Assignment of a single disulphide bridge in human α_2 -antiplasmin: implications for the structural and functional properties

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Human α_2 -antiplasmin (α_2 AP) is a serpin involved in the regulation of blood coagulation. Most serpins, unlike smaller serine proteinase inhibitors, do not contain disulphide bridges. α_2 AP is an exception from this generalization and has previously been shown to contain four Cys residues organized into two disulphide bridges [Lijnen, Holmes, van Hoef, Wiman, Rodriguez and Collen (1987) *Eur. J. Biochem.* **166**, 565–574]. However, we found that α_2 AP incorporates iodo[14 C]acetic acid, suggesting that the protein contains reactive thiol groups. This observation prompted a re-examination of the state of the thiol groups, which

revealed (i) a disulphide bridge between Cys⁴³ and Cys¹¹⁶, (ii) that Cys⁷⁶ is bound to a cysteinyl-glycine dipeptide, and (iii) and Cys¹²⁵ exists as either a free thiol or in a mixed disulphide with another Cys residue. The disulphide identified between Cys⁴³ and Cys¹¹⁶ appears to be conserved in orthologous proteins since the homologous Cys residues form disulphide bonds in bovine and possibly mouse α_2 AP. The conservation of this disulphide bridge suggests that it is important for functional aspects of α_2 AP. However, the structural and functional analysis described in this study does not support this conclusion.

INTRODUCTION

Some 60 different serpins have so far been identified from a range of organisms including viruses, plants, insects and animals, but not prokaryotes [1]. The best characterized members of the serpin family are found in human plasma, where they regulate the proteolytic cascades of coagulation, fibrinolysis, inflammation and complement activation [2].

Serpins are single-chain proteins composed of 370–390 amino acid residues, often flanked by N- or C-terminal extensions. The central part forms a scaffold, which supports the structure of the reactive-site loop (RSL) located in the C-terminus of the molecule. The RSL is exposed on the surface of the protein and is susceptible to proteolysis by non-target proteinases [3]. A single cleavage of any peptide bond within the RSL results in the transition of the inhibitory serpins to a more thermodynamically stable structure with no inhibitory capacity [2,4]. Complexes between serpins and proteinases are also thermodynamically stabilized [5,6]. The RSL of the inhibitory serpin is believed to be more flexible than that of the standard mechanism inhibitor, which is typically rigid, displaying a well-defined ‘canonical conformation’ [7]. An additional important difference between the serpins and non-serpin inhibitors is that serpin–proteinase complexes are stable to SDS, whereas non-serpin inhibitor–proteinase complexes dissociate after denaturation [8].

Serpin–proteinase complexes are cleared from the circulation by receptors in the liver. Two serpin receptors have been identified in mice (SR1 and SR2). SR1 recognizes a variety of serpin–proteinase complexes, whereas SR2 is specific for α_2 -antiplasmin (α_2 AP)–proteinase complexes [5,9].

In homologous proteins, Cys residues involved in disulphide bridges are generally highly conserved because of their contribution to the stability of the tertiary structure. Most standard-mechanism inhibitors contain many disulphides. In fact, in some cases the spacing and topology of the disulphides are the basis for the identification of family members [10]. In contrast, no

general disulphide-bridge pattern can be established among the members of the serpin family. Moreover, disulphide bridges are rare [4], and Cys residues and disulphide bridges are only conserved in orthologous members, e.g. anti-thrombin III from human and mouse [11,12]. The amino acid sequence of human [13], bovine [14] and mouse α_2 AP are known, and the Cys residues involved in the single disulphide bridge are conserved. (The cDNA sequence of mouse α_2 AP was obtained from the genEMBL database under accession number Z36774.)

The general lack of conserved disulphide bridges in the serpin family raises questions about the influence of this post-translational modification on the properties of serpins. In this study, we have determined the modification of the four Cys residues in human α_2 AP. In addition, the significance of these modifications is examined by comparing native and chemically modified α_2 AP, considering thermodynamic stability, inhibitory potency and receptor recognition.

MATERIALS AND METHODS

Materials

Bovine trypsin (treated with Tos-Phe-CH₂Cl), porcine trypsin and *p*-nitrophenyl *p*-guanidinobenzoate were from Sigma. Proline-specific endopeptidase was from Seikagaku. Benzyl-oxycarbonyl-arginine-*p*-nitroanilide was from Bachem Bioscience Inc. Human neutrophil elastase (HNE) was from ART Biochemicals. Human α_2 AP was purified as described by Wiman [15].

