6-mer Peptide Selectively Annels to a Pathogenic Serpin Conformation and Blocks Polymerization

IMPLICATIONS FOR THE PREVENTION OF Z α₁-ANTITRYPSIN-RELATED CIRRHOSIS*

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Conformational diseases such as amyloidosis, Alzheimer's disease, prion diseases, and the serpinopathies are all caused by structural rearrangements within a protein that transform it into a pathological species. These diseases are typified by the Z variant of α₁-antitrypsin (E342K), which causes the retention of protein within hepatocytes as inclusion bodies that are associated with neonatal hepatitis and cirrhosis. The inclusion bodies result from the Z mutation perturbing the conformation of the protein, which facilitates a sequential interaction between the reactive center loop of one molecule and β-sheet A of a second. Therapies to prevent liver disease must block this reactive loop-β-sheet polymerization without interfering with other proteins of similar tertiary structure. We have used reactive loop peptides to explore the differences between the pathogenic Z and normal M α₁-antitrypsin. The results show that the reactive loop is likely to be partially inserted into β-sheet A in Z α₁-antitrypsin. This conformational difference from M α₁-antitrypsin was exploited with a 6-mer reactive loop peptide (FLEAIG) that selectively and stably bound Z α₁-antitrypsin. The importance of this finding is that the peptide prevented the polymerization of Z α₁-antitrypsin and did not significantly anneal to other proteins (such as antithrombin, α₁-antichymotrypsin, and plasminogen activator inhibitor-1) with a similar tertiary structure. These findings provide a lead compound for the development of small molecule inhibitors that can be used to treat patients with Z α₁-antitrypsin deficiency. Furthermore they demonstrate how a conformational disease process can be selectively inhibited with a small peptide.

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Members of the serine proteinase inhibitor or serpin superfamília are characterized by an exposed 14-residue (P1+4) mobile reactive center loop and a dominant five-stranded β-sheet A (1, 2) (Fig. 1a). Biochemical and crystallographic studies have defined the marked flexibility of the reactive loop and have demonstrated its role in inhibiting the target proteinase (3–6). The proteinase binds to the serpin and cleaves the reactive loop at the P1–P1' bond. This cleavage initiates a profound conformational change in the serpin in which the reactive loop peptide inserts into β-sheet A to form a new central strand termed s4A (6, 7). The conformational change inactivates the proteinase by translacating it over 70 Å to the lower pole of the molecule and disrupting the catalytic site.

This reactive loop-β-sheet A interaction, while being essential for proteinase inhibition, also renders the serpin vulnerable to conformational disturbances that are associated with disease, the serpinopathies (8). Point mutations perturb the relationship between the reactive loop and β-sheet A to allow the sequential interaction between the reactive center loop of one molecule and β-sheet A of another (Fig. 1a). These loop-sheet polymers are active as proteinase inhibitors and are retained within the cell of synthesis. This process is best characterized for the deficiency variants of α₁-antitrypsin (9–13). α₁-Antitrypsin is synthesized in hepatocytes and circulates in the plasma. Most Europeans are M homozygotes, but 1 in 25 has the severe Z α₁-antitrypsin deficiency variant (E342K). This is at residue P17 (17 residues proximal to the P1 reactive center) at the head of strand 5 of β-sheet A and the base of the mobile reactive loop (Fig. 1). The mutation opens β-sheet A thereby favoring the insertion of the reactive loop of another α₁-antitrypsin molecule to form a dimer. This can then extend to form chains of polymers that accumulate in the endoplasmic reticulum of the liver to form inclusion bodies (9). These inclusions result in neonatal hepatitis, cirrhosis, and hepato-cellular carcinoma (14–16), and the lack of circulating proteinase inhibitor exposes the lungs to uncontrolled proteolytic attack and early onset emphysema (17).

An effective approach to treatment of the liver disease would be to inhibit polymerization of the Z α₁-antitrypsin and thus prevent the accumulation of the protein within hepatocytes. Previous studies have shown that synthetic peptides with homology to the reactive loop of α₁-antitrypsin and the related serpin antithrombin can anneal to β-sheet A of both M α₁-antitrypsin and antithrombin (18–21). Furthermore, binding of these 11–13-mer peptides prevents the polymerization of Z α₁-antitrypsin (9) (Fig. 1a). However, such peptides are promiscuous and can efficiently anneal to, and inactivate, both M and Z α₁-antitrypsin and other members of the serpin superfamily (20). Their size and lack of specificity precludes the use of these peptides as therapeutic agents or as lead compounds from which to develop mimetic drugs. Consequently other strategies are being developed to prevent polymerization using chemical chaperones (22, 23) and by targeting a hydrophobic pocket (24, 25) that is filled as polymers form (26).

