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Isolation, Chemical, and Physical Properties of α-1-Antitrypsin†

Piero Musiani and Thomas B. Tomasi, Jr.*

ABSTRACT: A method of isolation of α-1-antitrypsin (α-1-AT) in good yield from normal human plasma is described. A key step was affinity chromatography employing an antiserum which had been depleted of α-1-AT antibodies. The final preparations were homogeneous by immunological and physicochemical criteria. The specific activity of the purified α-1-AT was 0.363 mg of active bovine trypsin inhibited per 1.0 mg of inhibitor. Polyacrylamide gel patterns at both alkaline and acid pH of highly pure preparations frequently, but not invariably, showed multiple bands. Molecular weight studies by sedimentation equilibrium ultracentrifugation in aqueous buffer and in 6 M guanidine as well as sodium dodecyl sulfate polyacrylamide gel electrophoresis suggest that α-1-AT is a single polypeptide chain having a molecular weight of 49 500. Other physical and chemical properties of the inhibitor are described. A limited N-terminal sequence (Glu-Asp-Pro-Gln-Gly-Asx-Ala-Ala) was obtained. It was found that α-1-AT easily forms polymers and higher aggregates when exposed to denaturing agents such as 8 M urea and 6 M guanidine. The results suggest that aggregation is determined by both covalent and noncovalent forces.

Human serum has long been known to inhibit the activity of several proteolytic enzymes. The most important protease inhibitor in human serum is α-1-antitrypsin (α-1-AT)[1] designated as such by Schultze et al. (1962). This protein has approximately 90% of the trypsin inhibitory capacity of human serum and it has also been shown to be active against other proteolytic enzymes including chymotrypsin, collagenase, elastase, plasmin, and thrombin and a protease from human leukocytes (Schwick et al., 1966; Gans and Tan, 1967; Kueppers and Bearn, 1966).

Interest in α-1-AT has been stimulated by studies which suggest a possible role in several human diseases. The genetically determined severe α-1-AT deficient state is associated with pulmonary emphysema in certain families (Eriksson, 1965) and with infantile cirrhosis in others (Sharp et al., 1969) while in occasional families (Glasgow et al., 1973) both organs are affected.

Although several methods have been reported for the purification of α-1-AT (Bundy and Mehl, 1959; Schultze et al., 1962; Heimburger et al., 1971; Myerowitz et al., 1972; Crawford, 1973; Liener et al., 1973) in our experience these techniques have not always yielded pure preparations of α-1-AT, particularly when analyzed immunologically with certain potent antisera to whole human serum. We report here a method for the isolation of α-1-AT which yields highly purified preparations of this protein from human plasma. Some of the physical and chemical properties of the inhibitor are described, including a limited N-terminal sequence.

Materials and Methods

Isolation of α-1-AT. Citrated plasma obtained from outdated blood was dialyzed for 24 h against 10 volumes of 0.01 M calcium chloride in 0.01 M phosphate (pH 7.4) containing 0.14 M sodium chloride. The resulting serum was then precipitated with an equal volume of saturated ammonium sulfate. The 50% saturated ammonium sulfate super-

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[1] Abbreviations used are: α-1-AT, α-1-antitrypsin; $\bar{v}$, partial specific volume; PEC, *S*-β-(4-pyridylethyl)cysteine; TIC, trypsin inhibitory capacity; PCA, pyrrolidonecarboxylic acid.

natant was dialyzed for 48 h against several changes of buffer, concentrated by positive pressure in an Amicon cell with a PM 10 membrane, and applied to a 3 × 80 cm column of DE 52 (Whatman) equilibrated with 0.01 M phosphate buffer (pH 7.6). Elution was carried out by means of a linear gradient obtained with a starting buffer of 0.01 M phosphate (pH 7.6) and a final buffer of 0.2 M phosphate (pH 6.0). The fractions collected were tested by gel diffusion (Ouchterlony, 1958) against an anti-α-1-AT antiserum (Behringwerke) and antisera to whole human serum. Those fractions containing high concentrations of α-1-AT were pooled, concentrated, and applied to a Sepharose-bound concanavalin A column (2.5 × 30 cm). Unbound material was eluted with 0.01 M phosphate (pH 7.4) containing 0.5 M sodium chloride and the α-1-AT was subsequently eluted with 0.1 M solution of methyl α-D-glucopyranoside in the same buffer, and dialyzed overnight against buffer, concentrated, and chromatographed on a G-100 column (2.5 × 120 cm) in 0.1 M phosphate (pH 7.4). The α-1-AT containing fractions were pooled and finally applied to an immunoadsorbent column prepared as described below. The yields from the various steps are outlined in Table I.

