Human α-1-Antichymotrypsin: Purification and Properties†

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ABSTRACT: Human α-1-antichymotrypsin has been purified to homogeneity by the following sequential steps—(a) ammonium sulfate fractionation; (b) chromatography on Cibacron Blue Sepharose at pH 7.0; and (c) chromatography on SP-Sephadex C-50 at pH 5.5. The inhibitor has a molecular weight near 68 000 and contains approximately 26% carbohydrate. α-1-Antichymotrypsin has an amino-terminal arginine and a carboxy-terminal glycine. It also has some homology with α-1-PI based on amino-terminal sequence analysis of both proteins. Complexes of α-1-antichymotrypsin with human chymotrypsin and human leukocyte cathepsin G are stable in sodium dodecyl sulfate and have molecular weights near 90 000 suggesting 1:1 complex formation on a molar basis between inhibitor and enzyme.

Because of the original observations which showed a strong correlation between α-1-proteinase inhibitor (α-1-PI)[1] deficiency and the development of emphysema (Laurell & Eriksson, 1963), significant interest has been shown in determining the role of plasma proteinase inhibitors in controlling tissue proteolysis. To date, seven major inhibitors have been described in serum and plasma (Heimberger et al., 1971; Moroi & Aoki, 1976) many of which are believed to have specific functions such as in the coagulation system (antithrombin III), fibrinolytic system (α-2-plasmin inhibitor), and the complement pathway (c-1-inactivator). The high molar concentration of α-1-PI in plasma as well as its ability to inactivate serine proteinases with broad specificity would indicate that it is a general scavenger for tissue proteinases of this class. α-2-Macroglobulin, an inhibitor of very high molecular weight and apparent general specificity toward all classes of endopeptidases, clears all proteinases released either extra- or intravascularly through rapid elimination of α-2-macroglobulin–proteinase complexes. The inter-α-trypsin inhibitor has, in the past, been suggested as a precursor to the low molecular weight inhibitors found in seminal plasma (Hochstrasser et al., 1973) because of partial immunological identity. However, this has not been clearly demonstrated and the function of this inhibitor remains to be elucidated.

Human α-1-antichymotrypsin (α-1-Achy) is a glycoprotein first described by Heimburger & Haupt (1965). This protein has two unusual properties in that (a) it appears to be specific for chymotrypsin-like enzymes and (b) it is an acute phase protein whose plasma concentration may double within 8 h after tissue damage. These two facts would suggest a primary role for the inhibitor in the regulation of specific chymotrypsin-like enzymes, particularly those released during an inflammatory episode. This report describes a rapid procedure for the isolation of the inhibitor from whole plasma as well as some of the properties of the purified protein. A subsequent paper in this series will consider the interaction of α-1-Achy with chymotrypsin-like enzymes.

Experimental Section

Materials

Cibacron Blue Sepharose was prepared as described earlier (Travis et al., 1976) except that coupling of the dye took place at 90 °C using cross-linked Sepharose 6B. SP-Sephadex C-50 was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Antisera to specific plasma proteins were from Behring Diagnostics, Somerville, N.J. Human plasma was a gift of the American National Red Cross, Atlanta, Ga. *N*-Benzoyl-L-tyrosine ethyl ester (Bz-L-Tyr-OEt) was purchased from Sigma Chemical Co., St. Louis, Mo.

Human pancreatic trypsin and chymotrypsin and human α-1-PI were prepared by previously described procedures (Travis & Roberts, 1969; Coan et al., 1971; Pannell et al., 1974). Human leukocyte cathepsin G was isolated from neutrophil granules by the procedure of Baugh & Travis (1976) as modified in the following paper (Travis et al., 1978).

Methods

Enzyme and Inhibitor Assays. Chymotrypsin esterolytic activity was measured using Bz-L-Tyr-OEt (Travis & Roberts, 1969). Inhibitory activity was measured by mixing a fixed quantity of human enzyme with varying amounts of inhibitor in 0.05 M Tris-HCl, 0.05 M NaCl, pH 8.0. After incubation for 5 min at 25 °C the mixtures were assayed for esterolytic activity. Inhibitor units and specific inhibitory activity of α-1-Achy were determined using the procedure outlined during the purification of human α-1-PI (Pannell et al., 1974). An extinction coefficient of 6.2 (1% solution, 280 nm) determined experimentally with purified inhibitor (Babul & Stellwagen, 1969) was used for the determination of protein concentration of α-1-Achy.