We report here the use of reactive loop peptides to explore the structural differences between the pathogenic Z and normal M α₁-antitrypsin. This has allowed us to first define the pathogenic conformation of Z α₁-antitrypsin and then exploit this difference from M α₁-antitrypsin to target a 6-mer peptide specifically to Z α₁-antitrypsin to prevent polymerization.
The reactive center loop (Z) bridge, UK) and dissolved in water. The peptides were annealed to M or Leu-Glu-Ala-Ile-Gly-OH) was synthesized and purified by MWB (Cambridge, UK) and dissolved in water. The peptides were annealed to M or Z with a native molecule control, and the final data were obtained by subtraction of α1-antitrypsin incubated with peptide from native α1-antitrypsin alone. The data were fitted to a single exponential function using Grafit (Version 3.00, 1992, Erithacus Software Ltd).

RESULTS AND DISCUSSION

The 12-mer P14-3 peptide, corresponding to the reactive loop of antithrombin, was incubated in 100-fold molar excess with 0.1 mg/ml M and Z α1-antitrypsin at 37 °C. The binding of the reactive loop peptide to α1-antitrypsin was monitored on acrylamide/8 M urea gels and by intrinsic tryptophan fluorescence. Native α1-antitrypsin unfolded in the urea and was retarded by the gel. The binary complex of α1-antitrypsin with peptide was stable in 8 M urea and hence migrated further into the acrylamide. The peptide annealed at a much slower rate to Z α1-antitrypsin than it did to M α1-antitrypsin (Fig. 2, top). Analysis of intrinsic tryptophan fluorescence allowed a more detailed assessment of the rate of peptide annealing. The addition of peptide to M α1-antitrypsin resulted in a significant increase in fluorescence as the peptide annealed to β-sheet A, reflecting incorporation of the reactive loop peptide into α1-antitrypsin (Fig. 2, bottom). However, the Z variant accepted the 12-mer reactive loop peptide at less than half of the rate of M α1-antitrypsin. Note the presence of a small amount of polymers of Z α1-antitrypsin in the starting material. Bottom, intrinsic tryptophan fluorescence of M α1-antitrypsin and Z α1-antitrypsin (0.1 mg/ml) with 100-fold molar excess of the 12-mer peptide at 37 °C for 24 h.

EXPERIMENTAL PROCEDURES

Purification of M and Z α1-Antitrypsin—M and Z α1-antitrypsin were purified from the plasma of known homozygotes by 50 and 75% ammonium sulfate fractionation followed by glutathione and anion exchange chromatography as described previously (27). The proteins migrated as a single band on SDS-PAGE and had a normal unfolding profile on transverse urea gradient gel electrophoresis. Both M and Z α1-antitrypsin were functional as inhibitors of bovine α-chymotrypsin.

Assessment of Synthetic Reactive Loop Peptide Annealing to α1-Antitrypsin by PAGE—A synthetic Nα-acetyl peptide corresponding to the P14-3 sequence of the reactive loop of antithrombin (Ac-Ser-Glu-Ala-Ala-Ser-Thr-Ala-Val-Val-Ile-Ala-OH) was synthesized and purified by Genosys Biotechnologies Inc. (Cambridge, UK) and dissolved in 50 mM Tris, 50 mM NaCl, pH 7.4. The P14-3 sequence of α1-antitrypsin (Ac-Phe-Leu-Glu-Ala-Ile-Gly-OH) was synthesized and purified by MWB (Cambridge, UK) and dissolved in water. The peptides were annealed to M or Z α1-antitrypsin, or other serpins, by incubating at a final concentration of 0.1–0.5 mg/ml in 50 mM Tris, 50 mM KCl, pH 7.4 at 37 °C. The binary complex between α1-antitrypsin, and other serpins, and the reactive loop peptides was assessed on a 7.5% (w/v) non-denaturing polyacrylamide gel containing 8 M urea.1 The formation of reactive loop-β-sheet A polymers was assessed on the same gel without urea (28).

Assessment of Synthetic Reactive Loop Peptide Annealing to α1-Antitrypsin by Intrinsic Tryptophan Fluorescence—The kinetics of reactive loop peptide annealing to α1-antitrypsin were assessed by following the intrinsic tryptophan fluorescence of α1-antitrypsin in a PerkinElmer LS 50B spectrophotometer. Intrinsic tryptophan fluorescence of α1-antitrypsin was measured in 50 mM Tris, 50 mM KCl, pH 7.4 using an excitation wavelength of 295 nm and an emission wavelength of 340 nm. The excitation and emission slit widths were controlled to give the optimal emission signal as described previously (10). Each experiment was performed with a native molecule control, and the final data were obtained by subtraction of α1-antitrypsin incubated with peptide from native α1-antitrypsin alone. The data were fitted to a single exponential function using Grafit (Version 3.00, 1992, Erithacus Software Ltd).