Purification of Cys-containing peptides

14 C-radiolabelled α_2 AP was cleaved with 1% (w/v) CNBr in 70% formic acid for 12 h at 23 °C. The CNBr fragments were dissolved in 100 mM NH₄HCO₃ and digested with trypsin (1:100,

Abbreviations used: α_2 -AP, α_2 -antiplasmin; α_2 AP/RA, reduced and alkylated α_2 -antiplasmin; Cys, cysteine; PDMS, plasma desorption MS; HNE, human neutrophil elastase (EC 3.4.21.37); TUG, transverse urea gradient; RSL, reactive-site loop.

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w/w) for 4 h at 37 °C, after which the peptides were separated on a Beckman System Gold HPLC system using a Waters Nova-Pak C₁₈ column (3.9 mm × 150 mm). The column was equilibrated in 0.1 % trifluoroacetic acid and developed with a linear gradient from 0 to 90 % acetonitrile. Peptides were detected at 220 nm and collected manually. Subdigestions of peptides with chymotrypsin or proline-specific endopeptidase (molar ratios 10:1) were performed in 0.1 M NH₄HCO₃ at 37 °C for 30 min before the reaction products were separated by reverse-phase HPLC using a Vydac RP C₁₈ column (2.1 mm × 200 mm) as described above.

Reduction and alkylation

For detection of free cysteines, approx. 1.7 mg of α_2 AP was denatured in 6 M guanidinium chloride/250 mM Tris/HCl/1 mM EDTA, pH 7.5, containing 10 μ Ci of iodo[¹⁴C]acetic acid, and incubated for 20 min; 80 mM unlabelled iodoacetic acid was then added. After an additional 30 min incubation, the radio-labelled α_2 AP was desalted on a fast desalting column HR 10/10 (Pharmacia Biotech Inc.), and fractions containing free Cys residues were identified by liquid-scintillation counting in a Beckman LS 5000TD or by amino acid analysis.

As a control, 1 mg of α_2 AP was reduced with dithiothreitol (2 mM) and alkylated with iodoacetamide (20 mM) in 100 mM Tris/HCl/100 mM NaCl/20 mM CaCl₂, pH 8.0, without denaturing agents. The protein was desalted on a fast desalting column HR 10/10 column (Pharmacia Biotech Inc.). Potential unmodified Cys residues were identified by cysteic acid (Cya) detection in amino acid analysis.

Analysis of amino acid composition

Peptides were hydrolysed in 6 M HCl for 24 h at 110 °C [16]. For Cya detection, samples were treated with performic acid before hydrolysis [17]. The hydrolysates were dried in a Speed-Vac concentrator (Savant), and the amino acid compositions determined in a Beckman 6300 amino acid analyser.

Analysis of amino acid sequence

Edman degradation was carried out in an Applied Biosystems 477A automated protein sequencer with on-line analysis of the phenylthiohydantoin using an Applied Biosystems 120A HPLC. Samples were applied to Polybrene-treated precycled glass-fibre filters.

Plasma-desorption MS (PDMS)

Peptides were analysed by time-of-flight MS. Spectra were recorded using nitrocellulose targets [18] in an Applied Biosystems Biolon 20 plasma-desorption instrument [19]. The spectrum was accumulated for 1×10^6 fission events corresponding to approx. 10 min.

Radiolabelling of proteins

α_2 AP was radioiodinated by the chloramine-T procedure [20] using 10 μ g of protein/0.5 mCi of ¹²⁵I followed by desalting on a PD-10 column (Pharmacia Biotech Inc.).

Preparation of modified α_2 AP and α_2 AP–trypsin complexes

Proteolytically modified α_2 AP was prepared by incubation with HNE (molar ratio 2:1) in 50 mM Tris/HCl/100 mM NaCl,

pH 7.4, at 23 °C for 5 min. The reaction was stopped with 3,4-dichloroisocoumarin (5 μ M final concentration). ¹²⁵I- α_2 AP–trypsin complexes for use in mouse plasma elimination studies were prepared by incubating equimolar amounts for 2 min at 23 °C. α_2 AP–trypsin complexes for transverse urea gradient (TUG) gel analysis were prepared by mixing 100 μ g of α_2 AP with 88 μ g of trypsin (molar ratio 1:1.2).