Other Methods

Amino acid analysis, carbohydrate analysis, ultracentrifuge studies, immunoelectrophoresis, and amino terminal sequence analysis were performed as previously described (Pannell et al., 1974; Baugh & Travis, 1976). Gel electrophoresis was run in an Ortec apparatus (Ortec Inc., Oak Ridge, Tenn.) using the standard sulfate–borate alkaline procedure and the Na-DodSO$_4$ procedure outlined in Ortec Life Science Note 13.

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[1] Abbreviations used: α-1-Achy, α-1-antichymotrypsin; α-1-PI, α-1-proteinase inhibitor; NaDodSO$_4$, sodium dodecyl sulfate; Bz-L-Tyr-OEt, *N*-benzoyl-L-tyrosine ethyl ester.

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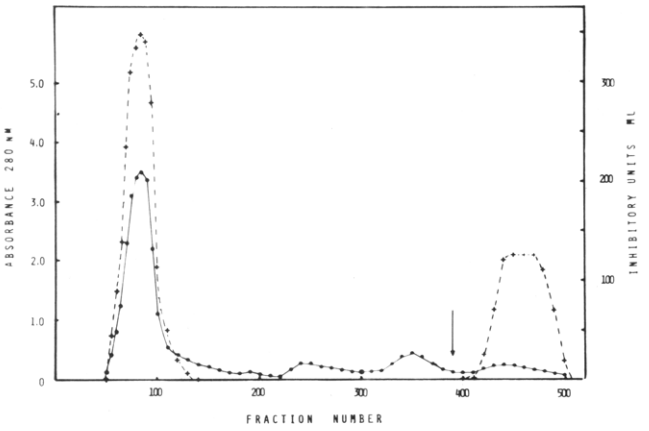

FIGURE 1: Chromatography of 0.5–0.8 M ammonium sulfate fraction of human plasma on Cibacron Blue Sepharose at pH 7.0. The column was equilibrated with 0.03 M sodium phosphate, pH 7.0. The arrow designates a change in buffer to 0.03 M sodium phosphate, 0.1 M NaCl, pH 7.0. Column dimensions: 5.0 × 50 cm; flow rate, 60 mL/h; fraction size, 10 mL. Curves are designated as follows: absorbance at 280 nm (●—●), left ordinate; inhibitory activity toward human chymotrypsin (+- - -+), right ordinate.

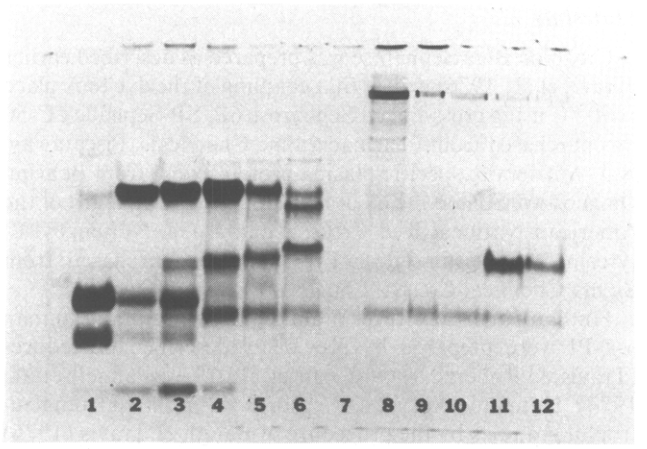

FIGURE 2: Gel slab electrophoresis of plasma fractions from cibacron blue Sepharose chromatography. The polyacrylamide gel was made in a discontinuous system from 4% to 8%; the running pH was 8.8. Staining was performed with Coomassie Brilliant Blue G. Direction of migration is from cathode (top) to anode (bottom). The fractions utilized were taken from the profiles shown in Figure 1. Slots 1–12 represent fractions 51, 60, 70, 100, 110, 150, 310, 350, 400, 425, 460, and 480, respectively.