Affinity Chromatography. A goat antiserum to whole human serum was depleted of antibodies to α-1-AT and coupled to Sepharose 4B by the method of Cuatrecasas et al. (1968). In order to adsorb α-1-AT antibodies from this antiserum, we employed a partially purified preparation of α-1-AT coupled to silica supports (Biomaterial supports-Corning Biological Products, Medfield, Mass.) with a 2.5% aqueous glutaraldehyde solution. The α-1-AT preparation employed for adsorption was obtained as previously described by Crawford (1973), but in addition affinity chromatography with an antialbumin antiserum was employed as a final step. This preparation contained over 90% α-1-AT, but three minor contaminants were detectable by the particular goat antiserum used for affinity chromatography. Four other antisera to whole human plasma did not detect these contaminants. Subsequently we have found one other antiserum which detects two of the three contaminants. By careful adsorption, as described above, we were able to specifically delete α-1-AT antibodies so that on subsequent gel diffusion analysis there was no reaction with α-1-AT. The contaminant lines, although slightly diminished, were still strong. The adsorbed antiserum, when used as an immunoadsorbent, yielded preparations which, when examined by gel diffusion using the same *unadsorbed* antiserum, detected a single α-1-AT precipitin band. Thus, although adsorption may have decreased the antibody titers to the contaminants to a certain extent, the depletion was not sufficient to prevent this antiserum from being used as an effective immunoadsorbent. The immunoadsorbent columns were eluted with 0.01 M phosphate buffer (pH 7.4) with 0.5 M NaCl. Regeneration of the columns was carried out with 1 M potassium thiocyanate solution in the same buffer.

Polyacrylamide gel electrophoresis was performed in 5% gels according to the method described by Ornstein and Davis (Ornstein, 1964; Davis, 1964). In some experiments the gels were prepared with or without 8 M urea and the samples were applied in 8 M urea. The gels were stained with Coomassie Blue in 12% trichloroacetic acid. For the urea acetic acid gel electrophoresis the method of Panyim and Chalkley (1969) was followed. Dodecyl sulfate polyacrylamide gel electrophoresis was performed in 5 and 10% polyacrylamide gels by the techniques described by Weber and Osborn (1969). Polyacrylamide gel diffusion was performed as previously reported (Tomasi and Hauptman, 1974). Briefly, petri dishes (60 × 100 mm) are filled with 18 ml of 2% noble agar (Difco) and allowed to cool to 50 °C. The unstained polyacrylamide gels are layered on the surface and the agar allowed to solidify. Troughs are then placed laterally and filled with antisera. Plates are developed at room temperature for 24–72 h and observed periodically for precipitin formation.

Table I: Yields of Trypsin Inhibitor during Isolation Steps.

	Total Protein[a] (mg)	Active Trypsin Inhibitor[b] (mg)	Percent Recovery of Inhibition
Defibrinated plasma (190 ml)	15 390	287.1	100
$(NH_4)_2SO_4$ (50–75%)	7 900	229.4	79.9
DEAE ion exchange	1 200	99.6	34.7
Concanavalin A-Sepharose	185	70.3	24.5
Sephadex G-100	100	60.2	21.0
Immunoadsorbent column	65	49.1	17.1

[a] Protein determined by Folin method (Lowry et al., 1951) using as a reference dried purified human albumin (Behringwerke AG, Marburg-Lahn, West Germany). [b] The values are obtained from the TIC data (Eriksson, 1965) assuming 23 800 as molecular weight of trypsin and 49 500 as molecular weight of α-1-AT. The trypsin preparation was 65.3% active as determined by the active site titration method of Chase and Shaw (1967).

Ultracentrifugation. Analytical ultracentrifugation was performed in a Spinco Model E at 52 640 rpm at 20 °C with schlieren optics. In some experiments a positive wedge window was employed. $s_{20,w}^0$ was calculated by correcting sedimentation coefficient to water at 20 °C and extrapolating to zero concentration. Molecular weights were determined in 0.1 M phosphate buffer (pH 7.4) and in 6 M guanidine by the high-speed sedimentation equilibrium method of Yphantis (1964).

The partial specific volume ($\bar{v}$) of α-1-AT was determined by ultracentrifugation in water and D_2O as described by Edelstein and Schachman (1967). Partial specific volumes were also calculated from the amino acid and carbohydrate composition data. The value of $\bar{v}$ in 6 M guanidine was assumed to be 0.01 cm^3/g less than that in aqueous buffer (Hade and Tanford, 1967).