PAGE

TUG–PAGE analysis was performed in 7 % gels containing a continuous 0–8 M urea gradient [5,21]. SDS/PAGE was performed in 5–15 % linear gradient gels using the glycine/2-amino-2-methylpropane-1,3-diol/HCl system described by Bury [22]. All experiments were repeated in triplicate.

Measurement of second-order association rate constants

The ability of native α_2 AP and reduced and alkylated α_2 AP (α_2 AP/RA) to inhibit trypsin was examined by kinetic analysis. The active concentration of porcine trypsin was determined as described by Chase and Shaw [23] using *p*-nitrophenyl *p*-guanidinobenzoate, and the concentration of α_2 AP was estimated by amino acid analysis. Kinetic experiments were designed to compare the second-order rate constants of native α_2 AP and α_2 AP/RA. The analyses were performed in 100 mM Tris/HCl/100 mM NaCl/20 mM CaCl₂, pH 8.0. To determine the steady-state rates of substrate hydrolysis, the change in *A*₄₀₅ over time was followed in a Beckman DU 640 spectrophotometer. Second-order rate constants for the association of trypsin and either α_2 AP or α_2 AP/RA were determined by incubating equimolar amounts (40 nM) of trypsin and α_2 AP for given periods of time before adding 5 μ l of 225 mM benzyloxycarbonyl-arginine-*p*-nitroanilide to the mixture (1005 μ l final volume) [24].

Plasma elimination studies

This procedure has been described in detail elsewhere [25]. In brief, approx. 1.0 μ g of radioiodinated α_2 AP was injected into the lateral tail vein of CD-1 mice. Blood samples of volume 25 μ l were collected at timed intervals via retro-orbital puncture. The initial time point, 5–10 s after injection, was considered to represent 100 % radioactivity in the circulation. Each study was performed at least in duplicate and the critical experiments (α_2 AP/RA and α_2 AP/RA–trypsin) were performed three times, and the results averaged.

RESULTS

Isolation of disulphide- and Cys-containing peptides

The four Cys residues in human α_2 AP have previously been suggested to be engaged in two disulphide bridges [13]. Consequently the protein contains no free thiol groups. In conflict with this prediction we found that human α_2 AP incorporates iodo[¹⁴C]acetic acid in the absence of reducing agents, indicating the presence of free thiols in the protein (Figure 1).

To identify the free Cys residue(s), α_2 AP was alkylated using iodo[¹⁴C]acetic acid. The labelled molecule was cleaved with CNBr to facilitate the subsequent trypsin digestion. The resulting peptides were purified by reverse-phase HPLC, and [¹⁴C]carboxymethylated Cys residues and Cys-containing peptides were identified on the basis of the presence of Cya in amino acid composition analysis and by counting the fraction in a liquid-scintillation counter (Beckman LS 5000 TA). Numbering is in accordance with the cysteine numbers found in human α_2 AP [26].

Figure 1 Incorporation of iodo[^{14}C]acetic acid into human $\alpha_2\text{AP}$

Denatured human $\alpha_2\text{AP}$ was incubated with iodo[^{14}C]acetic acid and desalted by gel filtration on G-25 FF Sepharose. All fractions were counted for ^{14}C . $\circ$ — $\circ$ A_{280} ; $\bullet$ — $\bullet$ ^{14}C radioactivity. $\alpha_2\text{AP}$ is eluted in the first peak (fractions 6–8) and excess iodoacetic acid in the second (fractions 15–20).

Analysis of Cys 76

The peptide Ser 68 –Leu 84 containing Cys 76 was analysed by Edman degradation and MS (Table 1). The mass of the peptide was 176.9 Da higher than the calculated theoretical mass (1757.1 Da), indicating an attachment of a cysteinyl-glycine dipeptide (176.2 Da) to Cys 76 . To confirm that the observed mass difference was due to modification of Cys 76 , the peptide was reduced and carboxyamidomethylated. The mass of the modified peptide was 1815.6 Da, and this is in accordance with the displacement of the dipeptide and the carboxymethylation (58.0 Da) of Cys 76 . We therefore conclude that Cys 76 of human $\alpha_2\text{AP}$ forms a mixed disulphide with cysteinyl-glycine.

