Interaction of Human α-1-Antitrypsin with Porcine Trypsin†

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ABSTRACT: These experiments were designed to examine the mechanism of the interaction of human α-1-antitrypsin with porcine trypsin by analysis of their reaction products. When a molar excess of α-1-antitrypsin is added to trypsin, the resultant complex has a molecular weight equal to or slightly less than the sum of the moleculur weights of the two reactants. The complex is only slightly degraded up to 24 h of continued incubation at 37 °C, but after 72 h degradation of the complex becomes more evident. A catalytically active component of porcine trypsin is not inhibited by α-1-antitrypsin. The component, which could account for the delayed degradation of the complex, may be an autolytic product of the trypsin. Inhibitors of serine proteases or 8 M urea eliminate the observed degradation. The amino acid composition of the complex is in accord with that predicted for a 1:1 molar ratio of enzyme to inhibitor. In addition, the complex has the amino-terminal residues observed for trypsin and native α-1-antitrypsin. The enzyme–inhibitor complex can be dissociated in alkaline solutions, with the release of a large fragment of α-1-antitrypsin which has an amino-terminal threonine residue and a molecular weight between 46 000 and 50 000, and a small peptide. During this base-catalyzed hydrolysis, a single new carboxyl-terminal lysine residue is formed. This residue must be the carboxyl-terminal residue of the amino-terminal peptide of α-1-antitrypsin. If the trypsin inhibitor, phenylmethanesulfonyl fluoride, is not added, catalytically active trypsin can be measured after α-1-antitrypsin–trypsin complexes are dissociated at high pH. These findings support the hypothesis that trypsin reacts with α-1-antitrypsin at a Lys-Thr bond and that no peptides of the reactants have been lost from the intact complex. Available evidence suggests that the α-1-antitrypsin–trypsin complex is analogous to the tetrahedral or acyl intermediates which form only transiently between trypsin and its substrates.

Individuals who have a genetically determined low concentration of α-1-antitrypsin in their blood usually develop pulmonary emphysema at an early age (Laurell and Eriksson, 1963). Since α-1-antitrypsin is known to be a protease inhibitor (Heimburger et al., 1971), emphysema in such patients might logically be attributed to the unrestrained action of an enzyme or several enzymes on pulmonary tissues (Cohen, 1976).

Few studies have investigated the mechanism of inhibition of trypsin by α-1-antitrypsin and conflicting views have emerged regarding the number of moles of enzyme inhibited per mole of α-1-antitrypsin (Johnson et al., 1974; Moroi and Yamasaki, 1974). This laboratory demonstrated that trypsin, chymotrypsin, and elastase bind to α-1-antitrypsin stoichiometrically (Cohen, 1975). Enzyme inhibition by α-1-antitrypsin is markedly decreased if substrate is added to the enzyme before addition of the inhibitor (Hercz, 1974). In addition, diisopropyl phosphofluoridate inactivated trypsin does not compete with native trypsin for inhibitory sites (Hercz, 1974; Cohen, 1973). These studies suggest that α-1-antitrypsin binds tightly to the catalytic site on enzymes. Studies at low pH, which have been valuable in elucidating the mechanism of action of other anti-proteases in nature, have not been successful for α-1-antitrypsin, since the inhibitor is inactivated below pH 4.0 (Heimburger et al., 1971; Hercz, 1974).

Attempts to elucidate the amino acids at the inhibitory site in α-1-antitrypsin by modification of specific amino acids have produced ambiguous results. Reagents which are reported to specifically modify arginyl (Cohen, 1973) and lysyl (Laskowski, 1972) residues eliminate the trypsin-inhibiting capacity. However, recent investigations have suggested that the arginyl-modifying agent, phenylglyoxal hydrate, also modifies some lysyl residues when it reacts with α-1-antitrypsin (Busby and Gan, 1976).