Extinction Coefficient. The optical density of protein solutions was determined at 280 mμ in a Cary 14 recording spectrophotometer. The extinction coefficient was calculated from the protein concentration obtained by integrating areas under peaks on the ultracentrifuge plates, employing a Gaertner microcomparator (Chicago). The specific refractive increment of human serum albumin of 0.00186 cm^2 per g per 100 ml was assumed (Armstrong et al., 1947).

The extinction coefficient in 6 M guanidine was determined using the protein concentration in aqueous buffer obtained with the above determined extinction coefficient. The solution was then made 6 M in guanidine and the optical density redetermined and the extinction coefficient recalculated.

Amino acid analysis was carried out on a Beckman 121-M amino acid analyzer using single column methodology. Hydrolysis was performed at 110 °C for 22, 48, and 72 h with constant-boiling HCl distilled through a glass apparatus. Tryptophan was measured spectrophotometrically

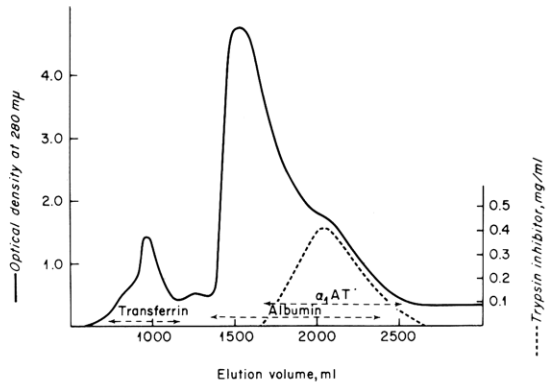

FIGURE 1: DEAE ion-exchange chromatography of an ammonium sulfate precipitate of defibrinated plasma. A linear gradient was obtained using a starting buffer of 0.01 M phosphate (pH 7.6) and a final buffer of 0.2 M phosphate (pH 6.0). Transferrin, albumin, and α-1-AT elution, indicated by the arrows, determined by immunodiffusion using specific antisera. Values of trypsin inhibitor obtained by the method of Eriksson (1965), indicated by dashed curved line.

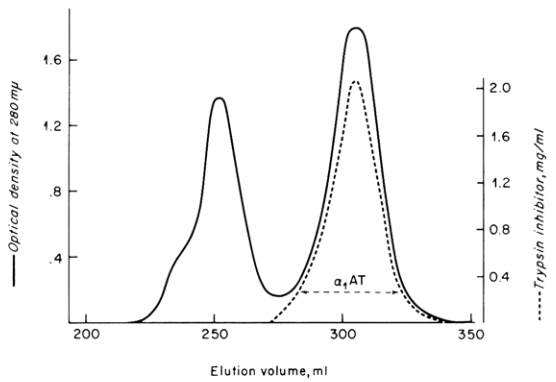

FIGURE 2: Sephadex G-100 chromatography of α-1-AT containing fractions obtained from concanavalin A column. Elution was carried out with 0.1 M phosphate buffer (pH 7.4). The α-1-AT, indicated by the arrow, was contained in the fractions of the second peak as determined by immunodiffusion using a specific antiserum. Values of trypsin inhibitor obtained by the method of Eriksson (1965), indicated by dashed curved line.

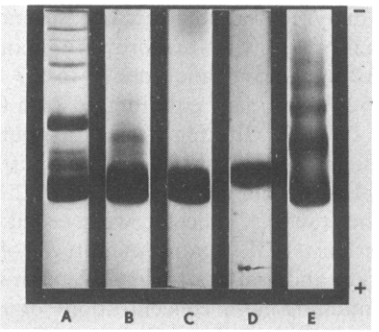

FIGURE 3: Polyacrylamide gel electrophoresis (alkaline pH). (A) α-1-AT containing fractions obtained from concanavalin A chromatography. (B) α-1-AT eluted in the second peak of G-100 Sephadex gel filtration (see Figure 2). (C) α-1-AT eluted from the immunoadsorbent column (final step). (D) Dodecyl sulfate gel of the same material as in C. (E) Electrophoresis of same sample as in C in 8 M urea at alkaline pH. Urea was present in sample and not in gel buffer.

