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THE CARBOXY TERMINAL SEQUENCE OF HUMAN ALPHA-1-PROTEINASE INHIBITOR

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Summary: The carboxy terminal residue of human α-1-proteinase inhibitor (α-1-PI) was found to be lysine by three independent techniques. These included digestion with carboxypeptidases B and A, hydrazinolysis, and sequence determination of the carboxy terminal peptide obtained from cyanogen bromide fragmentation. This structure was found to be GLY-LYS-VAL-VAL-ASN-PRO-THR-GLN-LYS. Carboxypeptidase C digestion indicated substantial degradation of α-1-PI by endopeptidases in the enzyme preparation. These results do not support the proposal of Cohen et al (Biochemistry (1978) 17 392) that H_2O^{18} incorporation into lysine in dissociating α-1-PI:proteinase complexes is indicative of a critical role of this residue in the reactive site of the inhibitor. We suggest that free trypsin, released from complexes, could readily activate the carboxy terminal lysine of α-1-PI, resulting in oxygen exchange with H_2O^{18} in the medium.

Human α-1-PI exhibits a broad inhibition spectrum towards serine proteinases and for this reason is believed to play an important role in controlling tissue proteolysis (1). A genetic deficiency of this protein in certain individuals has been correlated with the development of pulmonary emphysema (2) and for this reason the mechanism by which the inhibitor functions has been the subject of intense investigation (3-6). Results from this laboratory have suggested the interaction of an X-threonyl (X-seryl) peptide bond in α-1-PI with all serine proteinases inactivated by this protein (3) while catalytic amounts of papain effect peptide bond cleavage at the same position with concomitant inactivation of the inhibitor (4).

It has been known for some time that modification of the lysyl residues of α-1-PI by acylating reagents results in the abolishment of all inhibitory activity towards trypsin, chymotrypsin, and elastase (1) and this activity is rapidly regained if the blocking groups are removed (7). Such data would appear to be compatible with the involvement of a lysyl residue at the reactive center

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of this protein and Cohen and colleagues (5,6) have recently published data using H_2O^{18} incorporation into dissociated α-1-PI:trypsin complexes to confirm the importance of a lysyl residue at the reactive center of α-1-PI. This data, however, would only be consistent if the carboxy terminal of intact α-1-PI were any residue except lysine or arginine since trypsin catalyzed exchange with such a carboxy terminal amino acid would be expected to occur resulting in O^{18} incorporation into this residue. Since our laboratory as well as several others (8-11) have claimed lysine as the carboxy terminal amino acid in α-1-PI, while Cohen et al (6) indicate leucine, we decided to re-investigate the two sets of conflicting data.

Materials and Methods

Carboxypeptidase C was obtained from Boehringer-Mannheim; DFP-treated carboxypeptidase A and DFP-treated carboxypeptidase B were from Worthington Biochemicals; human α-1-PI was prepared as previously described (8).

Digestion of α-1-PI with carboxyeptidase C followed the exact conditions described by Cohen et al (6) using 12 mg of inhibitor and 0.4 mg of enzyme mixed in 0.05M sodium citrate, pH 5.3, at 37°. Aliquots of the incubation mixture were removed at specific time intervals and the reaction stopped by lowering the pH to 2.0 and freezing the samples prior to analysis. The samples were split with 10% being utilized for sodium dodecyl sulfate-gel electrophoresis experiments and the remainder subjected to amino acid analysis after precipitation of protein with trichloroacetic acid.

Digestion of α-1-PI with DFP-treated carboxypeptidase B and/or DFP-treated carboxypeptidase A was performed as previously described (8).

