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THE PRIMARY ROLE OF THE P_1' RESIDUE (SER^{359}) OF ALPHA-1-PROTEINASE INHIBITOR

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Summary: The replacement of ser^{359} with ala^{359} at the P_1' position in human α-1-proteinase inhibitor results in the production of a variant protein containing 15% of the inhibitory activity of the normal inhibitor. Separation of active from inactive inhibitor on anhydrochymotrypsin-sepharose yields a form which has a second order association rate with neutrophil elastase which is approximately one half that for the native protein. These data indicate that the P_1' residue is not of primary importance during the interaction of proteinases with α-1-proteinase inhibitor. Since substitution of alanine for serine causes the formation, primarily, of inactive inhibitor the major function of ser^{359} probably involves proper folding to give a functionally active inhibitory conformation.

Several of the proteins in the proteinase inhibitor superfamily referred to as Serpins (1) have either a serine or threonine residue in the P_1' reactive site suggesting an important role for a hydroxylated amino acid in this position. Included in this list is α-1-proteinase inhibitor (α-1-PI), the major proteinase inhibitor in human plasma, which functions to control proteolytic events mediated by neutrophil elastase (2). It has recently been shown that a naturally occurring altered form of the related proteinase inhibitor, antithrombin III (AT III), with a serine to leucine replacement at the P_1' position has significantly reduced inhibitory activity (3), further supporting a major role for the P_1' amino acid. However, other P_1' variants of AT III produced by recombinant DNA technology have been found to vary in their inhibitor activities (4),

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suggesting that this residue may play a more important role in maintaining proper inhibitor conformation, rather than being directly involved in inhibitor function. Since the three-dimensional structure of native α-1-PI is unknown the contribution of the P_1' ser^{359} residue to inhibitory function cannot be readily ascertained. By utilizing site-specific mutagenesis (5,6) we have been able to obtain a protein in which the P_1' residue of α-1-PI has been altered from ser^{359} to ala^{359}. In this report we describe the isolation and inhibitory properties of this protein.

MATERIALS AND METHODS

Human neutrophil elastase (HNE), human cathepsin G, and S. aureus V-8 proteinase were prepared as described previously (7-9). Porcine pancreatic elastase (PPE), porcine trypsin, bovine chymotrypsin, and chymostatin were purchased from Sigma, as were the trypsin substrate Bz-L-Arg-OEt, the chymotrypsin substrate, Bz-L-Tyr-OEt, the PPE substrate t-Boc-L-Ala-NP, and the active site titrant nitrophenyl-p-guanidinobenzoate. The HNE substrate t-Boc-L-Ala-L-Ala-L-Norval-SBz was a gift from Dr. James Powers.

The ala^{359} α-1-PI cDNA was constructed, expressed, and the protein isolated from a yeast system as previously described for two other mutants (4,5). Because it appeared that ala^{359} α-1-PI was composed of a mixture of active and inactive components further separation was accomplished by anhydrochymotrypsin-sepharose chromatography (10), chymostatin being utilized to desorb active inhibitor.

Second order association rate constants for the interaction of ala^{359} α-1-PI with several mammalian proteinases were determined by procedures developed previously in this laboratory (11). Equimolar activities of inhibitor and various proteinases were incubated at room temperature in 0.1M sodium phosphate, pH 7.4, 0.15M NaCl, for appropriate time periods followed by the addition of saturating concentrations of substrate specific for each proteinase. Residual enzyme activity was then determined and compared to a control containing enzyme alone. Final molarities of substrates used were 2.0 mM for HNE and PPE, 1.0 mM for chymotrypsin and cathepsin G, and 0.9 mM for trypsin. All association rates reported are average values with standard deviations of 20% or less.

RESULTS AND DISCUSSION

Characterization of Ala^{359} α-1-PI: The ala^{359} α-1-PI migrated as a single band after $NaDodSO_4$ gel electrophoresis with a Mr = 46,000 (compared to a Mr = 52,000 for the native plasma inhibitor) due to the lack of glycosylation in the yeast system utilized. However,

unlike other α-1-PI mutants previously investigated (met^{358}, val^{358} ala^{358}, and cys^{358} (5,6)) which contained between 65% and 90% functional inhibitor, the ala^{359} mutant had only 15% of the activity expected in the preparations tested. This low activity did not appear to be due to oxidation of met^{358} since dithiothreitol was present at all times in the purification of the ala^{359} inhibitor and dialysis with dithiothreitol containing buffers (4) did not increase inhibitory activity. Furthermore, each of the six ala^{359} α-1-PI preparations had the same low inhibitory activity immediately after purification which remained at that level for more than six months.

