The reactive-site loops of serpins are characterized by a defined mobility where the loop adopts a new secondary structure as an essential part of the inhibitory process. While the importance of mobility in the N-terminal region of the reactive-site loop has been well studied, the role of mobility in the C-terminal portion has not been investigated. The requirements for mobility of the C-terminal portion of the reactive-site loop of α1-antitrypsin were investigated by creating a disulfide bridge between the P8 residue and residue 283 near the top of strand 2C; this disulfide would restrict the mobility of the C-terminal portion of the reactive-site loop by locking together strands 1 and 2 of the C β-sheet. The engineered disulfide bond had no effect on the inhibitory activity of α1-antitrypsin, indicating that there is no requirement for mobility in this region of the molecule. Moreover, these results, coupled with those from molecular modeling, indicate that insertion into the A β-sheet of the intact reactive-loop beyond P12 is not rate-limiting for the formation of the stable complex. The engineered disulfide bond should also prove useful in the creation of more stable serpin variants; for example, such a bond in plasminogen activator inhibitor-1 would prevent it from becoming latent by locking strand 1C onto the C β-sheet.

α1-Antitrypsin (α1-AT) is a member of the serpin family of serine protease inhibitors. Members of this large group of inhibitors are involved in the control of proteolysis in many physiological processes including coagulation, fibrinolysis, complement activation, and inflammation. Serpins are glycoproteins with a molecular mass of approximately 50 kDa. They form a tight complex with the active site of cognate serine proteases through an exposed peptide loop of some 20 residues. Following the initial attack of the protease on the exposed peptide loop, serpins undergo significant conformational rearrangements resulting in the formation of an extremely stable bimolecular complex (1–4).

Early interactions between serpins and cognate proteases presumably resemble those of the proteases with their natural peptide and macromolecular substrates. Serpin reactive-site loops share many features with the cleavage sequences of natural substrates of serine proteases, including sequence, surface accessibility, and flexibility. The requirements for correct sequence and accessibility are relatively apparent; the need for flexibility is less obvious. It has been proposed that the structure of the transition state of good substrates of serine proteases is closely homologous to that demonstrated by the so-called “canonical” inhibitors, a class which includes the Kunitz, Kazal, and ovomucoid third domain inhibitors (5, 6). These inhibitors have reactive-site loops whose main-chain geometry is tightly restrained into a specific conformation shared by all members of these families between residues P9 and P39, both when free in solution and when bound to the enzyme (7). This canonical conformation allows a β-strand type of hydrogen bonding interaction between the active site of the protease and the inhibitor; this structure is believed to resemble the Michaelis complex formed early in the proteolytic process. Natural proteolytic sites do not normally exist in this configuration, yet must adopt it during the Michaelis step of the reaction. It has been proposed by Hubbard et al. (8) that the requirement for flexibility in proteolytic cleavage sites of natural substrates is due to the distortions required to allow the residues surrounding the scissile bond to adopt the Michaelis complex structure, i.e. the canonical conformation. It is suggested that these distortions would require substantial mobility of at least 5 residues on each side of the P1-P1′ bond (8).

The nature of the serpin-enzyme complex is still a matter of great debate. It has been variously proposed that a portion of the inhibitory loop of serpins adopts a configuration similar to that of the canonical inhibitors after docking with the protease (5, 9), that they resemble the tetrahedral intermediate (10), and that the P1-P1′ peptide bond is cleaved in the complex with the P-side of the reactive-site loop fully inserted into the A β-sheet of the serpin (11–13). Each of these hypotheses requires that the serpin reactive-site loop adopts, at least transiently, a Michaelis complex-like structure (i.e. similar to the canonical configuration), and, according to the hypothesis of Hubbard et al. (8) presented above, this requires mobility of at least 5 residues on each side of the scissile bond. The presence of a flexible reactive-site loop is both a conspicuous feature of

