb that increases the rate of polymerization in a dose-dependent manner (Figure 1E), shows a strong preference for polymer over the non-activated monomer (Figures 1B and 1C), does not affect native state stability against change to an activated intermediate (Figure 2B), and does not preferentially act on the barrier between activated monomer and polymer (Figure 3B). However, a stability-polymerization analysis [10] shows that the effect on the polymerization rate for both AT$_{WT}$ and AT$_{E342K}$ variants is greater than expected from negligible effects on native state stability (Figure 6A). Additionally, the antibody interacts with a species that is a hallmark of a polymerization intermediate (Figure 1C), induces polymerization at otherwise non-polymerization-permissive temperatures (Figures 3A and 4A) and increases the rate of polymerization of both the endogenously glycosylated M and Z variants of α_1-antitrypsin to a level comparable to that of non-glycosylated recombinant protein (Figure 3B). Monovalent interaction by the Fab fragment induces the wild-type variant to behave like that of the Z variant alone (Figure 4B).

With an epitope that appears to include Ser[47] and Glu[205] (Figure 5B), this antibody bridges two important regions of α_1-antitrypsin: Ser[47] lies adjacent to the Asn[46] glycosylation site, and Glu[205] is proximate to Thr[203], which forms an interaction with Glu[342], the site of the Z mutation. Given the unexpected coincidence of mAb$_{5E3}$-treated endogenously glycosylated plasma protein with the temperature-dependent rate of polymerization of non-glycosylated recombinant material, it is noteworthy that the

Asn[46] glycosylation site is also implicated in significant effects on altered H/D exchange in the vicinity of Glu[205] [34].

Collectively, these data are consistent with a mechanism in which mAb$_{5E3}$ acts in two distinct ways: (a) a stabilization of conformational changes that occur in the Ser[47]/Glu[205] region during transition between the native state and the polymerization intermediate through interaction with a cryptic epitope; and (b) in the context of the full antibody, an increase in the efficiency of the bimolecular association step by optimally orienting the molecules in a manner that reduces the proportion of non-productive interactions resulting from interference by the glycan chains. The latter characteristic is reflected by a lack of templating mechanism and changes in rates of, but not the energetic barrier to, polymerization.

Polymerization is a process that is under kinetic, not thermodynamic, control [10]. Thus, the basis of the susceptibility of the Z variant to polymerization is an increased rate of interchange between – but not the relative stability of – native and activated states [7]. In the context of the subsequent, essentially irreversible, oligomerization step, this increased flux translates overall into an enhanced rate of polymerization. The conformation-stabilizing effect of mAb$_{5E3}$ bears these hallmark characteristics. A mechanism consistent with the cumulative data from the present study is shown in Figure 6(B), in which mAb$_{5E3}$ binds in a reversible fashion to a cryptic epitope that is revealed in the transition state during activation to intermediate, increasing its stability and accordingly reducing the unimolecular activation energy barrier. Against a kinetic backdrop, as with the Z variant, the resulting increased flux translates into an acceleration of the overall polymerization process. In effect, the antibody causes the wild-type M variant to adopt the behaviour of the pathogenic Z mutant.

From this, a number of observations can be made about the polymerization process of α_1-antitrypsin. The data are consistent with a model in which the presence of glycan chains slow the inter-conversion between native and intermediate conformers, such that mAb$_{5E3}$ exerts a greater effect on glycosylated material. Glycans also increase the proportion of 'frustrated' bimolecular interactions, without affecting the energy barrier

to polymerization. In addition, the activated state of the Z variant shows a similar amenity to polymerization to that of the wild-type protein, indicating that there are no substantial structural differences induced by the E342K mutation once in this intermediate conformation. Finally, it is of note that the binding of mAb$_{5E3}$ to the intermediate fails to interfere with oligomerization; this indicates that there are structural changes associated with the cryptic epitope that persist from activated monomer to polymer.