Analysis of Cys 125

Three variants of the peptide Leu 124 –Arg 134 were identified as described above. The mass of the peptides are consistent with a

modification of Cys 125 by attachment of Cys (1297.5 Da), oxidation to Cys (1225.6 Da) or carboxymethylation (1235.5 Da) (Table 1). The carboxymethylation suggests that the Cys 125 contained a free thiol group before the chemical modification. The oxidation of Cys 125 to Cys probably occurred during the purification of the protein. Thus Cys 125 exists both as a free thiol and as a mixed disulphide with Cys.

Analysis of Cys 43 and Cys 116

A peptide with a mass of 2856.8 Da was purified. Edman degradation revealed the sequence corresponding to Leu 105 –Arg 123 in human $\alpha_2\text{AP}$ (Table 1). The theoretical mass of this peptide (2026.4 Da) is inconsistent with the observed mass. However, reduction and alkylation, followed by reverse-phase HPLC resulted in the dissociation of two peptides. The mass of the Leu 105 –Arg 123 peptide (2086.1 Da) is in agreement with carboxymethylation of Cys 116 . The N-terminus of the other peptide was blocked but the peptide was identified as Ser 38 –Arg 45 by amino acid analysis, and chymotrypsin subdigestion followed by Edman degradation and MS of the resulting products. The expected mass of the carboxymethylated Ser 38 –Arg 45 peptide was 859.9 Da. However, the observed mass (888.1 Da) was 28.2 Da higher than expected, suggesting the presence of a formyl group probably attached to the N-terminal serine during purification of the peptides. These data show that in human $\alpha_2\text{AP}$ Cys 43 and Cys 116 form a disulphide bridge.

In conclusion, our data show that Cys 43 and Cys 116 form the only disulphide bridge in human $\alpha_2\text{AP}$. Cys 76 is attached to a cysteinyl-glycine dipeptide and Cys 125 is found both as a free thiol and as a mixed disulphide with a free Cys residue. Thus the position of the disulphide bridge in human $\alpha_2\text{AP}$ corresponds to both the bridge in bovine $\alpha_2\text{AP}$ and the conserved Cys residues in the mouse protein [14].

TUG electrophoresis of $\alpha_2\text{AP}$

TUG gel electrophoresis was used to probe possible structural differences between native $\alpha_2\text{AP}$ and $\alpha_2\text{AP}/\text{RA}$. In these gels, the protein migrates more slowly as it unfolds since the unfolded state occupies a larger hydrodynamic volume. This results in visualization of an unfolding transition in the area of the gel between the folded and the unfolded state [5,6,21,27].

Table 1 Masses of Cys-containing peptides determined by PDMS

Calculated masses are derived from the amino acid sequence of the peptides. Mass measurement accuracy by PDMS is expected to be within 0.1%.

Peptide	Molecular mass (Da)			
	Original peptide		Reduced and alkylated peptide	
	Observed	Calculated	Observed	Calculated
Ser 68 –Leu 84	1934.0	1757.1	1815.6	1815.1
Leu 124 –Arg 134	—	1176.3	—	—
Addition of Cys	1297.5	1296.5	—	—
Oxidation to Cys	1225.6	1224.1	—	—
Carboxymethylation	1235.5	1234.3	—	—
Ser 38 –Arg 45 /Leu 105 –Arg 123	2856.8	2827.3	—	—
Ser 38 –Arg 45	—	801.9	888.1	859.9
Leu 105 –Arg 123	—	2026.4	2086.1	2084.4

Figure 2 Conformational stability of α_2 AP and α_2 AP/RA

Samples (100 μ g) of α_2 AP or α_2 AP/RA were analysed by TUG-PAGE (**A** and **B**), modified through RSL cleavage by HNE (**C** and **D**), or in complex with trypsin (88 μ g) (**E** and **F**). Only complexed and modified α_2 AP forms are stable, with no detectable differences between reduced and non-reduced forms of α_2 AP.