The abbreviations used are: α1-AT, α1-antitrypsin, also known as α1-proteinase inhibitor; HLE, human leukocyte elastase; PCR, polymorerease chain reaction; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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The nomenclature for residues of the reactive-site loop of serpins is based on that of Schechter and Berger (30) for substrates of proteases. The residues are numbered from the scissile bond as follows: P9′− ... −P1′−P1−P2−P3−P4−P5−P6−P7−P8−P9; for a substrate, cleavage occurs at the P1-P1′ bond; in α1-antitrypsin this is the Met256-Ser359 bond. The corresponding binding sites for the residues on the protease are S8− ... S3′, S2′, S1′, S0′ = S0′.
serpins and a notable point of difference between serine protease inhibitors of the serpin family and those of the canonical classes. Up to 15 residues on the N-terminal side of the P1–P1′ bond of the serpin reactive-site loop (residues P1 to P10) constitute a very mobile region of the molecule, some of which are believed to move from a solvent-accessible position to being at least partially buried within the α-sheet as an essential part of the inhibitory process (11–14). Interfering with this mobility has been demonstrated to adversely affect the inhibitory potential of the serpin (15–18). In contrast, little is known about the role of the P′ side of the reactive-site loop. In the crystal structure of cleaved α1-antitrypsin, this region forms strand 1 of the C β-sheet from P′5 to P′6. Naturally occurring mutations of strand 1C have been shown to interfere with normal serpin activity (19, 20). In the crystal structures of two uncleaved members of the serpin family, antithrombin (4) and plasminogen activator inhibitor-1 (21), it has been demonstrated that strand 1C can detach from the C β-sheet upon insertion of the P-side of the reactive-site loop into the α-sheet. In addition, molecular modeling indicates that the N-terminal region of the reactive-site loop could not insert extensively into the α-sheet without either cleavage of the P1–P1′ bond or the detaching of strand 1C from the C β-sheet (22).

The work presented in this manuscript investigates the requirement for strand 1C mobility in serpin inhibitory function, using α1-antitrypsin as a model. The mobility of strand 1C was restricted through the creation of a disulfide bridge between two mutated residues Pro361 → Cys and Ser283 → Cys, present in positions P′3 and in an adjacent position at the end of strand 2C, respectively (see Fig. 1). The disulfide bridge was successfully created and shown to have a minimal effect on α1-AT inhibitory function, consistent with there being no general requirement for mobility in strand 1C in the inhibitory mechanism of serpins.

EXPERIMENTAL PROCEDURES

Materials—The chomogenic substrate N-MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide, Staphylococcus aureus V8 protease, protease-free bovine serum albumin, polyethylene glycol (PEG), and isopropyl-1-thio-β-D-galactopyranoside were obtained from Sigma (Poole, United Kingdom). Human leukocyte elastase (HLE) was prepared as described previously (23). Restriction enzymes were obtained from New England Biolabs (Beverly, MA), and oligonucleotides were synthesized by members of the Dept. of Biochemistry, University of Cambridge. Rifampicin was obtained as Rifadin (Merion Merrell Dow Ltd., Uxbridge, UK). All other chemicals were of the highest grade commercially available.

Production of Recombinant α1-AT—All antitrypsins were produced in Escherichia coli BL21 (DE3) under the control of the T7 RNA polymerase promoter using the vector pAN15 previously described (24). The mutants were made by PCR, exploiting the Aval restriction site that lies at a position corresponding to the codons for residues 362 and 363, a PstI site that was engineered silently into the position corresponding to codon 362, and a BstXI site that lies upstream of Ser283. The Pro361 → Cys oligonucleotide used was: GAACTTGACCTCGGGGCAGATAGA-TCACCAAGTTCCTGGAAAATGAAGACAGAAGGTGTGCCAGCTTAC; the Ser283 → Cys oligonucleotide used was: CCAATGCAGACAGATAGA-TCACCAAGTTCCTGGAAAATGAAGACAGAAGGTGTGCCAGCTTAC; the mutated bases are underlined. PCR was performed with these oligonucleotides using antitrypsin cDNA containing the silent mutation Cys361-Cys283. The entire region amplified by PCR was sequenced. To construct the Cys361-Ser283 mutant, the Cys361-Cys283 codon by the wild-type Ser283 codon.

The chromogenicsubstrate 4-Methylumbelliferone was added to a final concentration of 0.1 μM. After 30 min, rifampicin was added to a final concentration of 100 μg/ml, and the culture was grown for an additional 3 h.