In conclusion, we have shown how an antibody raised against a kinetically unstable mutant can induce similar behaviour in the wild-type protein. This monoclonal antibody represents a tool that can be used in further studies to probe the effects of novel mutations and effector molecules on the polymerization intermediate and, in particular, oligomerization.

REFERENCES

1 Gooptu, B., Ekeowa, U.I. and Lomas, D.A. (2009) Mechanisms of emphysema in alpha-1-antitrypsin deficiency: molecular and cellular insights. Eur. Respir. J. **34**, 475–488 CrossRef PubMed

2 Graham, K.S., Le, A. and Sifers, R.N. (1990) Accumulation of the insoluble PiZ variant of human a1-antitrypsin within the hepatic endoplasmic reticulum does not elevate the steady-state level of grp78/BiP. J. Biol. Chem. **265**, 20463–20468 PubMed

3 Hidvegi, T., Schmidt, B.Z., Hale, P. and Perlmutter, D.H. (2005) Accumulation of mutant alpha1-antitrypsin Z in the endoplasmic reticulum activates caspases-4 and -12, NFkappaB, and BAP31 but not the unfolded protein response. J. Biol. Chem. **280**, 39002–39015 CrossRef PubMed

4 Ordóñez, A., Snapp, E.L., Tan, L., Miranda, E., Marciniak, S.J. and Lomas, D.A. (2013) Endoplasmic reticulum polymers impair luminal protein mobility and sensitise to cellular stress in α1-antitrypsin deficiency. Hepatology **57**, 2049–2060 PubMed

5 Fairbanks, K.D. and Tavill, A.S. (2008) Liver disease in alpha 1-antitrypsin deficiency: a review. Am. J. Gastroenterol. **103**, 2136–2141 CrossRef PubMed

6 Dickens, J.A. and Lomas, D.A. (2011) Why has it been so difficult to prove the efficacy of alpha-1-antitrypsin replacement therapy? Insights from the study of disease pathogenesis. Drug Des. Dev. Ther. **5**, 391–405 PubMed

7 Knaupp, A.S., Levina, V., Robertson, A.L., Pearce, M.C. and Bottomley, S.P. (2010) Kinetic instability of the serpin Z α1-antitrypsin promotes aggregation. J. Mol. Biol. **396**, 375–383 CrossRef PubMed

8 Dafforn, T.R., Mahadeva, R., Elliott, P.R., Sivasothy, P. and Lomas, D.A. (1999) A kinetic mechanism for the polymerization of alpha1-antitrypsin. J. Biol. Chem. **274**, 9548–9555 CrossRef PubMed

9 Haq, I., Irving, J.A., Faull, S.V., Dickens, J.A., Ordóñez, A., Belorgey, D., Gooptu, B. and Lomas, D.A. (2013) Reactive centre loop mutants of α-1-antitrypsin reveal position-specific effects on intermediate formation along the polymerization pathway. Biosci. Rep. **33**, e00046 CrossRef PubMed

10 Irving, J.A., Haq, I., Dickens, J.A., Faull, S.V. and Lomas, D.A. (2014) Altered native stability is the dominant basis for susceptibility of α1-antitrypsin mutants to polymerization. Biochem. J. **460**, 103–115 CrossRef PubMed

11 Lomas, D.A., Evans, D.L., Finch, J.T. and Carrell, R.W. (1992) The mechanism of Z alpha 1-antitrypsin accumulation in the liver. Nature **357**, 605–607 CrossRef PubMed

12 Lomas, D.A., Evans, D.L., Stone, S.R., Chang, W.S. and Carrell, R.W. (1993) Effect of the Z mutation on the physical and inhibitory properties of alpha 1-antitrypsin. Biochemistry **32**, 500–508 CrossRef PubMed

13 Mahadeva, R., Dafforn, T.R., Carrell, R.W. and Lomas, D.A. (2002) 6-mer peptide selectively anneals to a pathogenic serpin conformation and blocks polymerization. Implications for the prevention of Z alpha(1)-antitrypsin-related cirrhosis. J. Biol. Chem. **277**, 6771–6774 CrossRef PubMed