Both α_2 AP and α_2 AP/RA unfolded at a concentration of urea of approx. 3 M (Figures 2A and 2B). Thus reduction and alkylation of the α_2 AP disulphide bridge induces no detectable change in the stability of the native protein. Next, the importance of the disulphide bridge in the more stable proteolytically modified forms of α_2 AP and α_2 AP/RA were analysed (Figures 2C and 2D). Neither the native nor the reduced molecule unfolded, indicating that reduction of the Cys⁴³–Cys¹¹⁶ disulphide bond does not significantly decrease the thermodynamic stability of the modified serpin. The stabilities of the α_2 AP–trypsin and α_2 AP/RA–trypsin complexes were similarly analysed (Figures 2E and 2F). Again, no difference in the TUG gel signature of the complexes nor the unfolding of the serpin–proteinase complexes was observed. This finding suggests that both complexes are stable in 8 M urea. Thus the disulphide bond does not appear to influence the thermodynamic stability of any of the tested conformations of α_2 AP.

Complex-formation and association rates of α_2 AP and α_2 AP/RA with trypsin

The influence of the disulphide bridge on the inhibitory capacity was analysed by enzyme kinetics and by the ability of α_2 AP–

Figure 3 SDS-stable complexes of α_2 AP–trypsin and α_2 AP/RA–trypsin

Complexes between reduced and non-reduced α_2 AP and trypsin were analysed by SDS/PAGE. The protein bands were visualized by Coomassie Blue staining. Lane 1, α_2 AP; lane 2, α_2 AP/RA; lane 3, α_2 AP–trypsin complex; lane 4, α_2 AP/RA–trypsin complex; lane 5, α_2 AP modified by HNE cleavage; lane 6, α_2 AP/RA modified by HNE cleavage; lane 7, size marker.

Figure 4 Plasma elimination of α_2 AP–trypsin and α_2 AP/RA–trypsin complexes in mice

¹²⁵I-radiolabelled protein (1.0 μ g) was injected into the mouse, and blood samples were taken as described in the Materials and methods section. ●, Native α_2 AP; ○, native α_2 AP/RA; ■, α_2 AP–trypsin; □, α_2 AP/RA–trypsin.

trypsin complexes to resist dissociation in SDS/PAGE. Both native and α_2 AP/RA formed SDS-stable complexes with trypsin, suggesting that the native conformation of α_2 AP/RA was preserved (Figure 3). To confirm that the inhibitory capacity was maintained, association rate constants (k_{ass}) for the interaction and inhibition of trypsin by α_2 AP and α_2 AP/RA were measured. The k_{ass} was found to be essentially identical for α_2 AP and α_2 AP/RA (9.5×10^6 and 5.3×10^6 M⁻¹·s⁻¹). We therefore conclude that the inhibitory capacity of α_2 AP/RA is maintained even without the Cys⁴³–Cys¹¹⁶ disulphide bridge.

Clearance of α_2 AP–trypsin and α_2 AP/RA–trypsin complexes from the circulation in mice

The presence or absence of the single disulphide bridge in α_2 AP appears to be of minor importance for both the stability and inhibitory potency of the protein. Plasma elimination studies were performed to examine whether lack of the disulphide induced conformational differences, not detected by the other

methods. Radiolabelled native or trypsin-complexed α_2 AP and α_2 AP/RA were injected into the blood stream of mice and the clearance from the blood circulation was followed (Figure 4). As previously reported [9], native α_2 AP cleared slowly from the circulation with a half-life of several hours. This also appears to be the case for the reduced and alkylated form of the protein. When α_2 AP and α_2 AP/RA in complex with trypsin was injected into the bloodstream, clearance occurred more rapidly, and no significant difference in the half-lives of the reduced and un-reduced form was observed. The slight increase in the clearance rate of α_2 AP/RA and α_2 AP/RA–trypsin suggests that a small population of the α_2 AP/RA molecules have an aberrant structure and are removed from the circulation by another mechanism.

These data indicate that the conformations of α_2 AP and α_2 AP/RA in complex with trypsin are very similar. Alternatively, neither of the modified Cys residues are involved in maintaining the receptor-recognized conformation.

DISCUSSION

According to the neutral theory of evolution, only functionally or structurally important amino acid residues are conserved [28]. Consequently, Cys residues involved in disulphide bridges are among the most highly conserved residues [29]. Most serpins do not contain disulphide bridges, although Cys residues are quite common [4]. The chemical structure of these Cys residues has been examined for only a few serpins. Since serpins generally do not depend on disulphides to maintain their structural integrity, these conserved Cys residues are usually assumed not to be involved in the formation of disulphide bridges. Moreover, these Cys residues are often positioned within the predicted tertiary structure so that disulphide formation appears very unlikely.