Incubation of ala^{359} α-1-PI with HNE caused complex formation to occur as demonstrated by $NaDodSO_4$ gel electrophoresis (Figure 1). Significantly, however, complex degradation was found to occur at less than 20% saturation of inhibitor based on the protein concentrations of each component. This is in agreement with the determination of inhibitory activity using chymotrypsin which indicated that the purified inhibitor preparation was only 15% active, despite the fact that it migrated as a single component during $NaDodSO_4$ gel electrophoresis. In order to examine these results more thoroughly the purified protein was applied to a column of anhydrochymotrypsin-sepharose, equilibrated with 0.05M Tris-HCl, 0.5M NaCl, pH 8.0. Inactive inhibitor, which could be detected immunologically as being α-1-PI, passed directly through the column which the active component bound. The latter was subsequently eluted by addition of buffers containing chymostatin (0.1 mg/ml). When the inactive component was incubated with HNE (not shown) no complex was formed at any molar ratio used and the protein appeared to remain intact, except at very high elastase:protein molar ratios (10:1) where degradation could be detected. In contrast, the active fraction which was

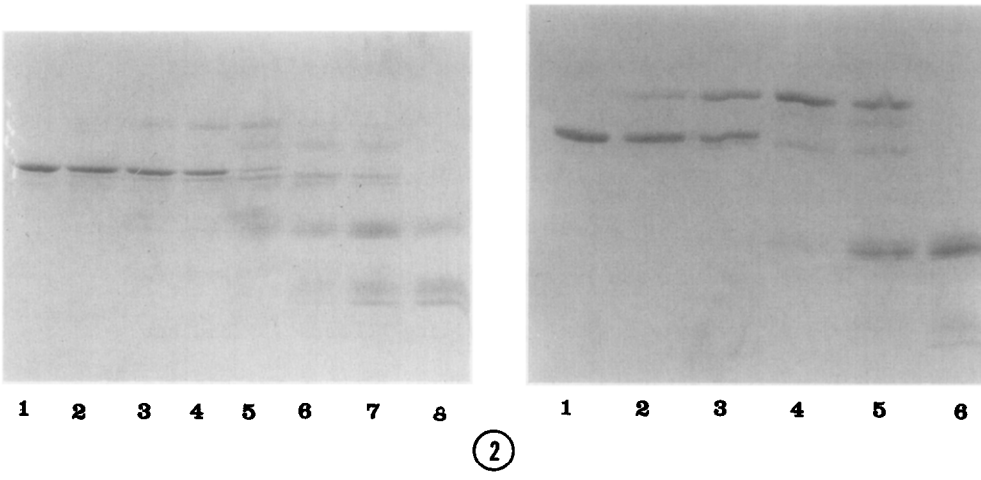

Figure 1. NaDodSO$_4$-PAGE of ala^{359} α-1-PI with human neutrophil elastase (HNE). Samples of inhibitor (12 ug) were allowed to react with various molar ratios of enzyme for one min. at room temperature, boiled in NaDodSO$_4$, and run in a 7-20% gradient gel system. Lanes are as follows: (1), inhibitor alone, (2-7), inhibitor + HNE at molar ratios of 50:1, 20:1, 10:1, 5:1, 2:1, and 1:1, respectively; (8), HNE alone.

Figure 2. NaDodSO$_4$-PAGE of the active fraction of ala^{359} α-1-PI with HNE. Samples of inhibitor (18 ug) were allowed to react with various molar ratios of HNE for one min. at room temperature and treated as described in Figure 1. Lanes are as follows: (1), inhibitor alone; (2-5), inhibitor + HNE at molar ratios of 20:1, 10:1, 5:1, and 2:1, respectively; (6), HNE alone.

eluted with chymostatin readily formed complexes with HNE (Figure 2, lanes 2-5), although some complex degradation did begin to occur before all of the inhibitor had reacted with free enzyme. Of the ala^{359} α-1-PI produced approximately 85% was found to be inactive and 15% functional.

Interaction of Ala^{359} α-1-PI with S. aureus V-8 Proteinase: In order to determine whether the ala^{359} α-1-PI preparation might contain a contaminating protein which had co-eluted with active inhibitor or, alternatively, might be a mixture of two identical proteins with substantially different conformations, inhibitor which had not been subjected to anhydrochymotrypsin-sepharose chromatography was treated with S. aureus V-8 proteinase and subjected to NaDodSO$_4$ gel electrophoresis. If the preparation was contaminated on could have expected