14 Yamasaki, M., Li, W., Johnson, D.J. and Huntington, J.A. (2008) Crystal structure of a stable dimer reveals the molecular basis of serpin polymerization. Nature **455**, 1255–1258 CrossRef PubMed

15 Yamasaki, M., Sendall, T.J., Pearce, M.C., Whisstock, J.C. and Huntington, J.A. (2011) Molecular basis of α1-antitrypsin deficiency revealed by the structure of a domain-swapped trimer. EMBO Rep. **12**, 1011–1017 CrossRef PubMed

16 Mallya, M., Phillips, R., Saldanha, S., Gooptu, B., Brown, S., Termine, D., Shirvani, A., Wu, Y., Sifers, R., Abagyan, R. et al. (2007) Small molecules block the polymerization of Z alpha1-antitrypsin and increase the clearance of intracellular aggregates. J. Med. Chem. **50**, 5357–5363 CrossRef PubMed

17 Goldsteins, G., Persson, H., Andersson, K., Olofsson, A., Dacklin, I., Edvinsson, Å., Saraiva, M.J. and Lundgren, E. (1999) Exposure of cryptic epitopes on transthyretin only in amyloid and in amyloidogenic mutants. Proc. Natl. Acad. Sci. U.S.A. **96**, 3108–3113 CrossRef PubMed

18 Rakhit, R., Robertson, J., Velde, C.V., Horne, P., Ruth, D.M., Griffin, J., Cleveland, D.W., Cashman, N.R. and Chakrabartty, A. (2007) An immunological epitope selective for pathological monomer-misfolded SOD1 in ALS. Nat. Med. **13**, 754–759 CrossRef PubMed

19 Dupont, D.M., Blouse, G.E., Hansen, M., Mathiasen, L., Kjelgaard, S., Jensen, J.K., Christensen, A., Gils, A., Declerck, P.J., Andreasen, P.A. et al. (2006) Evidence for a pre-latent form of the serpin plasminogen activator inhibitor-1 with a detached β-strand 1C. J. Biol. Chem. **281**, 36071–36081 CrossRef PubMed

20 Miranda, E., Pérez, J., Ekeowa, U.I., Hadzic, N., Kalsheker, N., Gooptu, B., Portmann, B., Belorgey, D., Hill, M., Chambers, S. et al. (2010) A novel monoclonal antibody to characterize pathogenic polymers in liver disease associated with α_1-antitrypsin deficiency. Hepatology **52**, 1078–1088 CrossRef PubMed

21 Haque, A., Andersen, J.N., Salmeen, A., Barford, D. and Tonks, N.K. (2011) Conformation-sensing antibodies stabilize the oxidized form of PTP1B and inhibit its phosphatase activity. Cell **147**, 185–198 CrossRef PubMed

22 Lee, H., Brendle, S.A., Bywaters, S.M., Guan, J., Ashley, R.E., Yoder, J.D., Makhov, A.M., Conway, J.F., Christensen, N.D. and Hafenstein, S. (2014) A cryo-electron microscopy study identifies the complete H16.V5 epitope and reveals global conformational changes initiated by binding of the neutralizing antibody fragment. J. Virol. **89**, 1428–1438 CrossRef PubMed

23 Sancho, J. (2013) The stability of 2-state, 3-state and more-state proteins from simple spectroscopic techniques . . . plus the structure of the equilibrium intermediates at the same time. Arch. Biochem. Biophys. **531**, 4–13 CrossRef PubMed

24 Layton, C.J. and Hellinga, H.W. (2010) Thermodynamic analysis of ligand-induced changes in protein thermal unfolding applied to high-throughput determination of ligand affinities with extrinsic fluorescent dyes. Biochemistry **49**, 10831–10841 CrossRef PubMed