The primary structures of human, bovine and mouse α_2 AP are known and these orthologous serpins all contain Cys residues [13,14]. Of the four Cys residues present in the human protein, three are conserved in bovine α_2 AP and two in mouse α_2 AP. The four Cys residues in human α_2 AP have previously been reported to be involved in two disulphide bridges [13]. In this study we re-examined the disulphide bridge pattern because we suspected that α_2 AP contains free Cys thiol groups (Figure 1). To investigate this discrepancy, we generated α_2 AP peptides and characterized the modifications of the individual Cys residues. These analyses identified only one disulphide bond in human α_2 AP (Cys⁴³–Cys¹¹⁶). Although the position of this disulphide conflicts with the disulphide bridge pattern previously reported in the human protein (Cys⁴³–Cys¹²⁵ and Cys⁷⁶–Cys¹¹⁶), it corresponds to the disulphide bridge found in bovine α_2 AP (Cys⁴⁹–Cys¹²²) [14], and the two conserved Cys residues in the mouse protein. Thus the Cys⁴³–Cys¹¹⁶ disulphide bond appears to be evolutionarily conserved. Cys¹²⁵ was found to exist both as a free thiol and as a mixed disulphide with Cys (Table 1). This residue is also conserved in the bovine protein (Cys¹³¹), and has likewise been identified as a partially free Cys residue. Hence the chemical structure of this residue is also conserved between the two species. Cys⁷⁶ is not conserved in bovine or mouse α_2 AP. In human α_2 AP the residue was identified as a mixed disulphide with a cysteinyl-glycine dipeptide. This modification is uncommon among plasma proteins where most Cys residues not involved in disulphide bridges are modified by attachment of either a free Cys or glutathione. The functional significance of preferential attachment of cysteinyl-glycine is unclear.

Considering the general importance of conserved Cys residues and the fact that disulphide bridges are rare among the serpins, one would expect the conserved Cys residues in α_2 AP to stabilize the native conformation and be of structural and/or functional

importance to the protein. We therefore compared several properties of α_2 AP and α_2 AP/RA to investigate the structural/functional role of the post-translational modifications of the Cys residues. The thermodynamic stability of α_2 AP and α_2 AP/RA were analysed by TUG gel electrophoresis, and the experiments demonstrated the characteristic serpin TUG signature of both α_2 AP and α_2 AP/RA. The virgin serpin is unstable and unfolds at approx. 3 M urea, whereas the modified and trypsin-complexed α_2 AP molecules are stable in 8 M urea (Figure 2). Thus the disulphide bond does not appear to influence the thermodynamic stability of any of the tested conformations of α_2 AP.

The urea-induced unfolding transition of the serpin was apparently unaffected by the reduction of the disulphide bond. The disulphide could, however, participate in maintaining the tight serpin–proteinase complex under native conditions. To investigate this possibility the second-order association rate constants for the interaction between trypsin and native α_2 AP or α_2 AP/RA were measured. These experiments failed to reveal any significant difference in the association rates. Moreover, both α_2 AP and α_2 AP/RA were able to form SDS-stable serpin–proteinase complexes (Figure 3). Taken together, these experiments indicate that the properties of the proteinase complexes are very similar and that the inhibitory capacity of human α_2 AP is unaffected by reduction and alkylation.

The mouse SR2 receptor specifically recognizes and eliminates human α_2 AP–proteinase complexes from the circulation [5,9]. The possible induction of more subtle changes in the function or conformation of α_2 AP by reduction and alkylation was therefore investigated by mouse plasma elimination studies (Figure 4). These experiments demonstrate that the recognition by the clearance receptor was similarly unaffected by disulphide bridge reduction of both native and α_2 AP–trypsin complexes.

Although the α_2 AP Cys⁴³–Cys¹¹⁶ disulphide appears to be evolutionarily conserved, it does not appear to have a profound influence on the structure or function of this serpin. Alternatively, it can be hypothesized that the Cys residues and the disulphide bridge influence events other than those examined here, such as *in vivo* binding to plasminogen or factor-XIII-mediated cross-linking of α_2 AP to fibrinogen. The disulphide may also affect the stability of α_2 AP so that the serpin retains inhibitory activity after advantageous proteolysis of the scaffold.

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