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Isolation and Properties of Human Plasma α-1-Proteinase Inhibitor†

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ABSTRACT: Human α-1-proteinase inhibitor has been purified to homogeneity in excellent yield (60%) by a simple four-step procedure. The protein has a molecular weight of 53,000 by sedimentation equilibrium centrifugation and contains four half-cystine residues. The N-terminal has been identified as either glutamic acid or glutamine. In contrast to the results of others, the molecule has only two sialic acid residues, indicating that differences in charge between various phenotypes of α-1-proteinase inhibitor cannot be due solely to differences in sialic acid content. Isoelectric focussing indicates that the protein is elicitied mainly as two major isoinhibitors. The protein inhibits 2 mol of trypsin or 2 mol of chymotrypsin, suggesting that it acts as a "multiheaded inhibitor" with overlapping inhibitory sites.

Human α-1-proteinase inhibitor (α-1-PI),[1] more commonly called α-1-antitrypsin, is a glycoprotein which has been shown to be responsible for approximately 90% of the trypsin inhibitory capacity of plasma (Heimburger *et al.,* 1971). The broad specificity and high concentration of this protein in plasma and tissue fluids reflect its important role in preventing tissue proteolysis. The high incidence of phagocyte mediated proteolysis of lung tissue observed in some α-1-PI deficient individuals correlates with this function (Laurell and Eriksson, 1963; Fagerhol, 1972). This has resulted in an increased interest in both the properties and function of this protein.

During the past several years many laboratories have published methods for the isolation of human α-1-PI in various states of purification (Moll *et al.,* 1958; Bundy and Mehl, 1959; Schultze *et al.,* 1962; Shamash and Rimon, 1966; Liener *et al.,* 1973; Murthy and Hercz, 1973; Crawford, 1973). Unfortunately, none of the procedures used are desirable because the isolated inhibitor is obtained either in low yield or in a partially inactive form.

The isolation of α-1-PI is complicated primarily by two other plasma proteins, albumin and orosomucoid (α-1-acid glycoprotein). Albumin has the same molecular weight behavior as α-1-PI in gel exclusion chromatography; its ionic properties, too, mimic those of α-1-PI so closely as to render them unresolvable by ion exchange chromatography, particularly with the high proportion of albumin present in plasma. Orosomucoid is poorly resolved from α-1-PI by disc electrophoresis (Ornstein, 1964); detection of this impurity is further obscured since it stains very weakly with conventional protein stains. Contamination with orosomucoid is also particularly inconvenient for sugar analysis since it contains some 40% polysaccharide.

Recently, we described a technique for the preparation of albumin-depleted plasma (Travis and Pannell, 1973). By using this procedure, together with other conventional steps, we have succeeded in purifying α-1-PI to homogeneity with good recovery of inhibitory activity. This report describes the techniques used for the isolation of the inhibitor as well as some of the properties of the purified protein.

Experimental Section

Materials

Human plasma was obtained from St. Mary's Hospital, Athens, Ga. Sepharose-Blue Dextran was synthesized by the method of Travis and Pannell (1973). The final product had a capacity to bind 8 mg of albumin/ml of packed derivative. Porcine and human trypsins were prepared as previously described (Travis and Liener, 1965; Travis and Roberts, 1969). Both trypsin preparations were titrated with *p*-nitrophenyl guanidinobenzoate (Chase and Shaw, 1967) to determine the number of active sites. The porcine and human enzymes were found to have 82.5 and 86.5% active sites, respectively. Bovine trypsin, bovine α-chymotryp-

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[1] Abbreviations used are: α-1-PI, α-1-proteinase inhibitor; NP, normal plasma; SDS, sodium dodecyl sulfate.