Results

Purification of Human α-1-Achy

1. Ammonium Sulfate Fractionation. Pooled plasma (500 mL) was diluted 1:1 with 0.03 M sodium phosphate, pH 7.0, and brought to 0.5 saturation by the addition of 313 g of solid ammonium sulfate with constant stirring. The resultant precipitate was removed by centrifugation and the supernatant treated with 210 g of ammonium sulfate to make the final concentration 0.8 saturation. The precipitate was recovered by centrifugation, dissolved in 400 mL of 0.03 M sodium phosphate buffer, pH 7.0, and dialyzed against 4 L of the same buffer for 24 h at 4 °C with three changes.

2. Chromatography on Cibacron Blue Sepharose. The yellow-colored solution obtained by salt fractionation (500 mL) was applied at room temperature to a column of Cibacron Blue Sepharose (5.0 × 50 cm) equilibrated in 0.03 M sodium phosphate, pH 7.0. Unbound protein was removed by washing with buffer until the A_{280nm} of the eluate was less than 0.050.

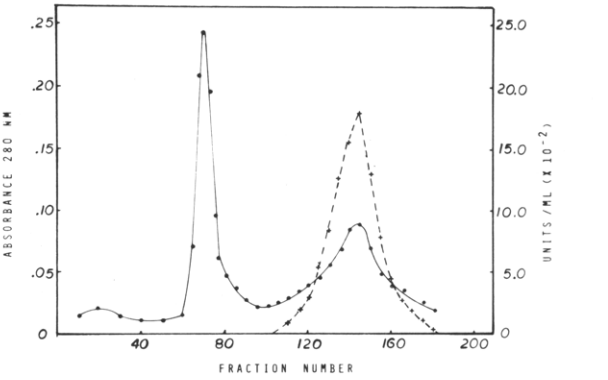

FIGURE 3: SP-Sephadex C-50 chromatography of α-1-antichymotrypsin fractions obtained from Cibacron Blue Sepharose chromatography. The column was equilibrated with 0.025 M sodium citrate, pH 5.5. The sample was applied and eluted with the same buffer. Column dimensions (5.0 × 90 cm); flow rate, 20 mL/h; fraction size, 5 mL. Curves are as designated in Figure 1.

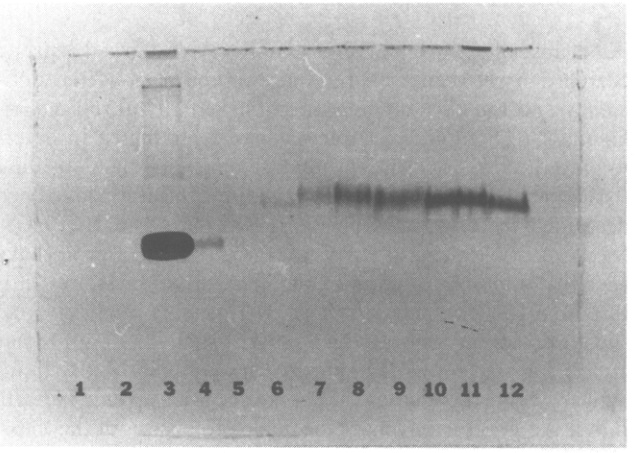

FIGURE 4: Gel slab electrophoresis of plasma fractions from SP-Sephadex C-50 column chromatography. Conditions are identical with those given for Figure 2. The fractions utilized were taken from the profile shown in Figure 3. Slots 1–12 represent fractions 40, 50, 60, 70, 80 90, 100, 120, 130, 140, 150, and 160, respectively.

Some inhibitory activity toward chymotrypsin was lost at this step due to the elution of α-1-PI. However, all α-1-Achy remained bound since the nonadsorbed proteins did not cross-react with anti-sera directed against this protein. When the column was subsequently washed with 0.03 M sodium phosphate, 0.1 M NaCl, pH 7.0, α-1-Achy, some albumin, and other plasma proteins were eluted. The elution profile is shown in Figure 1, while representative samples after acrylamide gel electrophoresis are presented in Figure 2. α-1-Achy can be easily seen in the last two samples in the latter electropherogram.

3. Chromatography on SP-Sephadex C-50. All α-1-Achy containing fractions from Cibacron Blue Sepharose chromatography were pooled and dialyzed against 0.025 M sodium citrate, pH 5.5, with several changes. After concentration by ultrafiltration to a final volume of 10 mL, the solution was applied at room temperature to a column of SP-Sephadex C-50 (5.0 × 90 cm) equilibrated with the citrate buffer. The column was then washed with the equilibrium buffer and fractions collected immediately. The elution profile is shown in Figure 3 and electrophoresis of representative fractions given in Figure 4.

