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α_1-Antitrypsin Polymerisation Can Occur by both Loop A and C Sheet Mechanisms

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A number of disease states are attributable to α_1-antitrypsin polymerisation within the endoplasmic reticulum of hepatocytes and subsequent plasma deficiency. Two distinct mechanisms describing the process of α_1-antitrypsin polymerisation have been proposed, the loop A-sheet and C-sheet mechanisms. We report fluorescence studies using α_1-antitrypsin covalently modified with pyrene maleimide. These results in conjunction with detailed molecular modelling studies, show that α_1-antitrypsin is capable of undergoing both loop A-sheet and loop C-sheet mechanisms of polymerisation, depending upon the *in vitro* buffer conditions.

Key Words: **α_1-antitrypsin; molecular modelling; polymerisation; Serpin.**

The inhibitory proteins which make up the serpin superfamily are involved in the regulation of many important biological processes such as fibrinolysis, inflammation, complement activation, coagulation and phagocytosis [1]. A particular serpin displays a unique function, while sharing a common tertiary structure with the rest of the family [2]. α_1-Antitrypsin is the archetypal member of the serpin family, and functions as an inhibitor of neutrophil elastase [3]. A number of well studied genetic variants of α_1-antitrypsin such as the Z ($Glu^{342}\rightarrow Lys$), M_{malton}(Phe^{52} deleted) and S_{iiyama}($Ser^{53}\rightarrow Phe$), undergo polymerisation which leads to retention within the hepatocyte and subsequently a decrease in the plasma concentration of active serpin. Hence, α_1-antitrypsin polymerisation can lead to liver disease and emphysema [4]. Many other serpin molecules also undergo polymerisation such as plasminogen activator-2 inhibitor, C1-inhibitor, α_1-antichymotrypsin and antithrombin (reviewed in Stein and Carrell, 1995[4]).

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An understanding of the mechanism by which serpins polymerise is of immense interest from both the therapeutical and structural perspective. The structural changes involved in polymerisation are poorly understood [5] however, two mechanisms of polymerisation have been proposed to explain the current data. The loop A-sheet mechanism involves insertion of the reactive loop residues of one serpin molecule into the A β-sheet of another [6] (Fig. 1a). Evidence for this mechanism is based upon the observation that synthetic peptides mimicking the serpin reactive loop inhibit polymerisation [7]. However, although it is proposed that the peptides insert into the A &betasheet, the actual position of the reactive loop peptide has never been experimentally determined. Both Z and S_{iiyama} variants of α_1-antitrypsin have been shown to undergo polymerisation by this mechanism ([8,9]. Loop C-sheet polymerisation involves the insertion of the reactive centre loop residues of one molecule into the vacant s1C position of another (Fig. 1b). Evidence for this mechanism came from the X-ray crystal structure of dimeric antithrombin [10]. Propagation of this polymer is made possible by the over-insertion of the reactive centre loop residues causing strand s1C to peel away from the C-sheet. This leaves the molecule, with its reactive centre loop available to donate to another molecule and a C β -sheet that is able to receive another reactive centre loop [11]. M_{malton} α_1-antitrypsin has been proposed to undergo C-sheet polymerisation, although the authors did not exclude the possibility of the A-sheet mechanism [12].

Experimentally distinguishing between the two mechanisms is extremely difficult [6]. The polymerisation products of both Z and M_{malton} α_1-antitrypsin variants (proposed loop A-sheet and C-sheet mechanisms, respectively) display different morphologies when studied by electron microscopy [12]. However, the electron micrographs do not show that the actual linkage between the monomers are different and so it does not necessarily follow that the polymers were created through different mechanisms. In this study the mo-