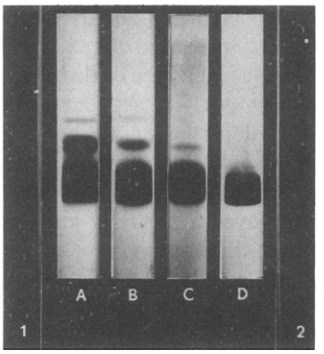

FIGURE 4: Polyacrylamide gel electrophoresis (alkaline pH) of purified α-1-AT from four different individuals (A–D). Gel diffusion patterns obtained with rabbit anti-α-1-AT serum are shown on outsides for gels A and D.

(Edelhoch, 1967). Methionine was determined as the sulfone derivative after performic acid oxidation according to the method of Hirs (1967). Cysteine was converted to *S*-β-(4-pyridylethyl)cysteine (PEC) by reduction with 0.1 M dithiothreitol in the presence of 6 M guanidine hydrochloride and alkylation with 4-vinylpyridine as described by Friedman et al. (1970).

Determination of Cysteine by Radioactive Labeling. [^{14}C]iodoacetamide at a specific activity of 0.6 mCi/mmol was used to quantitate half-cysteine residues as previously described (Miller and Metzger, 1965). Briefly, isolated α-1-AT at a protein concentration of 2 mg/ml was reduced in aqueous buffer and/or 6 M guanidine with 0.02 M dithiothreitol for 1 h at 37 °C in an atmosphere of N_2. Alkylation with [^{14}C]iodoacetamide was allowed to proceed for 30 min at room temperature in the dark with a 40% molar excess of iodoacetamide. Subsequently, the labeled protein was chromatographed over Sephadex G-25 and equilibrated in aqueous buffer or 6 M guanidine to remove the free [^{14}C]iodoacetamide. The void volume was then pooled and concentrated and the protein concentration determined. To 1 ml of this solution was added 15 ml of aquasol and counting was performed in an Isocap-300 (N-C) liquid scintillation counter. Results are reported as moles of SH (carboxyamidomethylcysteine) per mole of protein (α-1-AT) using the determined molecular weight.

Carbohydrate Determination. The hexose content was determined by the orcinol–H_2SO_4 method of Rosevear and Smith (1961) which is a slight modification of the one described by Winzler (1955). Standard curves were calculated using a mannose–galactose standard consisting of 20 mg % for each sugar over the range of 0–400 μg %.

Amino acid sequence studies were performed employing the Beckman automatic protein sequenator, Model 890C. The fast protein Quadral program was used (072172C). Phenylthiohydantoin derivatives of the amino acids were identified by gas chromatography on the Beckman GC65 with SP400 columns.

α-1-Antitrypsin Assays. Trypsin inhibition was measured by the trypsin inhibitory capacity (TIC) method described by Eriksson (1965) employing crystallized, salt-free trypsin (Worthington Biochemicals, Freehold, N.J.), benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA, Schwarz/Mann, Orangeburg, N.Y.) as substrate and soybean trypsin inhibitor (Worthington Biochemicals). The specific inhibitory activity was expressed as milligrams of trypsin inhibited per 1 mg of inhibitor.

Results

Isolation of α-1-AT. The elution diagram following DE 52 chromatography is shown in Figure 1. The fractions containing trypsin inhibitor were eluted in the position shown in Figure 1 after the major albumin peak. Albumin and sev-

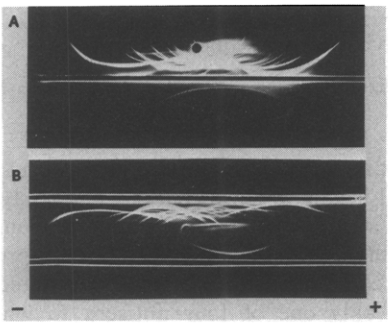

FIGURE 5: Immunoelectrophoresis of α-1-AT. (A) Top well: Normal human serum. Bottom well: Purified α-1-AT. Middle trough: Goat antinormal human serum. (B) Well: Normal human serum. Upper trough: Goat antinormal human serum. Bottom trough: Rabbit antiserum prepared against purified α-1-AT.

Table II: Physical Properties of α-1-AT.

