We have shown, by two independent criteria (protease and phospholipase sensitivity), that the elimination of the negatively charged transmembrane aspartate residue abolishes the signal for PI modification of Qa-2. These results indicate that at least one requirement for PI linkage of proteins is the presence of a weakly hydrophobic transmembrane segment.

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19. Monoclonal antibody to Thy-1.2 was from Dupont New England Nuclear. The American Type Culture Collection cell line J11d.2 was used to produce cell supernatants containing antibody to J11D. Other monoclonal antibodies used in this study have been described (*5, 8, 9*); these were cell supernatants from 20-8-4 cells for antibody to Qa-2, and from B22.249 for antibody to H-2D^b. Goat antiserum to mouse immunoglobulins IgM, IgG, and IgA, conjugated to fluorescein isothiocyanate, was from Cappel.
20. Papain was from Cooper Biomedical. This lot (77621M) had a specific activity of 21 U/mg and a concentration of 31.6 mg/ml.

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Alpha-2-Antiplasmin: A Serpin with Two Separate but Overlapping Reactive Sites

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Although the proteinase inhibitor alpha-2-antiplasmin (α_2AP) is known to control the activity of plasmin through rapid formation of stable complexes, it also efficiently inactivates chymotrypsin. These interactions are shown to occur at adjacent, overlapping sites so that plasmin attacks the inhibitor at an Arg^{364}-Met^{365} peptide bond, while chymotrypsin interacts at a Met^{365}-Ser^{366} sequence one residue downstream. Thus, a naturally occurring plasma serine proteinase inhibitor can have multiple specificities through interactions at adjacent sites. It also illustrates the potential flexibility of the reactive site loop in this class of inhibitors.

HUMAN α_2AP IS ONE OF SEVERAL homologous proteins that comprise the serpin (SERine Proteinase INhibitor) superfamily (*1, 2*). Kinetic studies on the interaction of this inhibitor with a series of proteinases have shown that its major target enzymes are plasmin and trypsin ($K_a = 1.0 - 4.0 \times 10^7 M^{-1}$ sec^{-1}) (*3–5*). Complex formation occurs through enzyme-inhibitor interactions at the Arg^{364}-Met^{365} peptide bond (*3, 6*), and this is in agreement with the specificities of both plasmin and trypsin. However, α_2AP can also form stable complexes with bovine α-chymotrypsin [$K_a = 6.7 \times 10^5 M^{-1}$ sec^{-1} (*3*)] which dissociate only very slowly [$K_d = 5.6 \times 10^{-5}$ sec^{-1} (*7*)]. Thus, such interactions are not simply a mechanism for chymotrypsin inactivation of α_2AP by limited proteolysis. Because the structure of the inhibitor loop of α_2AP includes the sequence beginning -Ala-Met-Ser-Arg-Met-Ser-Leu-Ser- (residues 361–368), with the Arg-Met peptide bond representing the inhibitory site for plasmin and trypsin inhibition (*3, 6*), many other adjacent peptide bonds in this region would appear to be more favorable for chymotrypsin binding and inhibition. We have, therefore, determined the site of attack of chymotrypsin during complex formation with α_2AP.

Complexes of α_2AP with bovine α-chymotrypsin were formed by incubation of equimolar quantities of inhibitor and enzyme at *p*H 7.4 for 1 minute at 25°C. Human α-2-macroglobulin (α_2M) (5*M* excess) was added to trap any chymotrypsin released from complexes, and the reaction mixture was incubated for 15 hours. Under these conditions more than 90% of the chymotrypsin activity was released. The large α_2M-chymotrypsin complexes were separated from mixtures of native and modified α_2AP by gel filtration chromatography on Sephadex G-100. The two forms of inhibitor were then purified by fast protein liquid chromatography (FPLC) on a mono-Q column (Pharmacia). Modified α_2AP, which represented more than 75% of the starting material, migrated as two components of 60 kD and 14 kD, on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). Preparative gel electrophoresis was used to isolate each, and both were then analyzed for NH_2-terminal sequences. The first ten residues are given below:

60 kD:
LysSerProProGlyValCysSerArgAsp **1**
14 kD:
SerLeuSerSerPheSerValAsnArgPro **2**

The sequence of peptide **1** indicates that chymotrypsin attacked α_2AP between residues 24 and 25 while analysis of peptide **2** shows cleavage between residues 365 and 366. The former reaction is presumed to be noninhibitory, representing only enzymatic hydrolysis of peptide bonds in the NH_2-terminal region and has been reported for other serpins (*8, 9*). However, the latter cleavage indicates that the interaction between chymotrypsin and α_2AP has occurred between Met^{365} and Ser^{366} in the reactive site loop and that the 14-kD fragment represents the cleavage peptide released during slow dissociation of this complex. Thus, α_2AP has two inhibitory sites that overlap, Met^{367} being in the P_1'-position for plasmin/trypsin inhibition and in the P_1-position for chymotrypsin inhibition (Fig. 2). Upstream, human neutrophil elastase inactivates α_2AP by cleavage between Ala^{361} and Met^{362} (*3, 10*). This reaction occurs even though there are two nearby Met-Ser se-

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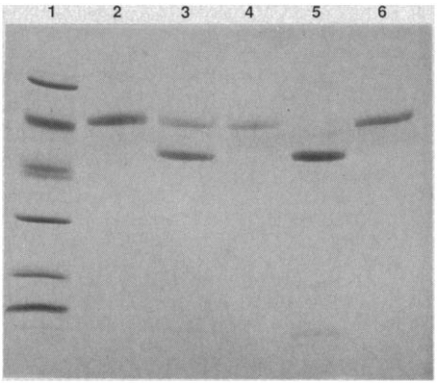

Fig. 1. SDS-PAGE of α_2AP after dissociation of complexes with bovine α-chymotrypsin. Samples were separated by electrophoresis on an 8 to 20% SDS-polyacrylamide slab gel with the Wyckof buffer system (*19*), and the gel was stained with Coomassie blue. Lane 1, protein standards; lanes 2 and 6, α_2AP control; lane 3, native and post-complex inhibitor after gel filtration; lane 4, active α_2AP after FPLC; lane 5, modified α_2AP and cleavage peptide after FPLC.