Two protein peaks were eluted from the SP-Sephadex C-50 column in this step. The first peak contained mainly albumin

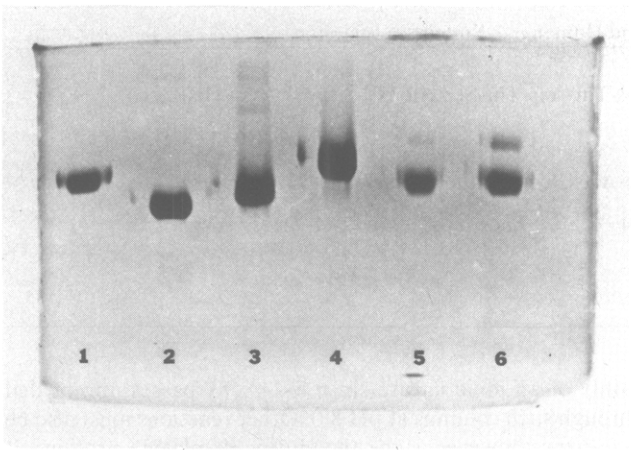

FIGURE 5: Gel slab electrophoresis of α-1-Achy, α-1-Achy-proteinase complexes, and protein standards. Samples of inhibitor and enzyme were incubated together for 5 min at pH 8.0. All samples were then boiled in 0.1% $NaDodSO_4$ and subjected to vertical slab gel electrophoresis. Slot 1, α-1-Achy (20 μg); slot 2, α-1-PI (20 μg); slot 3, human albumin (20 μg); slot 4, human transferrin (20 μg); slot 5, α-1-Achy (20 μg) + human chymotrypsin (5 μg); slot 6, α-1-Achy (20 μg) + human leukocyte cathepsin G (5 μg).

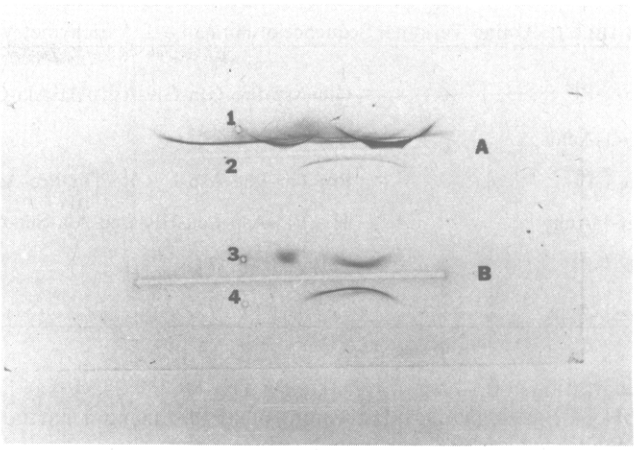

FIGURE 6: Immunoelectrophoresis of human α-1-Achy and normal human plasma. Electrophoresis was performed at 8 mA in 0.05 M sodium veronal, pH 8.6 for 2 h followed by immunodiffusion for 24 h at 4 °C. Samples were soaked in 0.9% saline and then stained with Coomassie Brilliant Blue G. Wells 1 and 3, normal human plasma (2 μL); wells 2 and 4, human α-1-Achy (1 μg); slot A, rabbit anti-whole human plasma; slot B, rabbit anti-human α-1-Achy.

but also traces of other proteins. The second peak was composed only of α-1-Achy and fractions containing this protein were immediately adjusted to pH 8.0 by dialysis against 0.05 M Tris, 0.05 M NaCl, pH 8.0. After multiple changes of dialysis buffer, preparations kept at 4 °C at this pH were found to remain stable for at least 1 month. Frozen preparations were found to be stable indefinitely. It should be noted that when the protein was concentrated by ultrafiltration, precipitation and, presumably, polymerization of the inhibitor occurred above 3 mg/mL.

From 500 mL of plasma approximately 45 mg of purified inhibitor could be easily obtained in less than 1 week.

Criteria for Purity of α-1-Achy

In order to obtain evidence for the purity of α-1-Achy, several experiments were carried out.