Purification of Recombinant Protein—The antitrypsins were purified from inclusion bodies as follows. The cells (10 g) were resuspended in 30 ml of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 10 mM EDTA, and 0.5% Triton X-100 and lysed by three passages through a French Press at 16,000 p.s.i. The inclusion bodies and cellular debris were collected by centrifugation at 5,000 × g for 20 min. This pellet was resuspended in 30 ml of the above buffer, vortexed vigorously, and centrifuged again. This procedure was repeated five times to wash the inclusion bodies, three times in the same buffer, and twice in buffer without Triton X-100. The pellet was dissolved in 10 ml of 50 mM Tris-HCl, pH 8.0, 8 mM guanidine hydrochloride, 100 mM Na-dithiothreitol (DTT); after purging with N2, the solution was incubated for 2 h. The antitrypsins were refolded by direct dropwise dilution into 1.6 liters of 50 mM Tris-HCl, pH 8.0, containing 5 mM DTT, at room temperature. The refolded protein was loaded directly onto a 1.6 × 25 cm column of Q-Sepharose column equilibrated in 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM DTT at 4 °C, and the bound proteins eluted using a 200-ml linear gradient from 50–250 mM NaCl. All buffers were N2-purged. The purity was assessed by SDS-PAGE, and the antitrypsin-containing fractions were purged with N2 and stored at −80 °C. For the preparation of the oxidized form of the Cys361-Cys283 mutant, the disulfide bond between Cys361 and Cys283 was allowed to form during removal of DTT by dialysis for 16 h against 50 mM Tris-HCl, pH 8.0, 50 mM NaCl (4 × 5 liters).

Sulphydryl Determination—The concentration of sulphydryl groups in antitrypsins was determined by the method of Ellman as described in Ref. 25. The recombinant wild-type and oxidized Cys361-Cys283 antitrypsins (0.03 μmol) were diluted into a final volume of 6 ml of 80 mM sodium phosphate buffer, pH 8.0, containing 130 mM EDTA and 2% (w/v) SDS, 3 ml of this was reacted with 100 μl of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 100 mM sodium phosphate buffer, pH 8.0, and incubated for 15 min at room temperature. After incubation, the absorbance at the 550-dithiobis(2-nitrobenzoic acid)-treated solution was measured at 410 nm against the untreated antitrypsin blank solution. The concentration of free sulphydryl in the antitrypsin was calculated using the molar absorption value of 13,600 M−1cm−1 for 2-nitro-5-thiobenzoate (25). Estimation of total cysteine content was performed by amino acid analysis of the Cys361-Cys283 antitrypsin at the Dept. of Biochemistry, University of Cambridge.

Determination of Inhibition Parameters—The protein concentrations and inhibitory titers were determined as described previously (17). All assays were performed at 37 °C in 30 mM sodium phosphate buffer, pH 7.4, containing 0.16 M NaCl, 0.1% polyethylene glycol (PEG), and 0.1% Triton X-100, and 500–600 μM N-MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide, using bovine serum albumin, polyethylene glycol (Mw = 8000) coated plastic cuvettes. Slow-binding kinetics were performed using a Hewlett-Packard diode-array spectrophotometer. Each progress curve experiment consisted of 7 assays, with 1 in the absence of serpin and 6 others with increasing serpin concentrations. After initiation of the reaction, the change in absorbance at λmax as a function of time was monitored. The absorbance at λmax was measured between 400 and 410 nm. Data points where substrate utilization was in excess of 10% of total substrate concentration were excluded from the analyses. Progress curve data were analyzed as described previously (17) to yield estimates for the association rate constant for the formation of the stable complex (Kns). The dissociation constants (K) for the inhibitors could not be determined under the conditions used; however, considering the conditions of the experiment, an upper limit of 1 μM could be placed on all reactions. Progress curve experiments were performed at least three times, and the values reported represent the weight mean of the determinations. Values of Kns required for the calculation of Kns were determined by standard initial velocity studies; an estimate of 85.5 ± 2.5 μM was obtained for N-MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide with HLE.

Analysis of Reaction Products by SDS-PAGE—The presence of the disulfide bond between Cys361 and Cys283 in the oxidized form of the Cys361-Cys283 protein was confirmed by analysis of the protein on SDS-PAGE before and after cleavage of the protein within the reactive-site loop. α1-AT and the mutant were incubated with S. aureus V8 protease in 50 mM NH4HCO3 buffer, pH 7.8. This reaction was terminated by the addition of 1 M 2-mercaptoethanol (Glu345) (26); when denatured, the C-terminal fragment was released giving a reduction in the apparent size of the protein on SDS-PAGE. In the double-cysteine mutant, however, the C-terminal fragment should remain attached to the main body of the molecule if the disulfide bond between Cys361 and Cys283 was intact.