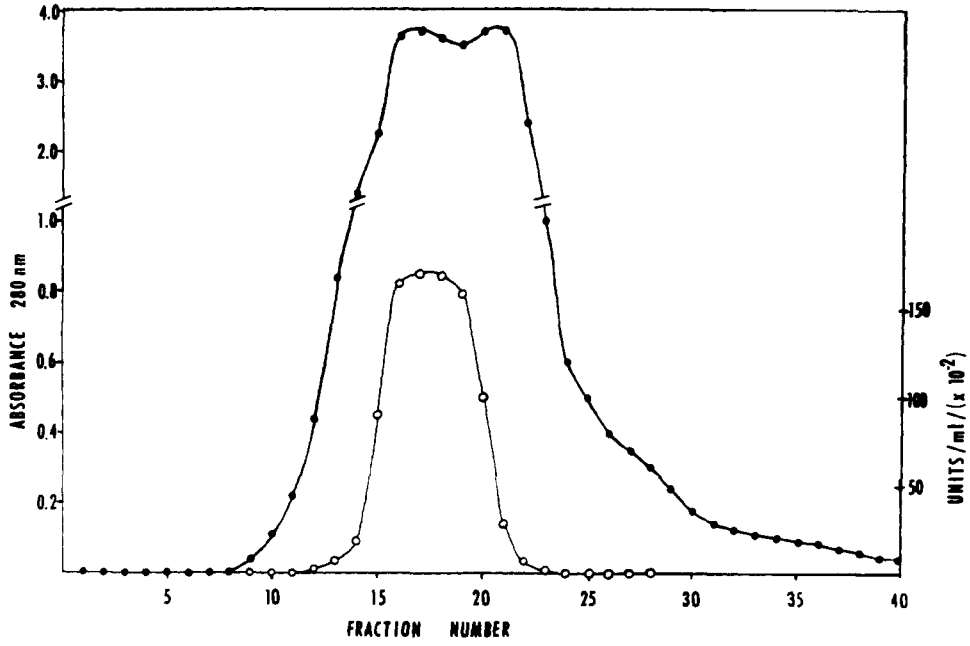

FIGURE 1: Chromatography of human plasma on Sepharose–Blue Dextran. The column (5.0 × 40 cm) was equilibrated with 0.05 M Tris-HCl–0.15 M NaCl (pH 8.0). After application of the sample (50 ml), the column was washed with equilibration buffer until the $A_{280\,nm}$ was less than 0.050. (●) $A_{280\,nm}$; (○) trypsin inhibitory activity.

sin, and porcine elastase were obtained from Worthington Biochemicals. Human pancreatic elastase was prepared by the method of Mallory and Travis (1973). Boar acrosin was a gift of Dr. W. L. Williams of this Department. Bovine thrombin was obtained from Sigma Chemical Co.

Methods

Enzyme and Inhibitor Assays. Trypsin esterase activity, using N-benzoyl-L-arginine ethyl ester as substrate, was measured by the procedure of Schwert and Takenaka (1955) as described by Mallory and Travis (1973). One unit of activity was defined as an absorbancy change of 1.0 optical density units per min at 253 nm.

α-1-PI activity was assayed by measuring loss of esterase activity at 253 nm after a sample of inhibitor was preincubated with a standard porcine trypsin preparation for 5 min at room temperature. One unit of inhibitory activity was defined as being equivalent to the loss of one unit of trypsin esterase activity. Specific activity was calculated as units of inhibitory activity per optical density unit at 280 nm. Acrosin and thrombin inhibition were measured by an identical procedure.

Chymotrypsin activity and inhibition were performed utilizing the procedure of Coan *et al.* (1971). Elastase was assayed by the method of Janoff (1969).

Carbohydrate Analysis. Total neutral sugars were determined on the intact protein using the phenol–sulfuric acid technique (Dubois *et al.*, 1956). Galactose was used as a standard. Hexosamine was determined by the technique of Good and Bessman (1964) after hydrolysis of the glycoprotein in 4 N HCl for 4 hr at 100°. Glucosamine was used as a standard.

Sialic acid was determined by the thiobarbituric acid assay method of Warren (1959) after hydrolysis of protein in 0.1 N H_2SO_4 at 80° for 1 hr. *N*-Acetylneuraminic acid was used as a standard.

Amino Terminal Analysis. The amino terminus of α-1-PI was determined by the dansyl technique according to the method of Gros and Labouesse (1969). Examination of the acid hydrolysate was performed as described by Woods and Wang (1967). Human serum albumin was used as a control. The results obtained were additionally confirmed by thin-layer chromatography on silica gel using a chloroform–*tert*-butyl alcohol–acetic acid system (6:3:1; v/v) which clearly separates Dns-glutamic acid from Dns-aspartic acid.

Other Analytical Techniques. Techniques for amino acid analysis, analytical ultracentrifuge studies, polyacrylamide electrophoresis, and immunoelectrophoresis were performed as described elsewhere (Coan *et al.*, 1971). The separation of α-1-PI from orosomucoid was readily demonstrated using the Ortec vertical gel slab electrophoresis system with a discontinuous gradient (4–8%) as described by the manufacturer.

Isoelectric focussing was performed according to the technique of Wrigley (1971).

Results

Purification of Human α-1-PI. All of the steps described in the purification of α-1-PI were performed at 4° with the exception of step 1, which was performed at room temperature. Although 50 ml of plasma was routinely used in developing the fractionation scheme, as much as 200 ml of plasma can be easily handled at one time using the conditions described below and increasing the size of both the columns and gradients.