Johnson and Travis (1976) have reported observations regarding the biochemical mechanism of α-1-antitrypsin which are at variance with other published literature. For example, 9.9×10^{-7} mol of trypsin was added to 5.7×10^{-7} mol of α-1-antitrypsin and left no residual tryptic activity. However, since there was no residual activity of the trypsin, the experiment was apparently performed at inhibitor excess. After the resulting complex was dissociated with benzamidine, the subsequently isolated fragment of α-1-antitrypsin had an amino-terminal threonine and a carboxyl-terminal serine-lysine dipeptide. The native α-1-antitrypsin preparation utilized also contained this dipeptide at the carboxyl-terminal end. If the experiment was performed at inhibitor excess, then the modified fragment of α-1-antitrypsin which was obtained should also have contained native α-1-antitrypsin, although probably in low concentrations. Furthermore, the carboxyl-terminal residues differ from those reported by others (Horng and Gan, 1974; Chan et al., 1976). If, however, the data are correct, they refute the hypothesis of Moroi and Yamasaki (1974), who suggested that the α-1-antitrypsin–trypsin complex is an acyl intermediate wherein trypsin is bound to α-1-antitrypsin through a new carboxyl-terminal arginine or lysine residue.

In this research we have reexamined some of the critical experiments of Moroi and Yamasaki (1974) and of Johnson and Travis (1976) and have performed additional experiments. These investigations indicate that most of the data of both investigators is reproducible. The new information obtained

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0006-2960/78/0417-0392$01.00/0

supports an alternative hypothesis which explains all of the existing data.

Experimental Procedure

Enzymes and Inhibitors. Carboxypeptidase B (treated with diisopropyl phosphofluoridate) was obtained from Worthington Biochemicals (Freehold, N.J.).

Porcine trypsin (three times crystallized; Miles Research Laboratories, Kankakee, Ill.) was used to avoid the instability characteristic of bovine trypsin (Bender et al., 1966).

It was necessary in these experiments to document the extent of autolytic cleavages in the preparation of trypsin employed. The amino-terminal residues of trypsin were isoleucine, serine, and valine. In some experiments, traces of aspartic acid were also observed. Digestion with carboxypeptidase B for 4 h released lysine in the amount of 50% and arginine in the amount of 5% of the molar quantity of trypsin present. These amino-terminal residues suggest that porcine trypsin undergoes autolytic cleavages similar to bovine trypsin (Keil, 1971; Dayhoff, 1972, 1976).

The carboxypeptidase C preparation from citrus leaves (Boehringer-Mannheim, Cleveland, Ohio) utilized in these studies had a specific activity of 790 mU/mg of enzyme at 37 °C, using *N*-carbobenzoxy-1-leucyl-L-phenylalanine as substrate (Moore, 1968). It was reconstituted from the lyophilized state to a protein concentration of 0.6 mg/mL with 0.05 M sodium citrate, pH 5.3. This enzyme is capable of cleaving all naturally occurring carboxyl-terminal amino acid residues, including proline (Tschesche and Kupfer, 1972).

In some experiments it was necessary to inactivate trypsin dissociated from enzyme–inhibitor complexes in order to examine the components of the complex in the absence of further cleavage by the trypsin which was released. Phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$)[1] (Sigma Chemical Co., St. Louis, Mo.) in isopropyl alcohol was added to achieve a final $PhCH_2SO_2F$ concentration of 1 mM. In experiments involving 4 h of incubation, one-half of the $PhCH_2SO_2F$ was added initially and the other half was added after 2 h.

Enzyme Assays. Enzyme reaction rates used for determining trypsin-inhibitory capacity were measured with a Radiometer pH Stat (Copenhagen, Denmark) by the procedure of Walsh and Wilcox (1970). The temperature was controlled with a water jacket and the titrant was 0.1 N sodium hydroxide. The substrate used to measure catalytic rates of trypsin was *p*-tosyl-L-arginine methyl ester (TAME) (Calbiochem, San Diego, Calif.) (Hummel, 1959). The TAME was dissolved in 0.01 M Tris-HCl at pH 7.75, with 0.1 M potassium chloride and 0.05 M calcium chloride. The enzyme and α-1-antitrypsin were preincubated for 10 min at 25 °C and added in 0.1-mL aliquots. The amount of substrate hydrolyzed was recorded for 3 to 5 min.