Cyanogen bromide fragmentation of α-1-PI (500 mg) was obtained by addition of a 25-fold molar excess of reagent to the protein dissolved in 70% formic acid. After 24 hrs at room temperature the mixture was lyophilized and the fragments subsequently separated by chromatography on Sephadex G-75. One low molecular weight peptide, devoid of homoserine by amino acid analysis, was obtained and this was further purified by chromatography on Dowex 50-X-2 using

TABLE I: CARBOXY TERMINAL AMINO ACIDS RELEASED FROM ALPHA-1-PROTEINASE INHIBITOR

	MOLES/MOLE PROTEIN											
	CPC							CPB		CPB + CPA		HYDRAZINOLYSIS
	TIME (MINUTES)											
AMINO ACID	30	60	120	240	360	480	1440	15	240	120	480	1440
LYS	0.1	0.2	0.4	1.3	1.9	2.8	3.8	0.5	0.6	0.6	0.6	0.4
LEU	0.1	0.3	0.6	1.4	2.1	3.2	4.7	-	-	0.1	0.3	-
PHE	0.1	0.3	0.5	1.2	1.6	2.2	3.0	-	-	-	-	-
"SER"	0.1	0.2	0.5	1.3	1.8	2.7	3.7	0.3	0.3	0.7	0.8	-
THR	-	0.1	0.1	0.4	0.6	0.9	1.6	-	-	0.3	0.4	-

a pyridine-acetate buffer system (12). Sequence analysis of this fragment was performed with a Beckman 890 sequencer using a dimethylallylamine program. Polybrene (13) was added to retain the peptide in the reaction cup. Analysis of fractions from the sequencer was performed by either back hydrolysis and amino acid analysis (14) or by high pressure liquid chromatography (15).

Results

The digestion of α-1-PI with carboxypeptidase C gave results similar to but not identical with that of Cohen et al (6) (Table 1) with leucine being released very rapidly. However, the recoveries of amino acids were far higher than one could have expected from the sequential removal of amino acids from the carboxy terminal of a protein as large as α-1-PI (52,000 daltons). In fact, as shown in Figure 1, what has certainly occurred is proteolysis of the inhibitor by contaminants in carboxypeptidase C as evidenced by the increasing quantities of low molecular weight fragments in the sodium dodecyl sulfate-polyacrylamide gel during the incubation. These endopeptidases are known to exist in commercial preparations of the enzyme (16) and have both chymotryptic and tryptic specificity. Since α-1-PI has approximately 20% of its composition due to phenylalanyl and leucyl residues (8) it is highly probable that a chymotrypsin-like contaminant

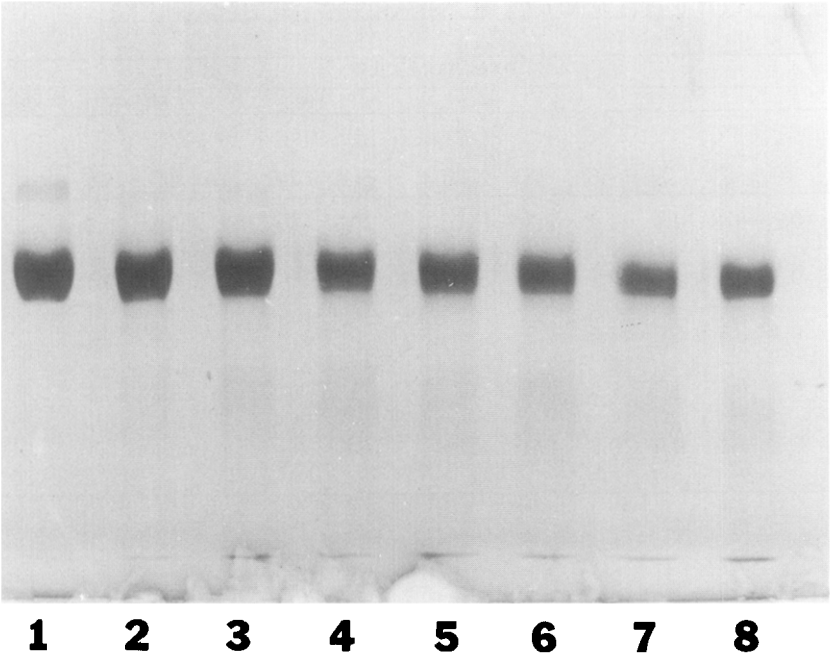

Figure 1. Carboxypeptidase C digestion of human α-1-proteinase inhibitor. α-1-PI (12 mg) and carboxypeptidase C (0.4 mg) were incubated at pH 5.3 and 40°. After various time intervals, aliquots were removed (20 µg), boiled in 1% sodium dodecyl sulfate for two minutes, and subjected to acrylamide gel electrophoresis in a 9% system. 1, zero time incubation; 2, 30 min.; 3, 60 min.; 4, 120 min.; 5, 240 min.; 6, 360 min.; 7, 480 min.; 8, 1440 min.

in carboxypeptidase C has cleaved peptide bonds exposing these amino acids as carboxy terminal residues which were subsequently released by the true exopeptidase.