(*1*) *Albumin Removal.* As described elsewhere (Travis and Pannell, 1973), a simple procedure for the preparation of albumin-depleted plasma has been developed by the use of Sepharose–Blue Dextran adsorption. The sample of clarified normal human plasma (50 ml) is applied to the top of a column of Sepharose–Blue Dextran equilibrated with 0.05 M Tris-HCl–0.15 M NaCl (pH 8.0). The same buffer is utilized to wash the column and fractions are collected until the $A_{280\,nm}$ is less than 0.050. The elution pattern is shown in Figure 1 and gel slab electrophoresis of representative fractions is demonstrated in Figure 2.

The results obtained show that not only is albumin adsorbed on the column but also that some fractionation due

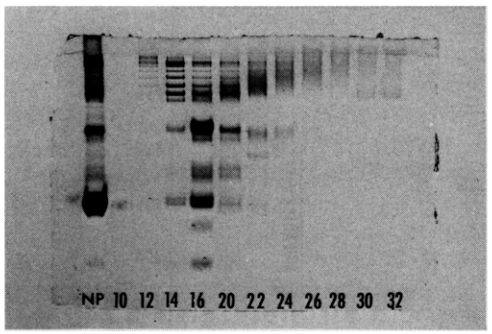

FIGURE 2: Gel slab electrophoresis of plasma protein fractions eluted from Sepharose–Blue Dextran. The gel was made in a discontinuous system from 4 to 8%. Running pH, 8.8. The gel was stained with 1% Amido Schwartz. Sample size, 10 μl for 1, 50 μl for remainder. Direction of migration is from cathode (top) to anode (bottom). The numbers under each slot are equivalent to the fraction numbers given in Figure 1.

to molecular weight differences as well as weak affinity for the derivative also occurs. The significant trailing shown in both Figures 1 and 2 is due to the interaction of immunoglobulins and lipoproteins with the Sepharose–Blue Dextran. This can be sharply reduced by using 0.5 M NaCl in the buffer system referred to above, if one wishes to recover all of the immunoglobulin fraction. However, no interference by either group of proteins in the fractionation of α-1-PI occurs under the conditions described here.

(*2*) *Ammonium Sulfate Fractionation.* The most active fractions from the preceding step are pooled and fractionated with solid ammonium sulfate. The precipitate obtained between 0.60 and 0.80 saturation is solubilized with 0.05 M Tris-HCl–0.05 M NaCl (pH 8.80) and the solution dialyzed with several changes of the same buffer.

(*3*) *DEAE-Cellulose Chromatography* (*pH 8.80*). The dialyzed fraction from the previous step is applied directly to a column of DEAE-cellulose equilibrated with 0.05 M Tris-HCl–0.05 M NaCl (pH 8.80). After the passage of unretarded protein, a linear gradient from 0.05 M NaCl to 0.15 M NaCl, both in 0.05 M Tris-HCl (pH 8.80) is initiated. The elution pattern is shown in Figure 3. The fractions

TABLE I: Purification of Human α-1-Proteinase Inhibitor.

Step	Total Protein (OD_{280})	Total Activity	Specific Activity (units/OD_{280})	Recovery (%)	Purification
Whole plasma	3210	500	0.016	100	1
Sepharose-Blue Dextran	2100	480	0.023	96	1.4
Sephadex G-75	350	368	1.05	73	6.2
DEAE-cellulose (pH 8.8)	30	340	11.3	68	70.0
DEAE-cellulose (pH 6.5)	16	300	20.0	60	125.0

having a specific inhibitory activity greater than 9.0 are pooled, concentrated to 25 ml by ultrafiltration using an Amicon UM-10 membrane, and dialyzed with several changes of 0.005 M sodium phosphate–0.05 M NaCl (pH 6.50).

(*4*) *DEAE-Cellulose Chromatography* (*pH 6.50*). The dialyzed inhibitor solution from the first DEAE-cellulose column is applied to a column of DEAE-cellulose equilibrated with 0.005 M sodium phosphate–0.05 M NaCl (pH 6.50). A linear gradient from 0.05 M NaCl to 0.20 M NaCl, both in 0.005 M sodium phosphate (pH 6.50), is then initiated. The elution pattern is shown in Figure 4. Virtually all of the active fractions have a specific activity near 20.0. These are pooled and the preparation concentrated to 25 ml by ultrafiltration. This material is used for the subsequent studies described below.