Both reactive loop peptide annealing to serpins and the formation of loop-sheet polymers occurs by intramolecular addi-

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1 A. Zhou, personal communication.
formed by annealing of P_{8-3} of the reactive loop to the lower part of \( \beta \)-sheet A (12, 30–32). The conformation adopted by Z \( \alpha_1 \)-antitrypsin is therefore similar to that produced by annealing a P_{14-4} 7-mer reactive loop peptide to antithrombin, which opens the top of \( \beta \)-sheet A to favor polymerization (33). It also approximates the conformation seen in our crystal structure of a naturally occurring mutant of \( \alpha_1 \)-antichymotrypsin that also readily forms polymers in vitro and in vivo (34).

The hypothesis that the pathogenic conformation adopted by Z \( \alpha_1 \)-antitrypsin is associated with partial insertion of the reactive loop was tested using a peptide targeted to the lower part of \( \beta \)-sheet A. A 6-mer peptide that was homologous to P_{7-2} of the reactive loop of \( \alpha_1 \)-antitrypsin was obtained from Dr A. Zhou and colleagues (Department of Hematology, University of Cambridge) who were undertaking a separate study on the structural requirements for peptide-\( \beta \)-sheet A blockage (Fig. 1b). This peptide was able to anneal to Z, but not to M \( \alpha_1 \)-antitrypsin (Fig. 3, top), and resulted in a 60% reduction in its inhibitory activity against bovine \( \alpha \)-chymotrypsin (data not shown). Furthermore, the rate of fluorescence increase when the peptide annealed was over 30-fold more rapid with Z \( \alpha_1 \)-antitrypsin than with M \( \alpha_1 \)-antitrypsin: \( 2.2 \times 10^{-8} \) and \( 0.07 \times 10^{-8} \) s\(^{-1}\) for Z and M \( \alpha_1 \)-antitrypsin, respectively (Fig. 3, middle). The importance of this interaction was highlighted by co-incubation of the 6-mer peptide with Z \( \alpha_1 \)-antitrypsin, which resulted in a complete inhibition of polymerization of Z \( \alpha_1 \) antitrypsin when incubated at 37 °C (Fig. 3, bottom) and 41 °C (data not shown). It is likely that the peptide also prevented polymerization of Z \( \alpha_1 \)-antitrypsin at peptide:protein ratios of less than 25:1, although accurate values were precluded by limited peptide solubility.

Peptide annealing was specific for Z \( \alpha_1 \)-antitrypsin as co-incubation of the 6-mer peptide with M \( \alpha_1 \)-antitrypsin for 3 days resulted in no significant binary complex formation (Fig. 3, top). Moreover it had no effect on inhibitory activity of M \( \alpha_1 \)-antitrypsin against bovine \( \alpha \)-chymotrypsin or the ability of M \( \alpha_1 \)-antitrypsin to form SDS-stable complexes with trypsin (data not shown). The specificity of the interaction of the 6-mer peptide with Z \( \alpha_1 \)-antitrypsin was underscored by the demonstration that it would not anneal to other members of the serpin superfamily that have the same tertiary structure (\( \alpha_1 \)-antichymotrypsin, plasminogen activator inhibitor-1, or \( \alpha \)-antithrombin) when incubated under physiological conditions (data not shown).

The implications of these findings are 2-fold. First, an effective approach to treat the liver disease associated with Z \( \alpha_1 \)-antitrypsin would be to inhibit polymerization of the Z protein and thus prevent the accumulation of the protein within hepatocytes. However, this approach may result in the release of inactive Z \( \alpha_1 \)-antitrypsin and would require intravenous replacement therapy with normal \( \alpha_1 \)-antitrypsin to replenish plasma levels to prevent emphysema. This makes it essential to specifically block \( \beta \)-sheet A of Z \( \alpha_1 \)-antitrypsin but not M \( \alpha_1 \)-antitrypsin or other proteins with a similar tertiary structure. We have shown that this is achievable in vitro. Second, these findings extend to other diseases that result from polymer formation. Loop-sheet polymerization is also recognized to underlie the deficiency of other members of the serpin superfamily: antithrombin (35), C1-inhibitor (36, 37), \( \alpha_1 \)-antichymotrypsin (34), and neuroserpin (38), which are associated with thrombosis, angioedema, emphysema, and an inclusion body dementia, respectively. All the mutations that favor these disease processes have been shown, or been predicted, to open \( \beta \)-sheet A and facilitate polymer formation (8, 38, 39). It is likely that peptides or synthetic mimetics can be created that

![Native vs Binary complex](image)
will bind specifically to these mutant serpins, prevent polymer formation, and so attenuate disease.

In summary, these findings offer the real prospect of selectively targeting Z\(\alpha_1\)-antitrypsin to prevent polymerization and so ameliorate the associated liver disease. The challenge for the future is to reconcile the requirements of a small molecule for specific inhibition of Z\(\alpha_1\)-antitrypsin with those properties needed for drug design and targeting to the endoplasmic reticulum.

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