	Present Study	Lit. Values
Molecular Weight		45 000[a]
		49 500;[b] 54 000[c,d]
Ultracentrifuge	49 500	55 000;[e] 59 900[f]
Sodium dodecyl sulfate	49 000	50 300[b]
Sedimentation coefficient	3.11	3.3;[e] 3.41;[a,c] 3.45[f]
Partial specific volume (cm^3/g)	0.725	0.646;[a] 0.726;[f] 0.728[b]
Extinction coefficient ($E_{1\ cm}(1\%)$)	5.0	4.4;[b] 5.0;[f] 5.3[a]

[a] Schultze and Heremans, 1966. [b] Crawford, 1973. [c] Bundy and Mehl, 1959. [d] Heimburger, 1972. [e] Shamash and Rimon, 1966. [f] Kress and Laskowski, 1973.

eral other contaminants were removed by concanavalin A chromatography. Gel filtration on Sephadex G-100 (Figure 2) separated α-1-AT in the second peak. As shown in this figure, using specific activity, the α-1-AT chromatographs with the characteristics of a single component. This preparation showed a small amount of several cathodally slower impurities on polyacrylamide gel electrophoresis (see Figure 3B). These contaminants were also demonstrated by immunoelectrophoresis and gel diffusion using one particular anti-whole human antiserum. It is important to note that several other anti-whole antisera did not detect these contaminants so that in analysis of purity a number of antisera must be employed. All of the remaining contaminants were removed completely by the immunoadsorbent column (see Materials and Methods).

Purity of α-1-AT. The final preparations of α-1-AT gave several types of patterns on polyacrylamide gels as illustrated in Figure 4. The preparation shown in Figure 4D, isolated from one serum, illustrates a single band on alkaline polyacrylamide gels. Three other preparations from individual sera have shown multiple banding as seen in Figure 4A–C. That each of these bands was α-1-AT is shown in Figure 4 by their immunological identity using the polyacrylamide gel diffusion technique. Ouchterlony gel diffusion analysis and immunoelectrophoresis of all four preparations showed a single precipitin arc when tested against several potent anti-whole antisera. No reactions were detected using antisera to α-1 acid glycoprotein, α-1-antichymotrypsin, or antithrombin III (obtained from Behringwerke, Marburg-Lahn, West Germany). When these preparations were injected into rabbits, the resulting antisera showed only antibodies directed against α-1-AT (Figure 5).

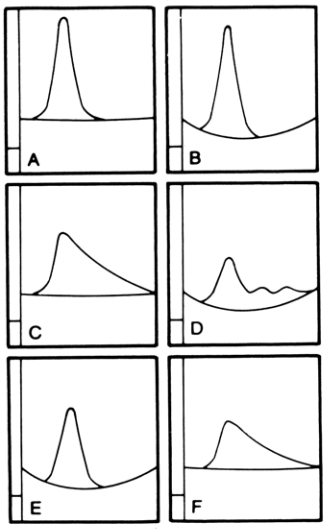

FIGURE 6: Schematic diagrams of analytical ultracentrifuge patterns of a purified preparation of α-1-AT in various buffers. (A) Purified α-1-AT in 0.1 M phosphate buffer at pH 7.4; (B) purified α-1-AT in 6 M guanidine; (C) sample in B dialyzed against 0.1 M Tris (pH 8.1) buffer; (D) sample in C after redialysis against 6 M guanidine; (E) sample in D reduced in 6 M guanidine with 0.02 M dithiothreitol and alkylated with 0.05 M iodoacetamide; (F) same sample in E dialyzed against 0.1 M phosphate buffer at pH 7.4.

Physical Properties. Similar physical properties were obtained on both types of α-1-AT preparations. The data presented in Table II are from the preparation shown in Figures 3C and 4D. Analytical ultracentrifugation of the purified α-1-AT at several different concentrations demonstrated a single boundary having a sedimentation coefficient at infinite dilution of 3.1 S. This preparation also showed a single symmetrical boundary when ultracentrifugation was carried out in 6 M guanidine.

The dodecyl sulfate polyacrylamide gels revealed only a single band (Figure 3D) and a molecular weight of 49 000 was calculated by this method.

Sedimentation equilibrium ultracentrifugation was performed at three different protein concentrations and a mean molecular weight of 49 500 ● 2 000 was calculated. A molecular weight of 49 800 was obtained when ultracentrifugation was performed in 6 M guanidine. The partial specific volume ($\bar{v}$) obtained by the D_2O technique was 0.725. The $\bar{v}$ calculated on the basis of the amino acid and carbohydrate concentration was 0.726. The extinction coefficient ($E_{1\ cm}(1\%)$) was 5.0 in aqueous buffer and 4.3 in 6 M guanidine.