The results of each fractionation step are summarized in Table I. Disc electrophoresis at each stage is shown in Figure 5.

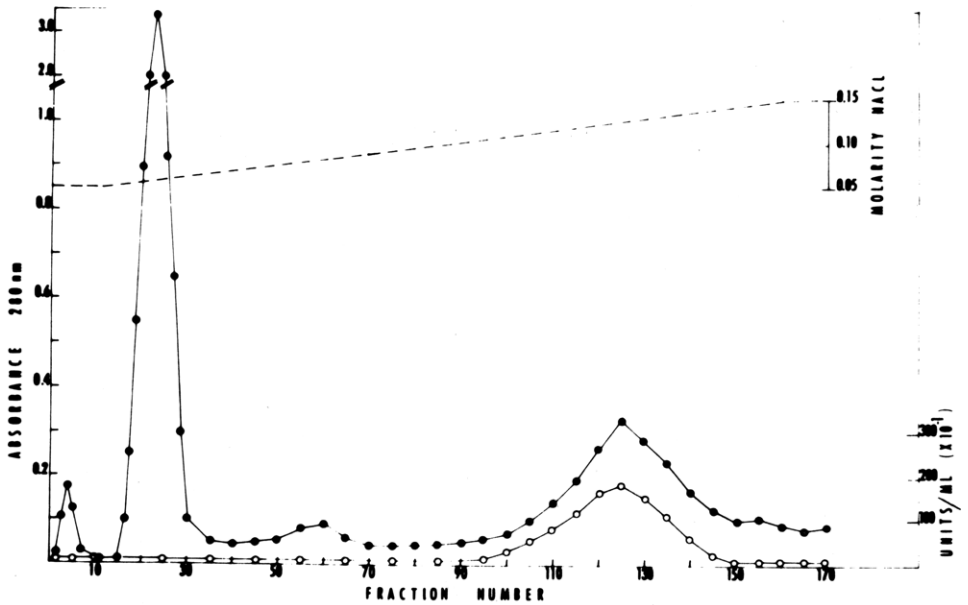

FIGURE 3: Ion-exchange chromatography of partially purified human α-1-PI on DEAE-cellulose (pH 8.8). The column (1.9 × 40 cm) was equilibrated with 0.05 M Tris-HCl–0.05 M NaCl (pH 8.8). After application of the sample the column was washed with equilibration buffer and a linear gradient initiated at fraction ten from 0.05 M NaCl to 0.15 M NaCl (500 ml total). (●) $A_{280\ nm}$; (○) inhibitory activity.

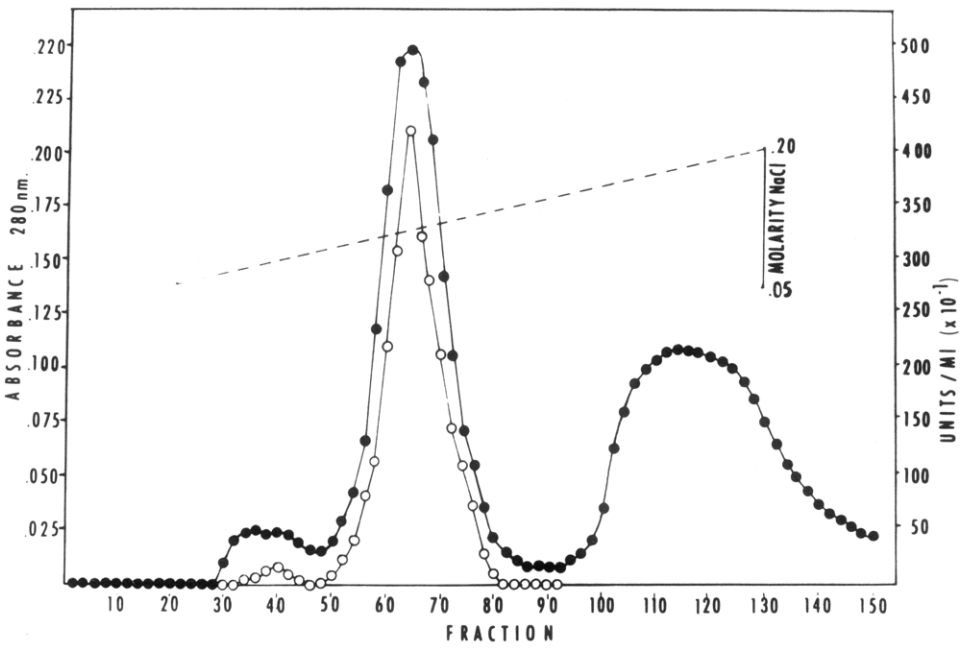

FIGURE 4: Ion-exchange chromatography of partially purified human α-1-PI on DEAE-cellulose (pH 6.5). The column (1.9 × 40 cm) was equilibrated with 0.005 M sodium phosphate–0.05 M NaCl (pH 6.5). After application of the sample the column was washed with equilibration buffer and a linear gradient initiated at fraction twenty from 0.05 M NaCl to 0.20 M NaCl (500 ml total). (●) $A_{280\ nm}$; (○) inhibitory activity.