The active sites of trypsin were determined using *p*-nitrophenyl-*p*-guanidinobenzoate hydrochloride (Nutritional Biochemicals Co., Cleveland, Ohio) (Chase and Shaw, 1970). The specific activity of carboxypeptidase B, assayed spectrophotometrically at 254 nm with hippuryl-L-arginine as substrate (Folk et al., 1960), was 163.3 units/mg (one unit = one μmol of substrate hydrolyzed per min).

Purification of α-1-Antitrypsin. α-1-Antitrypsin was purified by a modification of the previously described method (Cohen and Fallat, 1974). The acid alcohol precipitation step was changed to 50–75% ammonium sulfate fractionation. Ion-exchange chromatography was performed on 1-(diethylaminoethyl)-Sephadex A-50 by equilibrium chromatography as previously described, except that the column used had bed dimensions of 5 × 50 cm. The preparative disc electrophoresis step was carried out with a 6-cm column of polyacrylamide. When the preparation was not pure, a final chromatography step on Sephadex G-150 (2 × 100 cm) was executed. The inhibitor was immunologically monospecific on immunoelectrophoresis and had a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and on analytical disc gel electrophoresis at pH 8.2. It showed no precipitin band with antisera to orosomucoid, antithrombin III, antichymotrypsin, prealbumin, or albumin by double immunodiffusion analysis. Amino acid analysis was consistent with published data (Crawford, 1973; Kress and Laskowski, 1973). The only amino-terminal residue found was glutamic acid or glutamine (Glx). The molecular weight was 54 000, as determined on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The α-1-antitrypsin concentration was determined by absorbance at 280 nm ($E_{1\ cm}^{1\%} = 5.0$) (Schultze and Heremans, 1966).

Identification of Amino-Terminal Amino Acids. Amino-terminal amino acids were identified by the method of Woods and Wang (1967). All residues were proven by demonstrating that addition of standard dansylated amino acid gave no additional spot and addition of standards of neighboring residues gave additional spots.

Polyacrylamide Gel Electrophoresis. Analytic polyacrylamide disc gel electrophoresis was carried out in 7.5% acrylamide at pH 8.3 (Ornstein, 1964), and in sodium dodecyl sulfate, 8.3% acrylamide, for determination of molecular weight (Shapiro et al., 1967; Hokin et al., 1973).

Immunologic Identification of Constituents of Protein Bands in Polyacrylamide Gels. The α-1-antitrypsin in polyacrylamide gels was identified by Ouchterlony double immunodiffusion (Ouchterlony, 1967). Special Noble agar (Difco Laboratories, Detroit, Mich.) at 1% concentration was poured onto 3.25 × 4 in. glass plates. Unstained gels were cut into 2-mm segments, which were placed into wells in circular arrangement in the agar. Melted agar was added to form a seal between the polyacrylamide and the agar. Specific antiserum to α-1-antitrypsin was placed in the center wells. Immunoprecipitin lines were not formed until up to 5 days when polyacrylamide gels with sodium dodecyl sulfate were analyzed and compared to a maximum of 48 h for gels without sodium dodecyl sulfate. Negative controls excluded nonspecific precipitin lines caused by sodium dodecyl sulfate. In order to correlate an antigen identified in these plates to a band in the stained control gels, corrections were made for gel shrinkage during fixation.

Trypsin Iodination. Trypsin was labeled with ^{125}I (Amersham/Searle, Chicago, Ill.) by the chloramine-T method (Hunter and Greenwood, 1962) and the isotope was counted in a Nuclear-Chicago γ spectrometer (Amersham/Searle). Unbound ^{125}I was removed by passing the labeled protein through a 3-mL column containing Dowex 1 × 2 equilibrated with 0.9% NaCl.