Analytical ultracentrifugation of the purified α-1-AT in 6 M guanidine showed a single symmetrical boundary. When the guanidine was removed by dialysis against aqueous buffer (0.1 M Tris (pH 8.1)), the sedimentation pattern showed a diffuse boundary across the entire cell, indicative of aggregation. When this α-1-AT preparation was subsequently redissolved in 6 M guanidine, the broad boundary was replaced by three peaks suggesting polymer formation. Reduction resulted in the reappearance of a single symmetrical boundary. Finally, dialysis of the reduced preparation against aqueous buffer resulted in the reappearance of aggregation. These results are summarized schematically in Figure 6.

Chemical Properties. The amino acid and carbohydrate composition data are shown in Table III. The amino acid sequence for the N-terminal eight residues is illustrated in

Table III: Amino Acid Content of Human α-1-Antitrypsin.[a]

Amino Acid	Integral/mole of Residues
Lysine	32
Histidine	13
Arginine	6
Aspartic acid	42
Threonine	26
Serine	18
Glutamic acid	51
Proline	16
Glycine	24
Alanine	23
Half-Cysteine[b]	1
Valine	24
Methionine[c]	10
Isoleucine	17
Leucine	46
Tyrosine	5
Phenylalanine	26
Tryptophan[d]	2
Total	382

[a] A molecular weight of 42 400 was used to calculate amino acid composition. This molecualr weight was obtained from the experimentally determined molecular weight of 49 500 corrected for total carbohydrate content of 13.4% (5.3% hexose, as determined by the author, and 8.1% glucosamine and sialic acid, as determined by Crawford (1973)). [b] Determined as β-(4-pyridylethyl)cysteine (Friedman et al., 1970) and by radioactive labeling with [^{14}C]-iodoacetamide. [c] Determined as the sulfone derivative after performic acid oxidation (Hirs, 1967). [d] Determined spectrophotometrically (Edelhoch, 1967).

Figure 7. The hexose content obtained using the orcinol method was 5.3%.

Number of Chains. The following results support the hypothesis that α-1-AT is composed of a single polypeptide chain.

Analytical ultracentrifugation of the purified α-1-AT in 6 M guanidine hydrochloride showed a single symmetrical boundary and the sedimentation equilibrium molecular weight determinations in aqueous buffer and in 6 M guanidine were essentially identical. The molecular weight determination on dodecyl sulfate polyacrylamide gels following reduction also showed a single boundary with a molecular weight very close to that obtained by the ultracentrifugation. In addition, analytical polyacrylamide gels following reduction with 0.025 M MEA or 0.01 M dithiothreitol revealed only a single band. Finally, sequence analysis revealed a single N-terminal glutamic acid.

Activity. One milligram of the final preparation of α-1-AT inhibited 0.556 mg of trypsin in the test of Eriksson (1965). The bovine trypsin was found to be 65.3% active by the active site titration method of Chase and Shaw (1967). Therefore, the α-1-AT corrected specific activity is 0.363 mg of active bovine trypsin inhibited per 1 mg of inhibitor. Considering 49 500 as the molecular weight of α-1-AT and 23 800 for trypsin, this result shows that the α-1-AT preparation is 75.5% active, assuming 1:1 molar combination. Lyophilization of the α-1-AT preparation resulted in a loss of 13% of its inhibitory activity.

Discussion

Preliminary attempts at purification of α-1-AT using the procedures described in the literature yielded preparations that contained small amounts of three or four contaminating proteins, although we have not employed the method of

1 2 3 4 5 6 7 8
Glu-Asp-Pro-Gln-Gly-Asx-Ala-Ala

FIGURE 7: N-terminal amino acid sequence of human α-1-AT.

Kress and Laskowski (1973) and of Pannell et al. (1974), which recently came to our attention. The study of Pannell et al. (1974) was reported to give a homogeneous protein in excellent yields (60%) by a simple four-step procedure. In our studies, contaminating proteins were detected by gel diffusion by only one of four antisera to whole human serum, illustrating the necessity of testing purity using a number of different antisera. We were able to remove all of the contaminants by affinity chromatography employing this antiserum after it had been depleted of α-1-AT antibodies by adsorption with a partially purified preparation of α-1-AT. The antiserum employed for adsorption contained a relatively high content of anti-contaminant antibodies and the antigen preparations used for adsorption only small amounts of the contaminating antigens compared with the large amount of α-1-AT. Thus, the adsorption procedure, if carried out carefully in successive steps, removed primarily antibodies against α-1-AT and left the antiserum "contaminant specific". The final material obtained was homogeneous immunologically and by ultracentrifugation. However, polyacrylamide gel patterns frequently showed multiple bands, the origin of which is described below. When these preparations were injected into rabbits, only antibodies against α-1-AT were elicited.