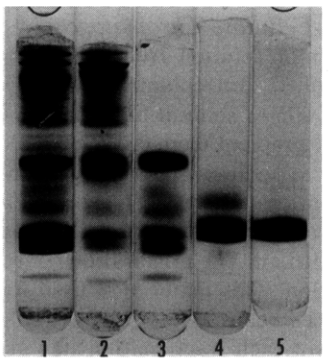

FIGURE 5: Disc electrophoresis of plasma fractions at each stage of purification of human α-1-PI at pH 8.9 in a standard 7.5% gel. Gels were stained in 1% Amido Schwartz. Direction of migration is from cathode (top) to anode (bottom). (1) Whole plasma (500 μg); (2) Sepharose-Blue Dextran treated plasma (200 μg); (3) $(NH_4)_2SO_4$ fraction (200 μg); (4) DEAE-cellulose, pH 8.8 (50 μg); (5) DEAE-cellulose, pH 6.5 (50 μg).

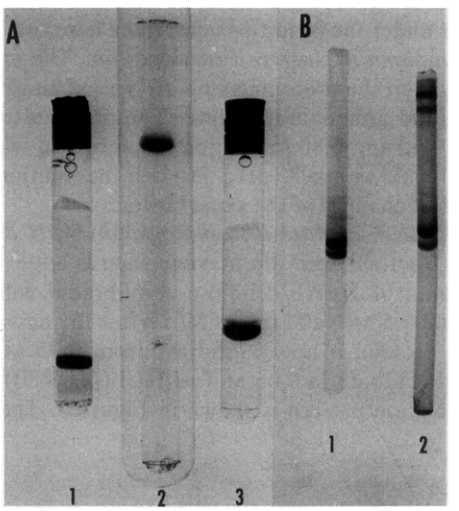

FIGURE 6: Gel electrophoresis of purified α-1-PI and α-1-PI-porcine trypsin complexes. (A) (1) pH 8.9, 7.5% gel (50 μg); (2) after mercaptoethanol–SDS treatment (15 μg) (Weber and Osborn, 1969); (3) pH 4.3, 7.5% gel (50 μg) (Reisfeld *et al.*, 1962). (B) Isoelectric focussing in acrylamide. Concentration of pH 4–6 ampholine, 2%; gel size, 0.6 × 15 cm. The pH gradient is from 6.0 (top) to 4.0 (bottom). (1) α-1-PI (50 μg); (2) mixture of α-1-PI (50 μg) and porcine trypsin (20 μg). Incubation time of mixture, 5 min at pH 8.0 at 25°.

Homogeneity of Human α-1-PI. Preparations of α-1-PI prepared in the manner described above were shown to be homogeneous by several criteria including elution as a single component with constant specific activity using both Sephadex G-75 chromatography and ion-exchange chromatography on DEAE-cellulose at pH 6.50. The latter was performed using the conditions given for the final step in the purification of this protein.

As shown in Figure 6A, α-1-PI migrated as a single component during normal alkaline disc gel electrophoresis and after SDS treatment in the presence of mercaptoethanol (Weber and Osborn, 1969). The latter results, using proteins of known size as standards, indicated that α-1-PI had a molecular weight of 52,000. In an acid gel system (Brewer and Ashworth, 1969), α-1-PI migrated as a major and minor component.

Isoelectric focussing of α-1-PI in a pH 4–6 ampholyte system resulted in a pattern of two major and three minor protein bands (Figure 6B). The major components were found, by gel scanning, to represent nearly 95% of the protein. The three minor components, however, are also proteinase inhibitors since addition of small quantities of porcine trypsin to the protein sample, prior to isoelectric focussing, causes all components to shift from their normal position to that of complexes with higher isoelectric points (Figure 6B). These results agree with the findings presented by others using crossed-gel electrophoresis (Kueppers, 1969) which indicate that the normal variant of α-1-PI is elicited as two major isoinhibitors.