When DFP-treated carboxypeptidase B alone, or together with DFP-treated carboxypeptidase A, was utilized only lysine and a residue eluting under serine on the amino acid analyzer were released. Hydrazinolysis for 24 hrs at 80° followed by amino acid analysis also yielded lysine as the carboxy terminal residue of α-1-PI.

The sequence of the single cyanogen bromide fragment obtained from α-1-PI which was devoid of homoserine was found to be as follows: GLY-LYS-VAL-VAL-ASN-PRO-THR-GLN-LYS. This result confirms that sequence also obtained for the carboxy terminal of α-1-PI by S.K. Chan (personal communication) and unequivocally provides evidence that lysine is the carboxy terminal of α-1-PI.

Discussion

It was originally believed by many groups, including ourselves, that a lysyl residue must be at the reactive site of α-1-PI because of the results of acylation experiments with this inhibitor (1,3,5,6). However, this indication lost considerable support when it was realized that the inhibitor could inactivate trypsin, chymotrypsin, and elastase at a common site (4) and, therefore, a lysyl residue need not be the key part of the reactive site. Furthermore, the results of Busby et al (17) which showed that methylation of lysyl residues in α-1-PI occurred without loss of inhibitor activity are certainly against the function of this residue in the active site. Finally, the principal physiological role of α-1-PI appears to be for the inactivation of elastolytic activity since a) there are several inhibitors of trypsin in serum but the only other elastase inhibitor is alpha-2-macroglobulin and b) except for extreme pathological states, it is doubtful that the inhibitor ever contacts trypsin. Therefore, a non-polar residue would be favored on a teleological basis over a polar residue.

In the past Cohen and colleagues have published results indicating lysine (5,6), arginine (18), and proline (19) at the reactive center of α-1-PI. Subsequently, the majority of this information was found to be incorrect and only recently has this group (6) confirmed our original observation that a threonyl (seryl) residue forms the P_1' binding site of this inhibitor (4). Indeed, the results presented here would tend to be dissuasive to their H_2O^{18} studies since a carboxy terminal lysine would interfere with a proper interpretation of the incorporation of H_2O^{18} into dissociated complexes of α-1-PI and trypsin. In fact, it seems almost certain that trypsin, released from α-1-PI:trypsin complexes would activate the carboxyl group of carboxy terminal lysine resulting in exchange of the oxygen atoms with the H_2O^{18} medium. Such a result has been well documented in the case of chymotrypsin (20). Although phenylmethanesulfonyl fluoride was used by Cohen et al (6) to inhibit free trypsin released, the pH (9.5) and temperature (40°) of the reaction buffer was far too high to stop instantaneous hydrolysis of the reagent as has been recently shown to occur (21).

Recently (D. Johnson and J. Travis, submitted for publication), we isolated a peptide fragment from papain digests of denatured α-1-PI. The structure of this peptide was found to overlap with that of the amino terminal sequence of modified inhibitor obtained from either proteinase:α-1-PI complexes (3) or from papain digests of native inhibitor (4). The sequence contained a methionyl-$\begin{matrix}\text{threonyl}\\\text{seryl}\end{matrix}$ linkage at what we believe to be the P_1-P_1' reactive center of α-1-PI. Since methionine has been implicated at the inhibitory site of other proteinase inhibitors (22) its presence here should not be deemed as unusual. What may prove important, however, is the fact that α-1-PI is readily inactivated by oxidizing agents which could be a result of specific oxidation of this critical methionyl residue. Investigations are continuing in an attempt to confirm this theory.

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