To limit autolysis, the labeled trypsin was cooled to 4 °C and passed through a column of Sephadex G-100 in 1 mM HCl. The trypsin-bound radioactivity was 98% precipitable in 4.5% sulfosalicylic acid.

Amino Acid Analysis. Analysis of amino acids was performed in an automated amino acid analyzer (HP-119; Beckman-Spinco, Fullerton, Calif.) by the method of Moore and Stein (1963). Half-cystine was determined on samples oxidized with performic acid (Moore, 1963), and tryptophan

[1] Abbreviations used: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; TAME, *p*-tosyl-L-arginine methyl ester; Glx, glutamic acid or glutamine.

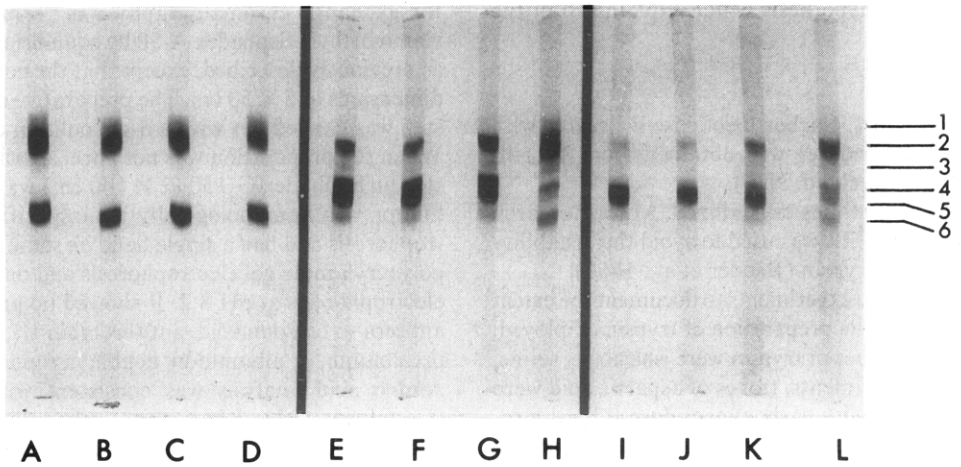


FIGURE 1: Effect of varying times and molar ratios of inhibitor to enzyme as examined with polyacrylamide gel electrophoresis. Solutions of inhibitor and enzyme (20 μg of total protein) were mixed and incubated at 37 °C for 1 min, 20 min, 4 h, and 24 h, from right to left, respectively, for each set of four gels. Migration is from the top (cathode) to bottom (anode). All gels shown were electrophoresed at the same time. The molar ratio of α-1-antitrypsin to trypsin was 1.5:1 (gels A–D), 1:1.5 (gels E–H), and 1:4 (gels I–L). Gels were run in duplicate to provide unstained gels for immunologic identification of α-1-antitrypsin. The stained gels were sectioned at 2-mm intervals and counted in a γ radiation spectrometer.

was determined by the method of Hugli and Moore (1972). Values for serine and threonine were determined by extrapolating to zero time from 24-, 48-, and 72-h hydrolysates.

To determine the residues released by carboxypeptidases B and C, the protein solutions were deproteinated with 4.5% sulfosalicyclic acid and the lyophilized supernatant was used for amino acid analysis. Control experiments indicated that the sulfosalicylic acid did not significantly alter the quantitation or position of eluted amino acids.

Gel Filtration Chromatography. Complexes and released peptides were isolated by gel filtration chromatography on 2 columns (1.5 × 100 cm) in series containing Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.) in an ammonium acetate buffer, 0.1 M, at pH 6.5. In some experiments, complexes were isolated on a 0.9 × 167 cm column containing Sephadex G-100 in 0.1 M Tris-HCl, pH 7.6, with 8 M urea.

^{35}S-Labeled $PhCH_2SO_2F$. $PhCH_2{}^{35}SO_2F$ was purchased from Amersham/Searle (Chicago, Ill.). Polyacrylamide gel slices containing the labeled inhibitor were solubilized in 30% hydrogen peroxide and Aquasol (New England Nuclear, Boston, Mass.) was added. Radioactivity was measured with an Isocap 300 scintillation spectrometer (Searle Analytical, Cherry Hill, N.J.).