The recovery of α-1-AT obtained by this method was about 17%. DE 52 chromatography was the purification step in which the major loss of trypsin inhibitor occurred; nevertheless, this step is found to be necessary in eliminating certain α and β globulins. The specific activity of α-1-AT was high (75.5%) when the α-1-AT assay was performed by the trypsin inhibitory capacity (TIC) method of Eriksson (1965), assuming 1:1 molar ratio between trypsin and α-1-AT. If a 1:2 molar ratio is assumed (Johnson et al., 1974; Pannell et al., 1974), the specific activity of the pure preparation falls to 37.8%. This latter value is considerably lower than expected, and our results seem, therefore, more consistent with a 1:1 molar ratio, although we have no direct experimental support for this. The specific activities of our α-1-AT preparations are within the range of the best preparations previously reported (Crawford, 1973; Kress and Laskowski, 1973).

It was found that α-1-AT easily forms polymers and higher aggregates if the inhibitor is exposed to a denaturing agent. In this study an α-1-AT preparation containing a single band, after exposure to 8 M urea and dialysis against buffer, showed multiple bands in polyacrylamide gels. These bands precipitated in a reaction of immunological identity in the Ouchterlony gel diffusion technique against an anti-α-1-AT antiserum, demonstrating that they represented various forms of α-1-AT. The ultracentrifuge analysis of α-1-AT after exposure to urea or guanidine hydrochloride and following reduction and alkylation (as summarized in Figure 6) suggests that aggregation is determined by both covalent and noncovalent forces. The higher molecular weight polymers which remained in 6 M guanidine and were reduced by dithiothreitol by a single symmetrical boundary were presumably disulfide bonded. The various disulfide polymers of α-1-AT were not separated and molecular weight data are therefore not available. However, the sedimentation patterns suggested at least two major

species with sedimentation coefficients higher than the monomer of α-1-AT, most likely representing disulfide bonded dimers and trimers.

The occurrence of disulfide bonded polymers of molecular weight higher than the dimer is difficult to reconcile with our sulfhydryl titration data. Both the [^{14}C]iodoacetamide method and the determination of cysteine as *S*-β-(4-pyridylethyl)cysteine suggested that there was a single cysteine residue per molecule of α-1-AT. If this were true of all α-1-AT molecules, it would not be possible for polymers larger than the dimer to be formed solely by disulfide linkages. One possible explanation is that the results obtained in the cysteine assays represent an average and that the α-1-AT preparations contain a mixture of molecules having 0, 1, and 2 cysteine residues. It is also possible that preparations obtained from different individuals might contain varying proportions of the different cysteine-containing molecules. This would explain the data in the literature in which values from 0 to 4 cysteine residues per mole of α-1-AT have been reported by different workers (Schwick et al., 1966; Crawford, 1973; Kress and Laskowski, 1973; Pannell et al., 1974). However, no evidence of such heterogeneity in cysteine content was obtained in this study. Four preparations from different individuals which included three with multiple bands and one with a single component on gel electrophoresis each contained a single cysteine residue.

The origin of the electrophoretic heterogeneity in α-1-AT preparations as shown by the alkaline gel patterns in this and other studies has not been elucidated. Most preparations of purified α-1-AT show in polyacrylamide gels a major anodal band and several (2–4) minor more cathodal bands. The major band represents 70–90% of the total protein in different preparations. Our studies, as well as others (Crawford, 1973), indicate that α-1-AT consists of a single polypeptide chain, so that mixed multimers formed by the combination of different subunits are an unlikely cause of heterogeneity. There are a number of possible explanations which can be entertained to explain electrophoretic heterogeneity in single chain molecules (reviewed in Williamson et al., 1973; Foster, 1968; Epstein and Schechter, 1968). The multiple bands observed on polyacrylamide electrophoresis represented different forms of α-1-AT and not contaminants as demonstrated by the immunological identity of the precipitin bands formed in the polyacrylamide gel diffusion technique. A similar banding pattern was also obtained in urea–acetic acid gels as well as the alkaline gels. Since these preparations were homogeneous by ultracentrifugation and in dodecyl sulfate gels and the banding pattern remained after reduction and alkylation and electrophoresis in 8 M urea, the multiple species seen did not represent a series of α-1-AT polymers or conformational isomers. The fact that neuraminidase treatment shifted the position of each of the bands cathodally to an equivalent extent suggested that the sialic acid content of each species was similar. PCA formation is certainly a possibility since the N-terminal residue was glutamic acid. Preparations showing both single and multiple bands on gels had an unblocked N-terminal. PCA formation from glutamic acid would, at the pH of our alkaline gels (pH 9.3), lead to a species which is more positively charged. This would not, however, explain more than one of the cathodal bands on the alkaline gels nor the multiple species noted in the acid–urea gels. If it is assumed that the minor bands represent post-secretion changes in the major most anodal species, then alterations such as amidation or enzymatic removal of acidic residues from the C terminus, which would produce a more basic molecule, are necessary.