Immunoelectrophoresis against either anti-whole human plasma or anti-human α-1-PI gave single precipitin lines as shown in Figure 7. In this regard, Ouchterlony immunodif-

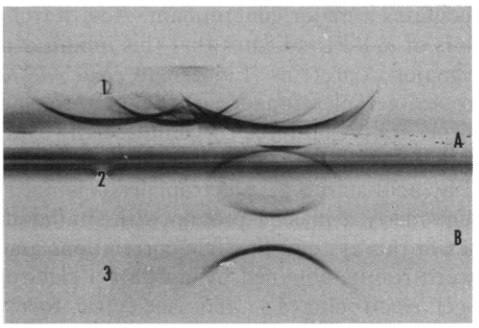

FIGURE 7: Immunoelectrophoresis of normal human α-1-PI and normal human plasma. Electrophoresis was carried out for 2 hr at 8 mA, in 0.05 M sodium veronal (pH 8.6) followed by immunodiffusion for 16 hr at 4°. Samples were soaked in 0.9% saline and then stained with 1% Amido Schwartz. Wells 1 and 3, normal human plasma (2 μl); well 3, normal human α-1-PI (0.25 μg); slot A, rabbit anti-whole human plasma; slot B, rabbit anti-human α-1-PI.

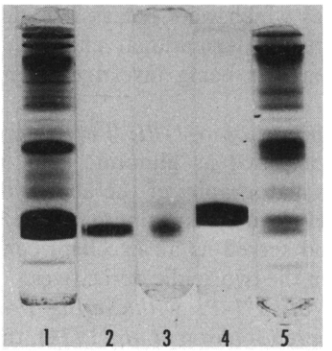

FIGURE 8: Disc electrophoresis of human plasma fractions. Conditions of run as in Figure 5. (1) Normal human plasma (5 μl); (2) human plasma albumin (50 μg); (3) peak two, DEAE-cellulose, pH 6.5 (orosomucoid, 200 μg); (4) normal human α-1-PI (50 μg); (5) Sepharose-Blue Dextran treated normal human plasma (5 μl).

TABLE II: Amino Acid and Carbohydrate Composition of Human α-1-Proteinase Inhibitor.

Residue	Residues/Molecule			
	A[a]	B[b]	C[c]	D[d]
Lysine	41	30	32	36
Histidine	13	12	13	12
Arginine	7	6	7	8
Tryptophan[f]	1	2	2	3
Aspartic acid	47	38	44	44
Threonine	26	26	31	28
Serine	23	18	22	21
Glutamic acid	55	46	50	52
Proline	23	15	16	19
Glycine	23	19	22	23
Alanine	25	22	24	24
Half-cystine[e]	4	0	1	2
Valine	25	21	23	27
Methionine[e]	6	7	8	8
Isoleucine	20	16	18	21
Leucine	48	39	45	51
Tyrosine	6	6	7	6
Phenylalanine	27	23	29	29
Hexosamine	17	10	10	14
Hexose	21	14	12	20[g]
Sialic acid	2	6	6	9[g]
Fucose		1	0	

[a] This paper. [b] Heimburger *et al.* (1971). [c] Crawford (1973). [d] Kress and Laskowski, Sr. (1973). [e] Average of 22- and 72-hr hydrolysate of oxidized sample. [f] Determined from 22-hr hydrolysate in presence of 4% thioglycollate. [g] Recalculated.

fusion experiments using antibodies prepared in response to albumin, orosomucoid, α-1-antichymotrypsin, antithrombin III, and group-specific components of plasma were all negative when tested against α-1-PI preparations, indicating that these proteins whose properties and migration rate in acrylamide gels are very similar to α-1-PI were not present as contaminants.

Molecular Weight Studies. Sedimentation velocity experiments run in 0.005 M sodium phosphate–0.05 M NaCl (pH 6.50) at a protein concentration of 7.0 mg/ml, and a speed of 60,000 rpm, indicated a single symmetrical component with a calculated $s_{20,w}$ of 3.60. An extinction coefficient ($E_{1\,cm,\,280nm}(1\%)$) was computed from ultracentrifuge studies using interference optics (Babul and Stellwagen, 1969) and found to be 5.30.

Sedimentation equilibrium experiments (Yphantis, 1964) on α-1-PI at an initial protein concentration of 0.15 mg/ml in 0.05 M Tris-HCl–0.05 M NaCl (pH 8.0) yielded a molecular weight of 53,000 using a partial specific volume of 0.743 calculated from the amino acid and carbohydrate composition. The linearity of ln C *vs.* r^2 plots indicated that the molecule was not associating into polymers as suggested by others (Heimburger *et al.*, 1971).