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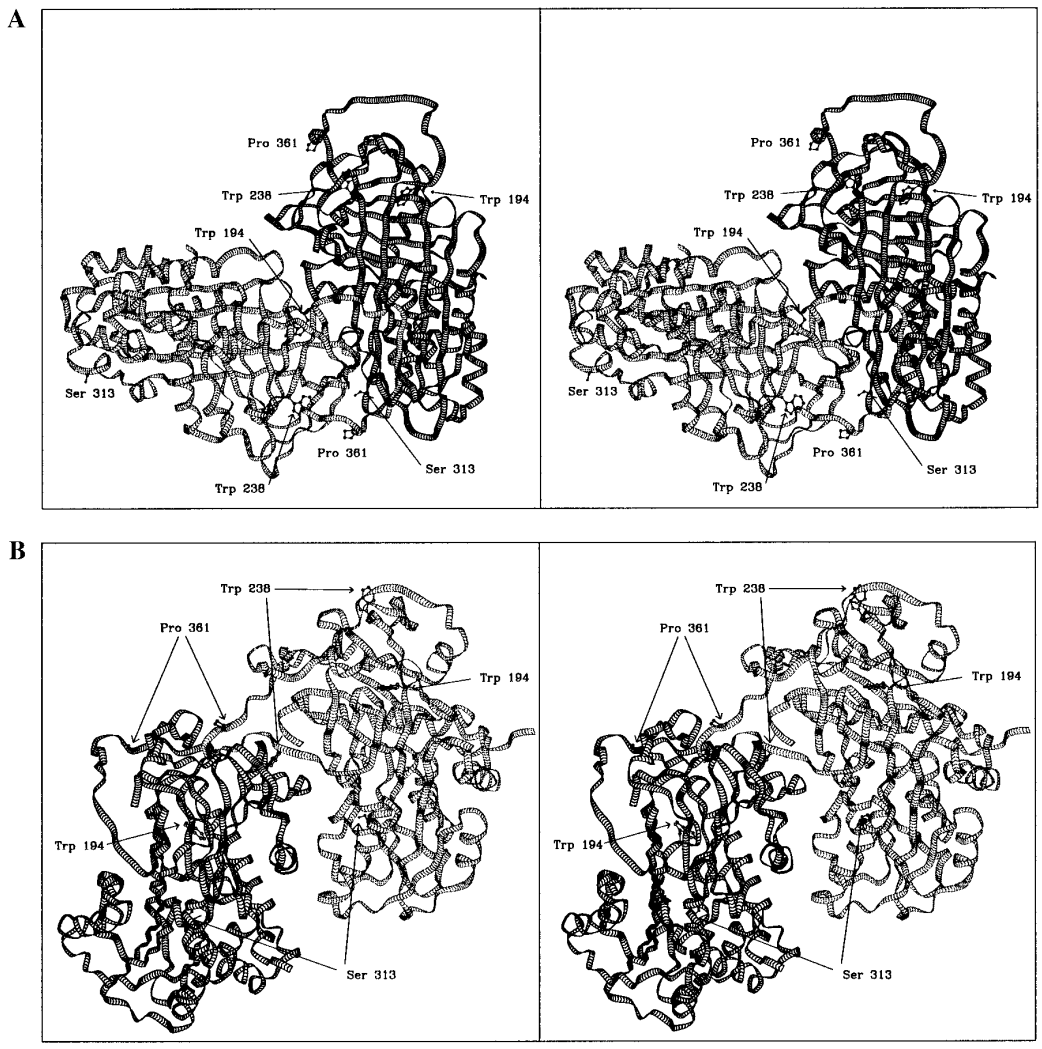

FIG. 1. Stereo diagrams illustrating the mechanism of Loop A-sheet and Loop C-sheet polymersation. (A) Loop A-sheet polymerisation was modelled using α_1-antitrypsin[11]. Shown is the minimum repeating unit of two serpin molecules (A-light grey, B-dark grey) which can propagate to form long chain polymers [11]. Residues 351–358 of the reactive centre loop are inserted into the A β-sheet of the adjacent molecule. Also highlighted are the P361 and S313 residues, which were mutated to cysteines, and the two tryptophan residues of each serpin. (B) Loop C-sheet polymerisation was also modelled using α_1-antitrypsin. Shown is the minimum repeating unit of two serpin molecules (A-light grey, B-dark grey) which can propagate to form long chain polymers [11]. Residues 351–358 of the reactive centre loop are inserted into the vacant s1C position of the C β-sheet.

lecular mechanism of polymerisation has been investigated using fluorescence spectroscopy and molecular modelling.