In order to determine whether the oxidized mutant was still capable
of making SDS-stable complexes, wild-type α1-AT and oxidized Cys361-Cys283 mutant (0.4 μM each) were incubated with an equimolar amount of HLE in a volume of 20 μl at 25 °C for 2 min. SDS-gel loading buffer (5 μl) containing 2.5% SDS was added, and the sample was heated at 100 °C for 3 min before analysis by SDS-PAGE.

RESULTS

Characterization of the Engineered Disulfide Bond in α1-AT—The sequence of α1-AT contains a single cysteine (Cys232). In order to restrict the mobility of the C-terminal portion of the reactive-site loop, a disulfide bond was engineered between the P3 residue (Pro361 → Cys) and a residue in strand 2C (Ser283 → Cys). Molecular modeling indicates that this disulfide bond was feasible (Fig. 1) and that it would lock the C-terminal end of the reactive-site loop including strand 1C against the reactive-site loop as in the crystal structure of intact antithrombin III (4). The Cys of residue P12 is shown as a filled circle.

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Inhibition of Human Leukocyte Elastase by Mutant Antitrypsins—Titration experiments with the oxidized form of the Cys361-Cys283 antitrypsin showed that the stoichiometry of inhibition of HLE was 1.1 relative to the wild-type. Serpins have been shown to function as suicide inhibitors as shown in Scheme 1 (11). After formation of an intermediate complex (E·I), the pathway partitions between the formation of the stable complex (E·I*) and the release of cleaved inhibitor (I*). The ratio of the rate constants for these two reactions determines the stoichiometry of inhibition. Thus, the fact that the stoichiometry of inhibition was not affected by the engineered disulfide bond indicates that the relative rates of the inhibition and cleavage pathways were not altered; i.e. the disulfide bond, which restricts the mobility of strand 1C, does not affect the partition of reaction products between the inhibitory and cleavage pathways. In contrast, mutations at the N-terminal end of the reactive-site loop can markedly affect this partitioning (15, 17, 18).

The Cys361-Cys283 mutant was still a potent slow tight-binding inhibitor of HLE; restriction of the mobility of strand 1C by the disulfide bond did not prevent the inhibitory process. The association rate constant (kass) for disulfide mutant was 0.21 × 107 M⁻¹ s⁻¹ (Table I); this value is about 5-fold lower than that of the recombinant wild-type protein (1.20 × 107 M⁻¹ s⁻¹). In order to investigate further the reason for the decrease in kass value, a mutant having only the Pro361 → Cys mutation was constructed. This mutant (Cys361-Ser283) also had a reduced kass value with HLE; the value obtained (0.32 × 107 M⁻¹ s⁻¹) was similar to that obtained for Cys361-Cys283 (Table I). This result demonstrated that the 5-fold decrease in the association rate constant of the double-cysteine mutant was not due to the restriction of the mobility of strand 1C by the disulfide bond, but was predominantly due to the replacement of the P3 proline by cysteine.

Analysis of Reaction Products by SDS-PAGE—Fig. 3 shows that the oxidized form of the Cys361-Cys283 mutant formed typical SDS-stable serpin-enzyme complexes, i.e. the disulfide bond does not prevent this. The gels also show no appreciable generation of reactive-center-cleaved α1-AT. Taken together with the stoichiometry of inhibition value of 1.1 and observed kass value, these data show that restricting the mobility of strand 1C has no appreciable effect on the inhibitory mechanism of α1-AT.

DISCUSSION

Although multiple lines of evidence have suggested that the mobility of strand 1C may be important to the function of
serpins (8, 19, 20), the data presented in this work indicate that, in α1-AT at least, restricting the mobility of strand 1C did not significantly affect the inhibitory activity of the molecule. The hypothesis presented by Hubbard et al. (8) concerning the requirement for mobility suggested that distortion of up to 5 residues on either side of the P1-P1′ bond is required for the formation of the canonical structure of the P2 to P3 residues. This would be required for any of the current models of the serpin-enzyme inhibition reaction: the formation of the Michaelis complex (10), the canonical form (5, 9), or cleavage of the reactive-site loop (11–13). However, it is apparent from the data presented here that in α1-AT mobility beyond residue P3 on the P′ side of the scissile bond is not required for the formation of the stable serpin-enzyme complex.