Results

Polyacrylamide Gel Electrophoresis. The effects of different molar ratios of α-1-antitrypsin to trypsin and varying incubation times, as analyzed by polyacrylamide gel electrophoresis, are shown in Figure 1. In this system trypsin migrates into the cathodal buffer chamber. α-1-Antitrypsin was incubated with trypsin in a molar ratio of 1.5:1 (gels A–D). The component designated as band 1 formed within 1 min, was stable for 24 h, and exhibited radioactivity from ^{125}I-labeled trypsin. Bands 1 and 6 reacted in double immunodiffusion analysis with antiserum to α-1-antitrypsin. Therefore, band 1 is the complex which forms between the enzyme and inhibitor and band 6 is native α-1-antitrypsin. When α-1-antitrypsin was incubated with excess trypsin in a molar ratio of 1:1.5 (gels E–H), four new bands were seen. Band 2 is suggested by the thickening of band 1, gel H, and by other experiments, e.g., Figure 2. Bands 2 and 3 developed by 1 min (gel H), along with a strong band at position 4 and a weaker band at position 5. Band 5 increased in intensity with continued incubation, while band 1 disappeared. Radioactivity was detected in bands 1, 2, and 4. Immunologic evidence of α-1-antitrypsin was obtained for bands 1, 2, 4, 5, and 6. The origin of band 3 was not defined by these methods. Therefore, the complex (band 1) is progressively degraded at enzyme excess. When α-1-antitrypsin was incubated with a fourfold molar excess of trypsin (gels I–L), band 1 was not seen, band 3 was transient, and bands 4 and 5 developed immediately. After 24 h of incubation (gel I), band 4 was absent and little was left other than band 5. Band 5 in gel I reacted with antiserum to α-1-antitrypsin but contained no radioactivity. Therefore, band 4 appears to be a partially degraded complex and band 5 a fragment of α-1-antitrypsin which has separated from the trypsin.

When a solution containing α-1-antitrypsin in 50% molar excess over trypsin (a molar ratio of 1.5:1) was chromatographed on Sephadex G-100, the first peak eluted contained material that reacted with antiserum to α-1-antitrypsin and it contained 85% of the radioactivity in the starting material. Polyacrylamide gel electrophoresis of the first peak eluted revealed the complex observed on polyacrylamide gel electrophoresis in Figure 1 (band 1). This peak, which had an elution volume corresponding to a molecular weight of 75 000 ± 15%, was lyophilized and prepared for amino acid analysis (Table I). Comparison of the results with the predicted amino acid ratios for 1:1 and 1:2 molar binding ratios of α-1-antitrypsin to trypsin indicated that observed molar ratios of amino acids in the complex were closer to 1:1 than 1:2 for 17 of the 18 measured amino acids. The alanine ratios differed in the second decimal place, within the range of error inherent in the method. These data indicate that, even in aqueous solutions, the α-1-antitrypsin–trypsin complex represents a 1:1 molar combination. Complexes isolated in this manner were not stable for prolonged periods. Therefore, in studies which required that complexes remain intact during several subsequent manipulations, such as for dansylation of the amino terminal residues, Sephadex G-100 columns were equilibrated with buffers containing 8 M urea and similar buffers were utilized to elute the complex (see below).

Separation of Components of the Complex at High pH. If the α-1-antitrypsin–trypsin complex is an acyl or tetrahedral intermediate, a mild nucleophilic attack on the complex might be expected to break the bond between the enzyme and inhibitor. Therefore, solutions containing a 50% molar excess of α-1-antitrypsin over trypsin were incubated at 26 °C for 10 min at pH 7.6, the pH was raised to 9.5 with potassium hy-

TABLE I: Amino Acid Composition of Isolated Complex.