METHODS

Materials. Pyrene maleimide was purchased from Molecular probes (Oregon, USA). Unless otherwise stated all reagents and enzymes were purchased from Sigma Chem. Co. (Dorset, UK). Restriction enzymes were obtained from New England Biolabs (Beverly, MA, USA) and used according to manufacturer recommendations. Oligonucleotides were purchased from Oswel Co. (Southampton, UK).

Mutagenesis and expression. Mutagenesis was performed on the α_1-antitrypsin expression vector pΔ N15 [13]. The production of $\alpha_1 AT_{(P3'=Cys)}$ had previously been described [14]. Mutagenesis to produce the $Ser^{313} \rightarrow$ Cys recombinant α_1-antitrypsin was performed using the mismatch primer method of Kunkel [15]. The expression and purification of the mutant from *E.coli* BL21(DE3) cells transformed with the appropriate expression vector was as described in [14].

Fluorescent labelling. Fluorescent labelling of $AT_{(P3'=Cys)}$ and $AT_{(S313=Cys)}$ was as described previously except the buffer used was 50mM Tris-HCl, pH 8.0, 100mM NaCl and 10μM pyrene maleimide [16].

Determination of inhibition parameters. The protein concentrations and inhibitory titers and association rate constants were determined as described previously [17].

Fluorescence experiments. All fluorescence spectra and measurements were performed on a Perkin Elmer LS50B at a constant

temperature of 25°C. Excitation and emission slits were set at 2.5nm for measurements.

RESULTS

Figures 1a and b diagrammatically illustrate the two contrasting polymerisation mechanisms. In order to distinguish between these two mechanisms we individually labelled the opposite poles of recombinant α_1-antitrypsin$_{(P1=Arg)}$ with the thiol-specific fluorescent reagent, pyrene maleimide. To achieve this we first produced a cysteine free α_1-antitrypsin$_{(P1=Arg)}$, which was constructed by replacing the native cysteine 232 with a serine residue. Two unique cysteine residues at positions P361 (P3′ using the nomenclature of Schechter and Berger 1967 [18]) and S313 were then inserted, to create AT$_{(P3'=Cys)}$ and AT$_{(S313=Cys)}$, respectively. Both AT$_{(P3'=Cys)}$ and AT$_{(S313=Cys)}$ were then covalently modified with pyrene maleimide. The stoichiometry of modification for both proteins was calculated using ϵ_{340nm}= 36000 M^1cm^{-1} and was found to be 0.9 mol/mol AT$_{(P3'=CysPM)}$ and 1mol/mol AT$_{(S313=CysPM)}$. This data also shows that the cysteine residues of both proteins are freely available for modification. The loss of cysteine 232 and the presence of the labelled cysteine residues at P3′ and S313 had little effect upon their inhibitory properties. The association rate constants with thrombin for AT$_{(P3'=CysPM)}$ (6.0×10^5 M^{-1} s^{-1}) and AT$_{(S313=CysPM)}$ (4.5×10^5 M^{-1} s^{-1}) were similar to that of α_1-antitrypsin$_{(P1=Arg)}$ (4.8×10^5 M^{-1} s^{-1}) [19].