Although mobility of the P3 residue was not required for complex formation, contacts between the active site of HLE and this residue appear to play a small role. Mutation of the P3 proline to cysteine led to a 4-fold decrease in the kmax value. A similar decrease was observed with the disulfide mutant. These results suggest that the decreases in kmax are due to loss of contacts with the P3 proline, since the chemical nature of the cystine in Cys361-Cys283 and cysteine in Cys361-Ser283 are different. Alternatively, the P3 proline could encourage a conformation of the reactive-site loop that facilitates rapid complex formation.

Biochemical evidence now suggests that while in the stable complex the hinge region of the serpin (residues P1 to P13) is at least partially inserted into the A β-sheet (14); the extent of the insertion is still a matter of debate, but it appears that insertion at least as far as P9 is required (15, 27). Recent modeling studies have indicated that insertion of the hinge region into the A β-sheet to P15 or beyond requires either the unlinking of strand 1C from the C β-sheet or cleavage within the reactive-site loop (22). Data presented in this work indicate that the uncoupling of strand 1C from the C β-sheet is not necessary for the inhibitory function of α1-AT. This observation may be interpreted in diverse ways: either extensive insertion of the hinge region beyond P9 is not a requirement for inhibitory function, or that insertion beyond P12 occurs and the P1-P1′ bond is cleaved in the complex, as suggested by two recent publications (12, 13). If the P1-P1′ bond is indeed cleaved in the complex, and the reactive-site loop is fully inserted into the A β-sheet as strand 4A (12, 13), then the data presented in this work indicate that cleavage occurs before insertion of the hinge region beyond P12. It is now widely accepted that serpins operate as suicide inhibitors (see Scheme 1), where after the formation of an initial complex, this complex may lead to the concurrent generation of either a stable serpin-enzyme complex (E1I*) or to the generation of reactive-site loop cleaved serpin and active enzyme (E + I′) (16–18, 27–29). Two processes are probably involved in the conversion of the initial complex to the final stable complex: insertion of the hinge region of the reactive-site loop into the A β-sheet and cleavage of the P1-P1′ bond (12, 13). Insertion of the hinge region appears to be the rate-limiting process in the formation of the stable complex; mutations in the hinge region of serpins reduce the rate at which the stable complex is formed from the intermediate EI, presumably by slowing down the rate of insertion of the hinge region into the A β-sheet (18, 27), and, thus, the insertion of the hinge region appears to be the rate-limiting step in the formation of the stable complex (EI*). The presence of a disulfide bond preventing the removal of strand 1C from the C β-sheet did not reduce the rate of formation of the inhibited complex. Although an attack on the P1-P1′ bond by the catalytic triad of the serine protease appears to be essential for the formation of the final stable serpin-enzyme complex, the data suggest that this attack is not a rate-limiting step and that it occurs after the rate-limiting step, i.e. after initiation of insertion of the hinge region into the A β-sheet.

The disulfide mutant described here has implications for pharmaceutical intervention in human medicine of the important regulatory systems controlled by serpins such as coagulation, fibrinolysis, inflammation, and complement activation. It has been discovered that serpins may loose activity by converting to a latent form where the reactive-site loop has inserted into the A β-sheet fully as strand 4A; this insertion is permitted because strand 1C has detached from the C β-sheet (22). The presence of a disulfide bond such as that described in this work could be used to abolish the formation of a latent form of a serpin such as plasminogen activator inhibitor-1 (21), or antithrombin (4), thus increasing their utility without necessarily affecting the inhibition of cognate proteases.

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FIG. 3. Non-reducing 10% acrylamide SDS-PAGE showing the generation of SDS-stable complexes between the oxidized form of the Cys361-Cys283 mutant and HLE. Antitrypsins and HLE were incubated at a concentration of 0.4 μM in buffer as described under "Experimental Procedures" and denatured in SDS after 2 min. Lane 1, oxidized Cys361-Cys283 mutant; lane 2, wild-type recombinant α1-AT; lane 3, wild-type recombinant α1-AT incubated with HLE; lane 4, oxidized Cys361-Cys283 mutant incubated with HLE.
Mobility of the Serpin Reactive-site Loop

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