Amino Acid and Carbohydrate Composition. The calculated amino acid and carbohydrate composition of human α-1-PI, based on the above molecular weight and extinction coefficient of the protein, is given in Table II. The composition of this protein, as reported by others, is also shown for comparison.

With the major exception of half-cystine content, these results indicate a reasonably strong similarity in amino acid composition to those reported by others. Of significant interest are the differences in carbohydrate content of the various preparations. These differences are almost certainly due to glycoprotein contamination, probably from orosomucoid, in the other preparations. This latter protein is difficult to separate unless appropriate conditions, such as those given in Figure 4, are followed. Orosomucoid stains poorly with Amido Schwartz or Coomassie Blue and, therefore, may not be easily visualized in impure preparation of α-1-PI. Furthermore, unless appropriate conditions are used during the gel electrophoresis, the orosomucoid may migrate with α-1-PI and not be detected at all. This is particularly well documented by the results shown in Figure 8 which compare the electrophoretic mobilities in a standard disc gel electrophoresis system of α-1-PI and the second peak (orosomucoid mainly) off of DEAE-cellulose at pH 6.5.

Stability of α-1-PI. The purified inhibitor was found to be stable at 4° for at least 6 months in the pH range 6.50–9.00. At pH values less than 4.0 all inhibitory activity was irreversibly lost. At room temperature α-1-PI retained full activity for at least 24 hr. Mercaptoethanol (0.001 M) had no activating or inhibitory effect on α-1-PI contrary to the results of Crawford (1973) who reported the need for this compound for stabilization of inhibitory activity.

Activity of α-1-PI preparations immediately after being brought to room temperature was somewhat lower than

that of samples which were not assayed for several hours, suggesting slow conformational changes in this protein. This phenomenon is being investigated in some detail at present.

Amino Terminus of α-1-PI. The N-terminal of α-1-PI was readily identified as glutamic acid or glutamine by thin-layer chromatography of the acid hydrolysates of the Dns-protein. Human serum albumin gave aspartic acid as N-terminal and served as an excellent control in differentiating between the two acidic derivatives.

Interaction of α-1-PI with Serine Proteases. As described elsewhere (Johnson *et al.*, 1974), the preparation of α-1-PI obtained by the above procedures was found to inhibit porcine trypsin at approximately at 1:2 molar ratio (inhibitor/trypsin). Similar results have now been confirmed with human and bovine trypsins and with bovine α-chymotrypsin. The preparation has also been found to inhibit human anionic trypsin and human chymotrypsin II, porcine and human pancreatic elastases, human granulocytic elastase, and boar acrosin. However, the stoichiometry of binding was not measured due to either instability and/or paucity of the enzyme preparation or lack of a good substrate for the measurement of available active sites. Significantly, no inhibition of thrombin esterase activity could be detected at 10:1 molar ratios of inhibitor to enzyme.

Discussion

The establishment of a procedure for the preparation of albumin-depleted plasma (Travis and Pannell, 1973) has resulted in the development of a rather simple series of steps leading to the isolation of a homogeneous preparation of α-1-PI. It is expected that many other plasma proteins as well as proteins from other sources which may be contaminated with albumin will now be much easier to purify. In fact, the simple chromatographic step at pH 8.8 results in the isolation of transferrin and prealbumin in homogeneous form, while the pH 6.5 chromatography yields both α-1-PI and orosomucoid as purified proteins.

Many other laboratories have presented evidence to indicate reasonable purity of their preparations of α-1-PI. However, in most cases the stoichiometry of protease binding on a molar basis was only 1:1 and no attempt was ever made to determine the actual number of available active sites in the enzyme preparations used in the assay. Indeed, this would tend to lower the activity of the inhibitor preparation. Furthermore, with the exception of one report (Chan *et al.*, 1973) none of the α-1-PI preparations were subjected to ion-exchange chromatography below pH 7.0. Thus, no attempt was made to remove orosomucoid from the inhibitor samples. As shown by the results given in Table I, the addition of this step resulted in a doubling in the specific inhibitory activity of α-1-PI. Unfortunately, the activity of our preparation cannot be compared with that of Chan *et al.* (1973) because the characterization of their product is poorly described and the preparation may still have been contaminated with either albumin or other components.