Loop A-sheet polymerisation. Serpin polymerisation can be induced by a number of factors, such as temperature and chaotropic agents. Heat induced polymerisation of α_1-antitrypsin$_{(P1=Arg)}$, AT$_{(P3'=CysPM)}$ and AT$_{(S313=CysPM)}$ was initially monitored by native PAGE as described by Lomas *et al.* [20]. α_1-Antitrypsin$_{(P1=Arg)}$, AT$_{(P3'=CysPM)}$ and AT$_{(S313=CysPM)}$ polymerised upon heating in 50mM Tris-HCl, pH 8.0 at temperatures >60° C (data not shown), no latent serpin molecules could be detected. Native PAGE analysis also showed that all three proteins (labelled and unlabelled) polymerised above 60°C indicating that the mutation and fluorescent label did not effect the propensity of the proteins to polymerise. The fluorescence emission spectra (λ^{ex} = 280nm) of native and polymerised α_1-antitrypsin$_{(P1=Arg)}$ is shown in Fig. 2a. The emission maximum of native α_1-antitrypsin$_{(P1=Arg)}$ is at 335nm. After polymerisation, the fluorescence emission peak remained at 335nm but the intensity increased by 20%. Under the conditions used, low protein concentration, the polymers were soluble as no changes in right-angle light scattering at 600nm were observed (data not shown). The fluorescence emission spectra of AT$_{(P3'=CysPM)}$ and AT$_{(S313=CysPM)}$ in their native and polymerised states (Fig 3b and c), also exhibited emission maxima at 335nm. An additional pair of emission

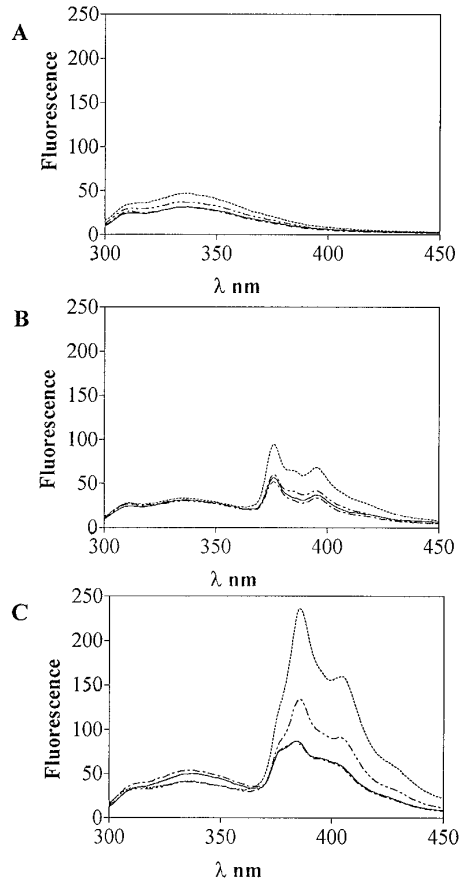

FIG. 2. Fluorescence emission spectra of native and polymerised α_1-antitrypsin$_{(P1=Arg)}$ and mutants. The emission spectra (λ^{ex} = 280nm) of 200nM (A) α_1-antitrypsin$_{(P1=Arg)}$, (B) AT$_{(P3'=CysPM)}$ and (C) AT$_{(S313=CysPM)}$ are shown. Fluorescence measurements were performed using 2.5nm slit widths and a scan speed of 20nm/min. The native structures were determined in either 50 mM Tris-HCl (—) or 0.7M citrate (– - –); similarly the polymerised structures were also determined in either 50 mM Tris-HCl (- - -) or 0.7M citrate (- – -). Polymers were formed by heating the serpins to 70°C for 15 minutes. The samples used for polymerisation were also checked by native PAGE analyis as described by Lomas *et al.* [20].

peaks (AT$_{(P3'=CysPM)}$ λ^{em} = 376, 395nm; AT$_{(S313=CysPM)}$ λ^{em} = 383, 402nm) were also detected due to pyrene labelling. This demonstrates that the tryptophan residues within the native proteins are capable of mediating pyrene fluorescence. Further, the tryptophan emission maxima of native α_1-antitrypsin$_{(P1=Arg)}$, AT$_{(P3'=CysPM)}$ and AT$_{(S313=CysPM)}$ were identical (335nm) suggesting that the cysteine replacement and modification did not significantly alter the serpin structure. Polymerisation of AT$_{(P3'=CysPM)}$ resulted in no increase in the fluorescence intensity at 335nm and a small enhancement (15%, measured at 380nm) in the fluorescence of the pyrene moiety (Fig. 2b). This increase in pyrene fluorescence is similar to the increase in tryptophan fluorescence seen upon polymerisation of α_1-antitrypsin$_{(P1=Arg)}$ (20%, measured at 335nm). Polymerisation of AT$_{(S313=CysPM)}$ caused a decrease

(26%, measured at 335nm) in the fluorescence of the tryptophan residues and an enhancement (65%, measured at 380nm) in the fluorescence of the pyrene moiety. This data shows that the distance between the probe at position S313 and the tryptophan residues (W194 and W238) of the adjacent serpin molecules (within the polymer) are within close enough proximity for additional resonance energy transfer to occur. The distance between P3′ and the additional tryptophan residues of the interlocking serpin molecules are further apart, resulting in far less efficient resonance energy transfer.