25 Sivasothy, P., Dafforn, T.R., Gettins, P.G. and Lomas, D.A. (2000) Pathogenic alpha 1-antitrypsin polymers are formed by reactive loop-beta-sheet A linkage. J. Biol. Chem. **275**, 33663–33668 CrossRef PubMed

26 Johnson, I. (2010) In The Molecular Probes Handbook: A Guide to Fluorescent Probes and Labeling Technologies, 11th edn, Life Technologies Corporation, Carlsbad

27 Chang, W.S., Whisstock, J.C., Hopkins, P.C., Lesk, A.M., Carrell, R.W. and Wardell, M.R. (1997) Importance of the release of strand 1C to the polymerization mechanism of inhibitory serpins. Protein Sci. **6**, 89–98 CrossRef PubMed

28 Dunstone, M.A., Rossjohn, J., Feil, S.C., Parker, M.W., Dai, W., Whisstock, J.C., Pike, R.N., Bottomley, S.P. and Le Bonniec, B.F. (2000) Cleaved antitrypsin polymers at atomic resolution. Protein Sci. **9**, 417–420 CrossRef PubMed

29 Schneider, C.A., Rasband, W.S. and Eliceiri, K.W. (2012) NIH image to ImageJ: 25 years of image analysis. Nat. Methods **9**, 671–675 CrossRef PubMed

30 Irving, J.A., Ekeowa, U.I., Belorgey, D., Haq, I., Gooptu, B., Miranda, E., Pérez, J., Roussel, B.D., Ordóñez, A., Dalton, L.E. et al. (2011) The serpinopathies: studying serpin polymerization in vivo. Methods Enzymol. **501**, 421–466 CrossRef PubMed

31   Mast, A.E., Enghild, J.J. and Salvesen, G. (1992) Conformation of the reactive site loop of alpha 1-proteinase inhibitor probed by limited proteolysis. Biochemistry **31**, 2720–2728 CrossRef PubMed

32   Zhou, A., Stein, P.E., Huntington, J.A., Sivasothy, P., Lomas, D.A. and Carrell, R.W. (2004) How small peptides block and reverse serpin polymerisation. J. Mol. Biol. **342**, 931–941 CrossRef PubMed

33   Ekeowa, U.I., Freeke, J., Miranda, E., Gooptu, B., Bush, M.F., Perez, J., Teckman, J., Robinson, C.V. and Lomas, D.A. (2010) Defining the mechanism of polymerization in the serpinopathies. Proc. Natl. Acad. Sci. U.S.A. **107**, 17146–17151 CrossRef PubMed

34   Sarkar, A. and Wintrode, P.L. (2011) Effects of glycosylation on the stability and flexibility of a metastable protein: the human serpin α1-antitrypsin. Int. J. Mass Spectrom. **302**, 69–75 CrossRef PubMed

35   Tsutsui, Y., Kuri, B., Sengupta, T. and Wintrode, P.L. (2008) The structural basis of serpin polymerization studied by hydrogen/deuterium exchange and mass spectrometry. J. Biol. Chem. **283**, 30804–30811 CrossRef PubMed

36   Nyon, M.P., Segu, L., Cabrita, L.D., Lévy, G.R., Kirkpatrick, J., Roussel, B.D., Patschull, A.O., Barrett, T.E., Ekeowa, U.I., Kerr, R. et al. (2012) Structural dynamics associated with intermediate formation in an archetypal conformational disease. Structure **20**, 504–512 CrossRef PubMed

37   Krishnan, B. and Gierasch, L.M. (2011) Dynamic local unfolding in the serpin α-1 antitrypsin provides a mechanism for loop insertion and polymerization. Nat. Struct. Mol. Biol. **18**, 222–226 CrossRef PubMed

38   Knaupp, A.S., Keleher, S., Yang, L., Dai, W., Bottomley, S.P. and Pearce, M.C. (2013) The roles of helix I and strand 5A in the folding, function and misfolding of alpha1-antitrypsin. PLoS One **8**, e54766 CrossRef PubMed