Polyacrylamide Gel Electrophoresis. As shown in Figure 4, alkaline gel electrophoresis of the purified inhibitor revealed a single band after staining. This protein had a mobility slightly slower than that of α-1-PI and became even slower in preparations aged at 4 °C. It should be noted that there was a slight decrease in electrophoretic mobility of the eluted fractions from the SP-Sephadex chromatography which may be related to the high sialic acid content of this protein causing the formation of isoinhibitors as occurs with α-1-PI. Frozen preparations of α-1-Achy were found to migrate mainly in the position of the freshly isolated protein after thawing but polymers of higher molecular weight were also detectable.

When α-1-Achy was subjected to electrophoresis after boiling in 0.1% $NaDodSO_4$, a single band was detected which migrated slightly slower than human albumin (Figure 5, slot 1). The apparent molecular weight of this protein, using albumin, transferrin, and α-1-PI as standards, was calculated to be 68 000. Similar results were found with α-1-Achy after prior reduction with 0.1 M mercaptoethanol and $NaDodSO_4$ treatment indicating this protein to be a single chain. Complexes of inhibitor with human chymotrypsin and leukocyte cathepsin G were found to be stable after $NaDodSO_4$ treatment and their molecular weights were both approximately 88 000 by this procedure (Figure 5, slots 5 and 6).

Immunoelectrophoresis and Immunodiffusion. Human α-1-Achy gave a single arc after immunoelectrophoresis using anti-whole human serum (Figure 6). Curiously, with commercial anti-sera to α-1-Achy (Behring Diagnostics), a major arc with a spur could be detected, while human plasma gave two well-separated lines. The nature of this result has not yet been established but could represent either an α-1-Achy complex or a form of desialylated inhibitor present in whole plasma.

Radial immunodiffusions of α-1-Achy against anti-sera directed toward α-1-PI, albumin, orosomucoid, GC-components, and transferrin were all negative indicating the absence of these proteins as contaminants.

TABLE I: Amino Acid and Carbohydrate Composition of α-1-Antichymotrypsin.

amino acid	residues/mole A[a]	B[b]
Lys	27	28
His	9	9
Arg	15	15
Asp	49	47
Thr	31	32
Ser	32	31
Glu	50	54
Pro	15	15
Gly	18	16
Ala	34	31
½-cystine[c]	2	0
Val	27	25
Met	11	12
Ile	23	20
Leu	56	54
Tyr	9	10
Phe	26	26
Trp[d]	4	3
neutral sugars	38–40	41
acetylhexosamine	30–35	25
sialic acid	11–14	14

[a] This paper. [b] Heimburger et al. (1971). [c] Average of 22-h hydrolysate of oxidized sample. [d] Determined from 22-h hydrolysate in presence of 4% thioglycollate.

Physical and Chemical Properties of α-1-Achy

Molecular Weight. Sedimentation equilibrium experiments (Yphantis, 1964) on α-1-Achy using an initial protein con-

TABLE II: Amino-Terminal Sequence of Human α-1-Antichymotrypsin and Human α-1-Proteinase Inhibitor.

	1	10	20
α-1-PI	Glu-Asp-Pro-Gln-Gly-Asn-Ala-Ala-Gln-Lys-Thr-Asp-Thr-Ser-His-His-Asp-Gln-Asp-His-		
			1
α-1-Achy			Arg-Gly-Thr-
		30	40
α-1-PI	Pro-Thr-Phe-Asn-Lys-Ile-Thr-Pro-Asn-Leu-Ala-Glu-*Phe-Ala-Phe-Ser-Leu-Tyr*-Arg-Gln-		
		10	20
α-1-Achy	His-Val-Asp-Leu-Gly-Leu-Ala-Ser-Ala-Asp-Val-Ser-*Phe-Ala-Phe-Ser-Leu-Tyr*-Lys-Tyr-		
	45		
α-1-PI	*Leu*-Ala-*Val-Thr-Gly*		
	28		
α-1-Achy	*Leu*-Val-*Val-Thr-Gly*		

centration of 0.14 mg/mL in 0.05 M Tris-HCl, 0.05 M NaCl, pH 8.0, gave a molecular weight of 65 000 using a partial specific volume of 0.715 calculated from the amino acid and carbohydrate composition. There was no evidence for aggregation into polymers. The molecular weight obtained is in close agreement with that determined after $NaDodSO_4$ gel electrophoresis.