	(Mol of each amino acid/ total amino acids per mol of complex) × 100[a]			
	Measured	Calcd 1:1 molar[b]	Calcd 1:2 molar[b]	Actual molar ratio[c]
Asp	11.03	10.85	10.65	1:1[b]
Thr	6.76	6.20	5.79	1:1
Ser	8.00	8.84	10.42	1:1
Glu	11.87	11.32	10.07	1:1
Pro	4.20	4.19	4.17	1:1
Gly	7.30	7.91	8.80	1:1
Ala	6.60	6.67	6.60	1:2[d]
½-Cys	1.74	2.17	3.24	1:1
Val	5.85	6.82	7.06	1:1
Met	1.65	1.55	1.39	1:1
Ile	4.70	4.80	5.32	1:1
Leu	10.50	9.92	9.03	1:1
Tyr	2.66	2.48	3.00	1:1
Phe	5.24	4.34	3.59	1:1
His	2.59	2.48	2.20	1:1
Lys	7.52	7.44	7.17	1:1
Arg	1.90	1.70	1.50	1:1

[a] % of each amino acid/mole of complex was calculated from amino acid analysis of α-1-antitrypsin and trypsin (trypsin not measured), assuming 1:1 or 1:2 molar ratio of α-1-antitrypsin to trypsin. [b] Moles of α-1-antitrypsin/mole of trypsin in the complex. [c] The ratio of moles of α-1-antitrypsin/mole of trypsin in the complex was determined by comparing measured with calculated values. [d] While the measured value is closer to the predicted value for a 1:2 complex, the differences are within the limits of the experimental methods.

TABLE II: Summary of Identity of Bands from Electropherograms.

Figure	Trypsin (contains ^{125}I)	Trypsin and α-1-antitrypsin	α-1-Antitrypsin (precipitated with antisera to α-1-antitrypsin but had no ratioact.)	Unknown
1		1, 2, 4[a]	5, 6[a]	3[a]
2		1, 2, 4[a]	5, 6[a]	3[a]
3	23 000[b]	73 000[b]	54 000[b]	
		62 000[b]	50 000[b]	

[a] Band designations on figures. [b] Daltons.

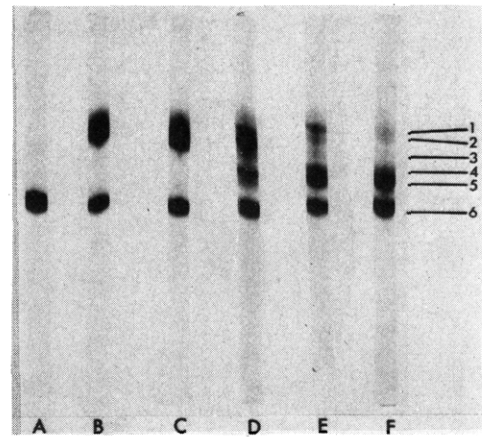


FIGURE 2: Effect of high pH on the α-1-antitrypsin–trypsin complex as examined with polyacrylamide gel electrophoresis. Solutions of α-1-antitrypsin and trypsin in 1.5:1 molar ratio were incubated at 26 °C for 10 min and then at 37 °C, pH 9.5, for the specified times and 20 μg of protein was added to each gel. Gel A contained α-1-antitrypsin alone. Gels B–F contained solutions incubated for 1 min, 20 min, 1 h, 2 h, and 4 h, from left to right, respectively.