Loop C-sheet polymerisation. It was previously noted by Lomas and colleagues [20] that the polymers of α_1-antitrypsin formed in 0.7M citrate buffer displayed a different morphology (suggested to be C-sheet polymers) than those formed in Tris-HCl buffer, when examined by electron microscopy. Native PAGE analysis of α_1-antitrypsin$_{(P1=Arg)}$, $AT_{(P3'=CysPM)}$ and $AT_{(S313=CysPM)}$ polymerised in 0.7M citrate showed no latent form present and again the presence of the pyrene group did not effect the ability of $AT_{(P3'=CysPM)}$ and $AT_{(S313=CysPM)}$ to polymerise (data not shown). Interestingly, an increased temperature of >70°C was required to induce polymerisation compared to a temperature of 60°C when Tris-HCl buffer was used. Similar findings were observed with plasma antitrypsin [20]. The fluorescence emission spectra obtained for both native and polymerised α_1-antitrypsin$_{(P1=Arg)}$ (45% increase measured at 335nm) was essentially the same as in Tris-HCl buffer except the quantum yield was increased approximately 2 fold. The polymers were again soluble as no changes in right angle light scatter were observed. The fluorescence emission spectra obtained for $AT_{(S313=CysPM)}$ (20% decrease and a 160 % increase measured at 335 and 383 nm, respectively) in 0.7M citrate, were qualitatively similar to those measured upon polymerisation in Tris-HCl (Fig. 2a and b). Again like α_1-antitrypsin$_{(P1=Arg)}$ in citrate buffer, the quantum yields were increased by approximately 2 fold. However, $AT_{(P3'=CysPM)}$ polymerisation in 0.7M citrate buffer resulted in an increase in pyrene fluorescence of 77% (measured at 383nm) which is 5 fold greater than when polymerisation was performed in Tris-HCl (Fig. 2c). Further the fluorescence intensity at 335nm was decreased by 40%, indicating that the increased fluorescence of the pyrene moiety was produced by resonance energy transfer. Therefore, the distance between the tryptophan residues of adjacent serpin molecules and the pyrene molecule was significantly decreased in comparison with polymers formed in Tris-HCl buffer.

Mechanism of serpin polymerisation. Figures 1a and b show models of dimers of the loop A-sheet and loop C-sheet polymerisation mechanisms respectively [11]. They also show the positions of residues P3′ and S313 and the two tryptophan residues W194 and W238. From these models of loop A- and C- sheet polymers the intermolecular distances between the tryptophan residues and the pyrene group were measured (Fig. 1a and b; Table 1). When the fluorescence probe is situated at the base of the molecule (as in $AT_{(S313=CysPM)}$), the tryptophan residues of the interlocking serpin molecules come within approximately 30Å of the probe in both the A and C sheet models. This is approximately 15Å shorter than in the native state. Thus, the increased probe fluorescence seen upon polymerisation of $AT_{(S313=CysPM)}$ is consistent with both models. However, the distances between P′3 and the tryptophan residues of the adjacent serpin molecules in the C-sheet model is greatly reduced due to polymerisation (13 and 27Å; a decrease of approximately 18Å compared to the native state). However, in the loop A-sheet model, the interlocking tryptophan residues are further away (an average distance of 48Å) from the probe at P3′ and therefore these additional tryptophan residues would contribute only weakly to the pyrene fluorescence. A large increase in pyrene fluorescence is only observed when $AT_{(P3'=CysPM)}$ is polymerised in citrate buffer. Therefore, we propose that heat induced polymerisation of α_1-antitrypsin *in vitro* can proceed by both previously suggested polymerisation mechanisms. In Tris-HCl it is the A-sheet mechanism but in citrate the polymers are formed by the C-sheet mechanism.