Perhaps the best evidence for the degree of purity of our α-1-PI preparation is shown by the isoelectric focussing experiments. The results indicate that in a pH 4–6 ampholyte system, α-1-PI migrates mainly as two major components. Crawford (1973) found three major and several minor components in the α-1-PI preparation which he isolated. The discrepancy between our results and his can be interpreted in two ways. Either we have lost one of the forms of α-1-PI during our purification or the preparation of Crawford (1973) contains a major contaminant. Acid starch gel electrophoresis of α-1-PI indicates that this inhibitor is elicited as three major isoproteins (Lieberman *et al.*, 1972). However, crossed-gel electrophoresis suggests that only two major forms are precipitated by commercially available antisera to this protein.It may well be that one of the proteins detected by acid starch gel electrophoresis is not a form of α-1-PI but merly a plasma protein with similar migratory properties in this system. Possible correlations and anomalies between results obtained by starch gel electrophoresis, crossed-gel electrophoresis, and isoelectric focussing are currently under investigation.

The carbohydrate composition of our preparation of α-1-PI has important ramifications. It has been suggested (Bell and Carrel, 1973) that differences in the migration rate of genetic variants of α-1-PI are due to differences in sialic acid content with the least acidic variant (type ZZ) containing less sialic acid than the normal variant (type MM). Considering the fact that the preparation of α-1-PI described here contains only two sialic acid residues, it is doubtful that differences between type MM α-1-PI, type ZZ α-1-PI, and all of the other genetic variants with intermediate mobilities could be due to the content of this sugar derivative.

With regard to the levels of α-1-PI in human plasma, the current values in the literature are all based on 1:1 binding. As we have shown elsewhere (Johnson *et al.*, 1974) 2 mol of trypsin are bound/mol of inhibitor. Therefore, the normal concentration of α-1-PI in plasma is probably only about 100 mg %. The fact that two inhibitory sites have been found in α-1-PI is not at all unusual. For example, it has been shown that chicken ovoinhibitor is able to inhibit both 2 mol of trypsin and 2 mol of chymotrypsin simultaneously (Tomimatsu *et al.*, 1966). Very recently it was shown that quail and chicken ovomucoids have two internal homologous regions which are also homologous with the Kazal pancreatic secretory inhibitory (Kato *et al.*, 1974). Curiously, these proteins are, like α-1-PI, glycoproteins with broad inhibitory specificity.

Throughout this paper we have referred to the protein which we have isolated as α-1-proteinase inhibitor, whereas in the literature it is consistently referred to as α-1-antitrypsin. Since the function of this molecule *in vivo* is not to act as an antibody and since it inhibits many other proteinases besides trypsin, its common name is truly a misnomer. It is suggested that the term, α-1-proteinase inhibitor, describes much more succinctly both the localization of this molecule during electrophoresis of plasma as well as its natural function.

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High-Anti Conformation in *o*-Azanucleosides. The Crystal and Molecular Structure of 6-Azacytidine†

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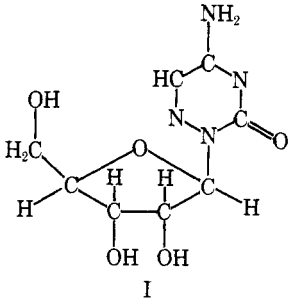
ABSTRACT: The crystal and molecular structure of 6-azacytidine has been determined from counter X-ray data. The nucleoside crystallizes in the space group $P2_12_12_1$ of the orthorhombic system with four molecules in a cell of dimensions $a = 7.623$ (6), $b = 6.993$ (7), and $c = 19.622$ (14) Å. Full matrix least-squares refinement using 1557 data has yielded a final value of the R factor (on F) of 0.031. The nucleoside adopts a glycosyl torsional angle, χ, of +99.1°, which is outside of the conventional syn and anti ranges and is in the "high-anti" region. The sugar pucker is the C-3′-endo (^3E) envelope conformation. The conformation about the extracyclic bond C-4′–C-5′ is the commonly occurring gauche–gauche. CNDO/2 molecular orbital calculations show that there is no residual charge on the aza atom N-6. The observed "high-anti" conformation may explain some of the known biochemical properties of 6-azacytidine.

6-Azacytidine (I) is an important carcinostatic agent



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(Sorm and Veseley, 1961) which interferes with the *de novo* synthesis of uridine through its inhibitory action on orotidylic acid decarboxylase. The inhibitory action of the nucleoside proceeds *via* two routes: (1) by the direct action of its 5′-nucleotide on the enzyme and (2) by first a deamination to 6-azauridine and then inhibition by the 5′-nucleotide of the latter (Skoda, 1963). Thus, 6-azacytidine and 6-azauridine are quite similar in their inhibition of orotidylic acid decarboxylase. Another similarity between the two nucleosides is seen in the ribosome binding experiments (Skoda, 1969). Thus, although GUU and GUC are the codon trip-

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