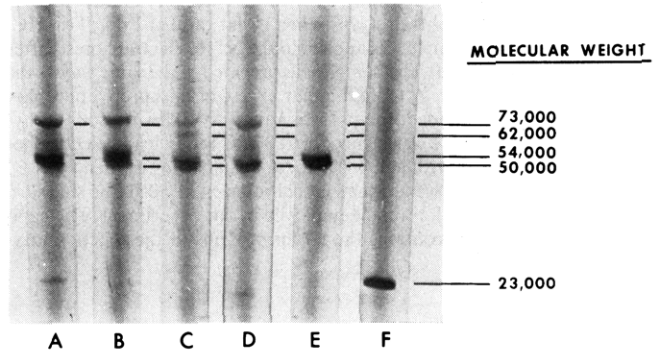


FIGURE 3: Electropherograms of α-1-antitrypsin and trypsin in polyacrylamide gels with sodium dodecyl sulfate. All gels contained a 50% molar excess of α-1-antitrypsin over trypsin and 30 μg of protein. All samples were incubated at 25 °C for 10 min. $PhCH_2SO_2F$ was added to samples A and B, then potassium hydroxide was added to samples B and C, and incubation was continued for 4 h before electrophoresis. Gel D represents the α-1-antitrypsin–trypsin reaction at neutral pH. Gel E contained α-1-antitrypsin alone and gel F contained trypsin alone.

droxide, the temperature was raised to 37 °C, and incubation was continued for varied times. These conditions do not change the electrophoretic pattern of α-1-antitrypsin in the absence of trypsin. Polyacrylamide gel patterns representing the results are shown in Figure 2. Gel A contained α-1-antitrypsin alone. Gels B–F represent incubation times of 0.33, 0.5, 1.0, 2.0, and 4.0 h, respectively, before electrophoresis. The changing pattern which developed in the presence of potassium hydroxide is identical with that which developed when α-1-antitrypsin was combined with a molar excess of trypsin (Figure 1). These data suggest that free, active trypsin is liberated by incubation of the complex with a low concentration of potassium hydroxide. Similar results have been produced by incubation with ammonium hydroxide at pH 9.5 (gels not shown). On return of the solution to pH 7.6, trypsin esterolytic activity was observed.

The effects of potassium hydroxide and $PhCH_2SO_2F$ on the complex were studied by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 3). The control samples, α-1-antitrypsin alone and trypsin alone, are shown in gels E and F, respectively. In the presence of a 50% molar excess of α-1-antitrypsin at neutral pH, a single complex of 73 000 daltons was seen (gel D). When the solution was incubated for 4 h with potassium hydroxide (gel C) as described above, a 62 000-dalton band formed which contained both α-1-antitrypsin (by immunodiffusion analysis) and trypsin (by radioactivity measurement). This band may correspond to a degraded complex (band 2 or 4, Figure 1) (see Table II). In addition, a protein band of 50 000 daltons was seen. This band reacted with antiserum to α-1-antitrypsin and it is the α-1-antitrypsin fragment liberated when the enzyme–inhibitor complex is split by potassium hydroxide (Table II). When $PhCH_2SO_2F$ was added to the solution (gel B), the 62 000-dalton complex failed to form but the 50 000-dalton component did form, indicating that $PhCH_2SO_4F$ effectively prevented further hydrolysis by the released trypsin. The absence of esterolytic activity was confirmed in the pH stat. The pattern of protein bands formed in the presence of $PhCH_2SO_2F$ alone (gel A) did not differ from the control pattern (gel D).

After incubation of the complex at pH 9.5 for 24 h, in the absence of $PhCH_2SO_2F$, trypsin activity could again be measured. Earlier times were not examined for tryptic activity,