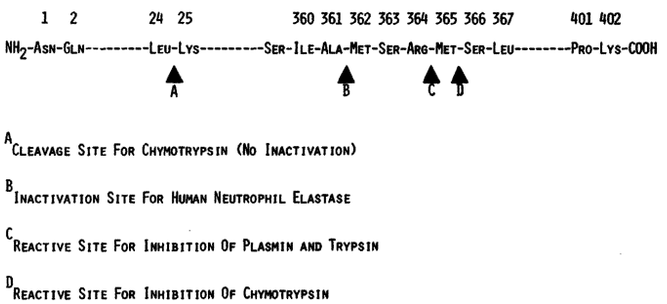

Fig. 2. The structure of the reactive site of human α_2AP. Complex formation with plasmin and trypsin occurs one residue upstream from that occurring with chymotrypsin. Neutrophil elastase inactivates α_2AP by hydrolysis of an Ala-Met peptide bond. Note that the inhibition of human leukocyte elastase by α_1-proteinase inhibitor occurs by attack at a Met-Ser peptide bond (*11*), two of which are present in the α_2AP reactive site loop, yet apparently ignored by this enzyme.

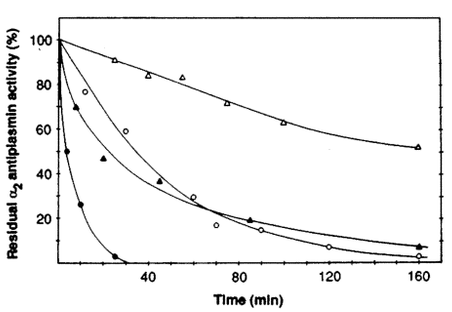

Fig. 3. Differential loss of α_2AP inhibitory activity after modification of arginine residues. The α_2AP (1.5 μ*M*) was incubated in the presence of 1,2-cyclohexanedione (7.5 m*M* and 75 m*M*) in 0.1*M* sodium borate buffer, *p*H 8.8, at 37°C. Aliquots were removed at specific time intervals and assayed for residual chymotrypsin and trypsin inhibitory activities. Inhibitor and enzyme were preincubated for three minutes at *p*H 8.0 prior to activity measurements. Substrates used were Suc-Ala-Ala-Pro-Phe-pNA for chymotrypsin and Bz-Ile-Glu-Gly-Arg-pNA for trypsin. (△ and ▲) chymotrypsin inhibitory activity; (○ and ●) trypsin inhibitory activity. Open and closed symbols represent low and high reagent concentrations, respectively.

quences, either of which might be considered equivalent to the reactive site of α_1-proteinase inhibitor (*11*), which is the controlling inhibitor for this enzyme.

Significantly, there was no evidence for chymotrypsin attack at the Arg^{364}-Met^{365} trypsin/plasmin inhibitory site. When the modified inhibitor obtained from chymotrypsin complexes was digested with carboxypeptidase (Cpase) A (Worthington) for 24 hours only methionine was released, while digestion with Cpase B gave only lysine, which is the COOH-terminus of native α_2AP (*12*). If, however, Cpase A treatment was followed with Cpase B, both lysine and arginine were found, as would be expected from the reactive site sequence. Confirmation of the presence of two inhibitory sites in α_2AP has been obtained through chemical modification experiments. When α_2AP was incubated with either a 5,000 or 50,000 molar excess of cyclohexanedione (Aldrich) at *p*H 8.8 there was rapid loss of trypsin inhibitory activity (Fig. 3) (*13*). However, much longer incubation times were required to reduce chymotrypsin inhibitory activity. We would have expected parallel losses of both activities if inhibition of either enzyme was occurring at a single site.

Serpins have a reactive center which is exposed on a strained loop near their carboxyl terminus (*2, 14*). While sequences at either end of this loop are highly conserved in all of the proteins of this class so far examined, within the reactive site loop there is considerable heterogeneity (*15, 16*). This has previously been shown in the ovomucoid inhibitor family (*17*), even though the reactive site peptide bond was rigidly specified. In α_2AP, however, the reactive site can apparently shift, depending on the enzyme being complexed, as shown in this report and also in data obtained with natural and recombinant derived mutants of α_2AP, where insertions and deletions in the reactive site loop caused the elimination of inhibitory activity or changes in specificity (or both) (*5, 18*). Since most of the serpins tested can form complexes with more than one proteinase, albeit at very different rates, it is likely that other members of this family will have properties similar to those found with α_2AP, particularly if inhibition is rapid and the complexes formed are stable. Examination of loop sequences alone, however, is not likely to be useful since potential reactive sites are not always recognized. Otherwise, we would expect α_2AP to inhibit neutrophil elastase. Obviously, other contact regions in serpins besides the putative P_1 residues must be important in dictating inhibitor specificity.

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