In this study we have shown that α_1-antitrypsin, *in vitro*, has the propensity to polymerise by two different mechanisms. The fluorescence data fits both polymerisation models that have been constructed to illustrate the loop A-sheet and loop C-sheet polymerisation products. It is important to note that we have examined the final products of serpin polymerisation therefore the

TABLE 1

Inter- and Intra-molecular Distances between Tryptophan Residues and the Fluorescence Probe

				Polymers	
			Monomer	A→B	B→A
A-Sheet	P361	W194	24.4	38.4	53.0
		W238	17.3	58.2	55.8
	S313	W194	44.0	27.1	77.8
		W238	42.4	34.1	68.4
C-Sheet	P361	W194	37.2	13.2	60.9
		W238	32.5	26.9	53.9
	S313	W194	43.9	30.0	72.8
		W238	42.1	45.8	71.4

Note. Distances (Å) between the two tryptophan residues (194 and 238) of the serpin and the pyrene moiety (at positions P3′C and S313C) were measured using the polymerisation models described earlier (Fig. 1a and b). The monomer distances refer to the intramolecular distance between the probe and the tryptophan residues of an individual serpin molecule within the polymer. The A→B and B→A refers to the direction in which the measurements were made within the repeating unit of the polymer structure (Fig. 1a and b).

actual molecular mechanisms involved are still unknown. A major distinguishing feature between the two proposed models (Fig. 1a and b) is that the loop A-sheet antitrypsin polymers require the reactive centre loop (of the donor serpin) to be fully expelled and s1C to be partially peeled away from its C β-sheet. The loop C-sheet polymerisation mechanism requires insertion of the reactive centre loop (of the donor serpin) into its own A β-sheet to remove strand s1C from the C β-sheet thus leaving space for the incoming strand. Therefore, when α_1-antitrypsin polymerises the conformation of the reactive centre loop may act as a deciding factor at some branch point along the polymerisation pathway. If sufficient reactive centre loop insertion into the A β-sheet has occurred then C-sheet polymerisation is preferred. However, if there is insufficient or no insertion of the reactive centre loop into the A β-sheet then A-sheet polymerisation proceeds. The conformation of the reactive centre loop will be dependent upon many factors including solvent composition and mutations within the serpin framework. Therefore, in Tris-HCl buffer (and elevated temperatures) the reactive centre loop adopts a conformation which hinders insertion into the A β-sheet, whereas in citrate buffer (and elevated temperatures) the reactive centre loop is inserted to some extent which then predisposes the serpin to polymerisation via the C-sheet pathway.

This branching in the polymerisation pathway of serpins also explains why some natural variants of α_1-antitrypsin (and other serpins) exhibit different polymerisation products (Table 2). Z ($Glu^{342} \rightarrow Lys$) antitrypsin, which polymerises via the A-sheet pathway, possesses a mutation within the amino terminal portion of the reactive centre loop [8]. This mutation hinders entry of the reactive centre loop into the A-sheet and this favours A-sheet polymerisation. Whereas the loss of Phe^{52} as in Mmalton α_1-antitrypsin disturbs the packing of helix B beneath the A β-sheet, which allows the reactive centre loop to over-insert and hence favour polymerisation via the C-sheet pathway [20].

Based on the data presented here, we propose that α_1-antitrypsin can polymerise by two pathways. The choice of polymerisation mechanism is dependent upon reactive centre loop conformation, which is sensitive to both solvent and mutation of the serpin body. Mutations and solvent that increase the chance of reactive centre loop insertion into the A β-sheet are likely to polymerise via the loop C-sheet mechanism. Whereas conditions and mutations which prevent reactive centre loop insertion into the A β-sheet polymerise via the loop A-sheet pathway. This information should facilitate the rationale design of therapeutics that may prevent polymerisation and aid in the treatment of polymerisation induced diseases.

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