A similar type of heterogeneity in serum albumin has been revealed by gel electrophoresis or isoelectric focusing on polyacrylamide gels (reviewed by Janatova, 1974). The origin of this heterogeneity, which also persists after reduction and alkylation and electrophoresis in urea, remains unexplained (Salaman and Williamson, 1971). Post-synthetic changes such as acetylation or amidation and deamidation have been suggested as likely candidates in the case of albumin (Spencer and King, 1971). Williamson et al. (1973) state that "deamidation may well turn out to be one of the most common causes of heterogeneity of pure proteins" and this remains a possibility for the heterogeneity observed in α-1-AT preparations.

Several "components" of α-1-AT have been noted in whole human sera using acid–starch gel electrophoresis followed by antigen–antibody crossed agarose electrophoresis (Fagerhol and Laurell, 1967). The various patterns obtained from different sera have been shown to be genetically determined, although the structural basis for the multiple boundaries seen on crossed electrophoresis has not been defined. The problem is that the genetic data obtained to date suggest a single genetic locus with multiple alleles (Fagerhol and Laurell, 1970). If this is true, then there should be no more than two different allelic proteins in the serum of a single individual. If the various electrophoretic species noted here and on crossed immunoelectrophoresis of whole sera are related and represent different structural proteins controlled by different alleles, it would suggest the existence of several genetic loci. We believe it is still not completely excluded that there are several closely linked loci in which crossing over and recombinational events are very rare and have not, therefore, been noted in the genetic analyses thus far performed. Obviously, further attempts to isolate and identify the structural differences in the various forms of α-1-AT are needed.

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The Role of 16S rRNA in Ribosomal Binding of IF-3†

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ABSTRACT: The binding of initiation factor IF-3 to *Escherichia coli* 30S ribosomal subunits has been found to be inhibited by rRNA ligands such as ethidium bromide, polyamines, and monovalent alkali metals. The order of effectiveness of the polyamines (spermine > spermidine > putrescine) and alkali metals ($Li^+ > Na^+ > K^+$) in inhibiting the ribosomal binding of IF-3 parallels their degree of affinity for the RNA. Furthermore, the binding of IF-3 to 30S subunits chemically modified by photooxidation with rose bengal, nitration with tetranitromethane, and reaction with kethoxal, monoperphthalic acid, and *p*-chloromercuribenzoic acid was studied. Results obtained after the direct treatment of the 30S subunits with the above chemical reagents or upon reconstitution of 30S particles having a modified rRNA or ribosomal proteins indicate that the IF-3 binding site is preferentially lost when the rRNA becomes modified. It was found that IF-3 could bind normally to 30S subunits lacking protein S1 or proteins S11, S12, S19, and S21 (and perhaps S14) which had been cross-linked to IF-3 in other laboratories.

Previous studies directed toward identifying the nature of the ribosomal binding site for initiation factor IF-3 have suggested that this factor binds to a segment of the 16S rRNA and that the ribosomal proteins (r-proteins)[1] confer specificity to the binding (Gualerzi and Pon, 1973). Since IF-3 has recently been cross-linked to some r-proteins, the most prominent being S12 (Hawley et al., 1974) or S12 and S11 (Traut et al., 1974; R. R. Traut, personal communication), the question has been raised as to whether these r-proteins are directly involved in the binding of IF-3 or whether the cross-linking simply reflects the proximity of these proteins to the ribosome-bound IF-3. This problem is of particular relevance, since the involvement of protein S12 in the initiation of R17 coat protein translation has been suggested (Held et al., 1974), while the role of IF-3 in directing the binding of mRNA to 30S subunits (Haselkorn and Rothman-Denes, 1973), and the importance of both S12 and S11 in controlling the codon–anticodon interaction on the ribosomes have long been recognized (Nomura et al., 1969).

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[1] Abbreviations used are: r-protein, ribosomal protein; $C(NO_2)_4$, tetranitromethane; *p*-ClHgBzO, *p*-chloromercuribenzoic acid.