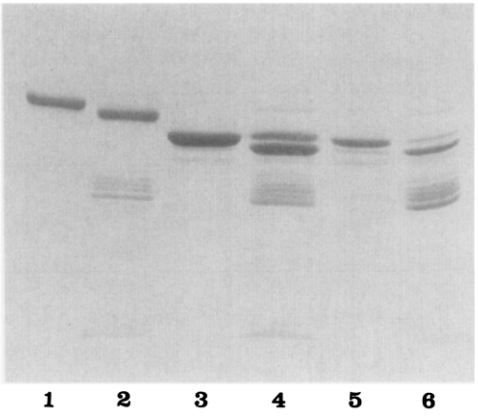

Figure 3. $NaDodSO_4$-PAGE of V-8 proteinase treated α-1-PI variants. Plasma α-1-PI (5 ug), ala^{358} α-1-PI (15 ug), and ala^{359} α-1-PI (17 ug) were incubated with V-8 proteinase (I:E molar ratio of 5:1) for 10 min. at room temperature and treated as described in Figure 1. Lanes are as follows: (1), plasma α-1-PI; (2), plasma α-1-PI + V-8 proteinase; (3), ala^{358} α-1-PI alone; (4), ala^{358} α-1-PI + V-8 proteinase; (5), ala^{359} α-1-PI alone; (6), ala^{359} α-1-PI + V-8 proteinase.

a very different gel pattern in comparison with controls containing either plasma α-1-PI or ala^{358} α-1-PI. Similarly, if significant conformational differences in active vs non-active inhibitor were present one might again have noted differences in peptide fragment patterns since 85% of the purified protein was inactive. However, as shown in Figure 3 there were no significant alterations in the peptide patterns obtained among the three forms of the inhibitor tested. In fact, as previously demonstrated (12), all three inhibitors were converted, primarily, to a slightly lower Mr form presumably by cleavage between the P_4 glu^{354} and P_3 ala^{355}, with minor cleavage at other positions.

Association Rate Measurements for Ala^{359} α-1-PI: The second order association rate constants for the interaction of ala^{359} α-1-PI with various proteinases are shown in Table 1, together with those constants previously determined for native human plasma α-1-PI (11) and the ser^{359} wild type protein (5). These data indicate that the ala^{359} mutant reacts slightly more slowly with most proteinases tested

Table 1: Association Rate Constants for the Interaction of Ala^{359}-1-PI with Serine Proteinases[1]

Enzyme	Inhibitor		
	Plasma	Ser^{359}	Ala^{359}
HNE	6.5×10^7	1.9×10^7	1.0×10^7
PPE	1.0×10^5	7.0×10^4	5.9×10^4
Chymotrypsin	5.9×10^6	-	8.8×10^5
Trypsin	4.2×10^4	-	8.2×10^4
Cathepsin G	4.1×10^5	7.0×10^4	2.7×10^4

[1] $moles^{-1} sec^{-1}$

in comparison with either the plasma form or the analogous recombinant derived protein. Therefore, the conformationally active form of ala^{359} seems to be unaltered by its conversion from ser^{359}. In contrast, however, a marked negative effect is had on the proportion of functional inhibitor obtained.

The results of these experiments suggest that the primary function of the P_1' residue in α-1-PI does not directly involve proteinase inhibition but rather the folding of the inhibitor into a functionally active state. By altering this residue to ala^{359} other inactive conformations are apparently favored, although some inhibitor still appears to fold correctly. It is likely that a similar pattern occurs in AT III variants since substitutions in the P_1' residue yielded, at most, 25% active material and in some cases only inactive protein (4). Clearly, natural or site-directed mutations in the P_1' region of Serpins do not favor the production of active inhibitor.

REFERENCES

1. Carrell, R. and Travis, J. (1985) Trends In Biochem. Sci. 10, 20-24.
2. Travis, J. and Salvesen, G. (1983) Ann. Rev. Biochem. 52, 655-709.
3. Stephens, A.W., Thalley, B.S., and Hirs, C.H.W. (1987) J. Biol. Chem. 262, 1044-1048.
4. Stephens, A.W., Siddiqui, A., and Hirs, C.H.W. (1988) J. Biol. Chem. 263, 15849-15852.
5. Travis, J., Owen, M., George, P., Carrell, R., Rosenberg, S., Hallewell, R.A., and Barr, P.J. (1985) J. Biol. Chem. 260, 4384-4389.
6. Matheson, N., Gibson, H., Hallewell, R.A., Barr, P.J., and Travis, J. (1986) J. Biol. Chem. 261, 10404-10409.
7. Baugh, R. and Travis, J. (1976) Biochemistry 16, 836-848.
8. Travis, J., Bowen, J., and Baugh, R. (1978) Biochemistry 17, 5651-5656.
9. Drapeau, G.R. (1978) J. Microbiol. 136, 607-613.
10. Dreschsel, D., Karic, L., and Glaser, C.B. (1984) Anal. Biochem. 143, 141-145.
11. Beatty, K., Bieth, J., and Travis, J. (1980) J. Biol. Chem. 255, 3931-3934.
12. Potempa, J., Watorek, W., and Travis, J. (1986) J. Biol. Chem. 261, 14330-14334.