Amino Acid and Carbohydrate Composition. The amino acid and carbohydrate composition of α-1-Achy is given in Table I. With the exception that two residues of cysteine were present in our preparation, the general composition is in agreement with previously published results (Heimburger & Haupt, 1965).

Amino-Terminal Sequence Analysis. In order to determine whether any sequence homology between α-1-Achy and α-1-PI exists at the amino-terminal region, both proteins were subjected to sequence analysis in a Beckman Model 890C sequencer using a 0.1 M Quadrol program. The Pth-amino acids were identified by both HPLC (Zimmerman et al., 1977) and by back hydrolysis followed by amino acid analysis (Mendez & Lai, 1975). In each case sequence analysis was performed on at least three different preparations of inhibitor, using approximately 200 nmol. Initial yields were 54% for α-1-PI and 48% for α-1-Achy. The repetitive yields were approximately 94% for both proteins.

The results obtained are shown in Table II. Although there was no sequence homology in the first several amino acids in each protein, a strong correlation in the structure of the two proteins became evident between residues 16–27 of α-1-Achy and residues 33–45 of α-1-PI. No extensive homology of this type was found with the amino-terminal sequence of antithrombin III (Kurachi et al., 1976). However, until more sequence data are available, the possible relationship in structure of all the plasma proteinase inhibitors cannot be considered. One curious note should be mentioned in that the unusual amino-terminal arginine in α-1-Achy is also shared by the inter-α-trypsin inhibitor (Steinbuch, 1976).

Carboxy-Terminal Sequence Analysis. Digestion of α-1-Achy (100 nmol) with 20 μg of carboxypeptidase Y at pH 7.2 for 10, 20, 30, and 60 min (Hayashi, 1976) consistently yielded equal quantities (40 nmol after 60 min) of both serine and glycine after brief acid hydrolysis (2 N HCl, 4 h, 105 °C) of the trichloroacetic acid soluble products released. However, hydrazinolysis (100 nmol) for 22 h at 80 °C followed by amino acid analysis yielded only glycine (30 nmol). These results indicate the carboxy-terminal sequence of human α-1-Achy is Ser-Gly.

Discussion

The purification procedure described here for the isolation of α-1-Achy is primarily dependent on a weak interaction between the inhibitor molecule and Cibacron Blue Sepharose at pH 7.0 at room temperature. This interaction is almost certainly of an ionic nature since α-1-Achy passes unretarded through such columns at pH 8.0. Other reactions must also be occurring, however, since the elution of plasma proteins of similar charge as α-1-Achy (e.g., α-1-PI) is not affected by the pH shift from 8.0 to 7.0. Presumably, ionic and hydrodynamic interactions are also occurring during the last purification step at pH 5.5 on SP-Sephadex C-50. It is unlikely, however, that any of these interactions are due to the large carbohydrate moiety present in α-1-Achy since proteins such as α-1-acid glycoprotein (orosomucoid) which contains about 40% carbohydrate, are unretarded on either Cibacron Blue Sepharose at pH 7.0 or SP-Sephadex C-50 at pH 5.5. Both of the chromatographic steps are extremely temperature dependent and at 4 °C it is necessary to use a buffer containing 0.2 M NaCl for the elution of the inhibitor in each procedure.

The isolated protein has essentially all of the characteristics of the inhibitor originally isolated by Heimburger & Haupt (1965). However, the purification procedure described here is much less complicated and 50 mg of the purified inhibitor may be readily isolated by three rapid steps from 500 mL of normal plasma. These yields may be highly variable since α-1-Achy is an acute phase protein whose concentration may differ significantly depending on the donor of the plasma utilized for its isolation.

The sequence given for α-1-PI is nearly identical with that reported by Morii et al. (1978) and, in fact, extends it by seven residues. A comparison with the sequence of α-1-Achy suggests that at least some degree of homology is shared by the two proteins. Unfortunately, little is known with regard to the reactive site of either inhibitor (Johnson & Travis, 1976) and it will be much more interesting to examine sequence homology when these sites are better delineated. Recent structural studies (D. Johnson & J. Travis, unpublished results) indicate that a methionyl residue forms the P_1 reactive site on α-1-PI for trypsin, chymotrypsin, and elastase. Furthermore, the molecule is rapidly inactivated by both *N*-bromosuccinimide and *N*-chlorosuccinimide (Shecter et al., 1975), both of which are specific oxidants of methionyl residues. However, it is doubtful that a methionyl residue is directly involved in the activity of α-1-Achy since neither oxidizing agent has any apparent inactivating effect.

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Human α-1-Antichymotrypsin: Interaction with Chymotrypsin-Like Proteinases†

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ABSTRACT: The interaction of human plasma α-1-antichymotrypsin with serine proteinases from different tissues has been investigated. The protein was found to form stable complexes with pancreatic chymotrypsin, leukocyte cathepsin G, and mast cell chymotrypsin. No inhibition of pancreatic trypsin or leukocyte elastase could be demonstrated. With mixtures containing both α-1-antichymotrypsin and α-1-proteinase inhibitor, it was found that the former preferentially inactivated leukocyte cathepsin G, while the latter showed a strong preference for pancreatic chymotrypsin. However, leukocyte elastase was specifically inactivated by α-1-proteinase inhibitor even in 1:1 mixtures with chymotrypsin. All of these results taken together suggest that one of the primary functions of α-1-antichymotrypsin is to inactivate leukocyte cathepsin G, while α-1-proteinase inhibitor controls the activity of other serine proteinases, particularly leukocyte elastase.

The mechanism by which many of the plasma proteinase inhibitors function has received a great deal of attention in recent years due to their possible role or relationship with the disease states. In particular, intense studies on α-2-macroglobulin (α_2M),[1] α-1-proteinase inhibitor (α-1-PI), and antithrombin III (AT III) have been initiated due to their respective links with cystic fibrosis (Shapira et al., 1977), pulmonary emphysema (Laurell & Eriksson, 1963), and thrombotic episodes (Harpel & Rosenberg, 1976). However, very little, if any, attention has been given to the function of α-1-antichymotrypsin (α-1-Achy), a major early stage acute phase plasma protein (Aronsen et al., 1972) which tends to be localized in high concentration in bronchial fluid (Ryley & Brogan, 1972). When one considers that the granule fractions of human neutrophils and basophils contain chymotrypsin-like enzymes (Mounter & Atiyeh, 1960) which may be utilized during phagocytosis, a potential role of α-1-Achy in controlling the degradative function of these proteinases must also be envisioned. In addition, other types of cells, such as mast cells, also contain a high concentration of chymotrypsin-like enzymes (Yurt & Austen, 1978), the function of which, although unknown, must also be regulated.

In the previous paper in this series (Travis et al., 1978), we described the properties of α-1-Achy isolated from human plasma in three rapid, uncomplicated steps. We now present data which indicate that α-1-Achy is a controlling protein for the chymotrypsin-like activity present in phagocytic cells. These results conclusively support both our preliminary report (Baugh et al., 1976) as well as that of Ohlsson & Akesson (1976) in which it was suggested that α-1-Achy was a more effective inhibitor of leukocyte cathepsin G than α-1-PI.

Materials and Methods

Human leukocytes were obtained from healthy donors as well as from patients with chronic granulocytic leukemia. The isolation of both cells and granules has been previously described (Baugh & Travis, 1976).

Dog mastocytoma tissue was generously provided by Dr. C. Rawlings, College of Veterinary Medicine, University of Georgia. Partial purification of the chymotrypsin-like enzyme present in this tissue was obtained by extraction with 0.05 M Tris-HCl, 1.9 M NaCl, pH 8.0, followed by chromatography on DEAE-cellulose at pH 8.0 in 0.05 M Tris-HCl. The bound enzyme was subsequently eluted with 0.5 M NaCl by stepwise elution and found to be approximately 85% pure as judged by $NaDodSO_4$ gel electrophoresis. The properties of this enzyme will be described separately (P. Robertie & J. Travis, manuscript in preparation).

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[1] Abbreviations used: α_2M, α-2-macroglobulin; α-1-PI, α-1-proteinase inhibitor; AT III, antithrombin III; α-1-Achy, α-1-antichymotrypsin; $NaDodSO_4$, sodium dodecyl sulfate; Bz-L-Tyr-OEt, benzoyl-L-tyrosyl ethyl ester; Tos-PheCH_2Cl, tosyl-L-phenylalanyl chloromethyl ketone.

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