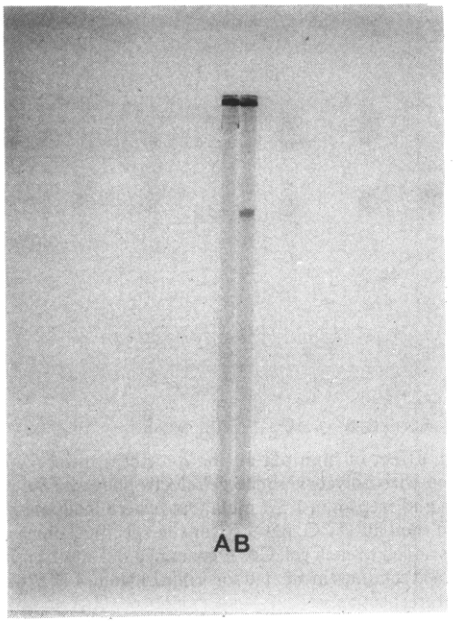


FIGURE 4: Electropherograms of the products of complex formation and destruction in sodium dodecyl sulfate with 10% polyacrylamide. (Gel A) Trypsin was added to α-1-antitrypsin in a molar ratio of 1:4 in Tris-HCl buffer, pH 7.6. The sample was applied to the gel for electrophoresis after 10 min at 26 °C. (Gel B) This sample was prepared in the same way as the sample shown in gel A. A tenfold molar excess of diisopropyl phosphofluoridate was then added, the pH was raised to 10.0 with sodium hydroxide, and the sample was incubated at 37 °C for 72 h and electrophoresed simultaneously with gel A. The peptide band (lowest band, gel B) had a lower molecular weight than cytochrome *c* (11 500), the standard with the lowest molecular weight employed in this experiment. The band at the top of the gels contains the larger proteins in the reaction mixture.

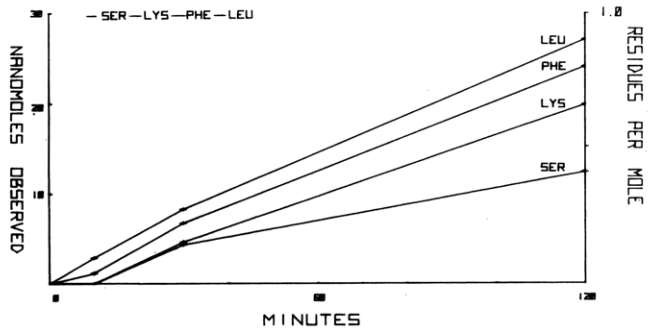


FIGURE 5: Carboxypeptidase C digestion of α-1-antitrypsin. Quantitative release of amino acids during digestion of native α-1-antitrypsin by carboxypeptidase C was measured at different intervals. Details of the methods are provided in the text.

but tryptic activity has been observed after 4 h of incubation at high pH. (See the discussion of Figure 2, above.)

The large fragment of α-1-antitrypsin which has been removed from the complex has clearly lost a small peptide. In order to determine if the peptide was lost during formation of the complex or during subsequent spontaneous or base-catalyzed splitting of the bond between the enzyme and the inhibitor, the following experiment was carried out. α-1-Antitrypsin was added to trypsin in a 4:1 molar ratio. Diisopropyl phosphofluoridate was added after 10 min of incubation at 26 °C at pH 7.6. The pH was raised to 10 for 72 h at 37 °C. A control sample was prepared by adding α-1-antitrypsin to trypsin in 4:1 molar ratio and incubating the solution for 10 min at 26 °C at pH 7.6. Then both samples were electrophoresed in 10% polyacrylamide gels with a ratio of acrylamide to *N,N'*-methylenebisacrylamide of 10:1 and with sodium dodecyl sulfate. The results (Figure 4) show that the small peptide is released during the splitting of the complex and not during the process of formation of the complex.

Amino-Terminal and Carboxyl-Terminal Residues of α-1-Antitrypsin. In order to evaluate the ensuing experiments it was necessary to have a quantitative knowledge of the carboxyl-terminal arginyl and lysyl residues of the α-1-antitrypsin and trypsin preparations and a qualitative knowledge of the amino-terminal residues on both proteins. Data for trypsin is presented under Experimental Procedure. The amino-terminal residue of α-1-antitrypsin was Glx, as determined by the dansyl method. Since the carboxyl-terminal residue on α-1-antitrypsin was controversial (Chan et al., 1976; Horng and Gan, 1974; Johnson and Travis, 1976), we determined the carboxyl-terminal sequence of α-1-antitrypsin with carboxypeptidase C by the method of Tschesche and Kupfer (1972). Briefly, 6.4 mg of α-1-antitrypsin in 0.05 M sodium citrate buffer, pH 5.3, was added to 0.21 mg of carboxypeptidase C in the same buffer (100:1 molar ratio, substrate:enzyme). At selected times aliquots equivalent to 30 nmol of α-1-antitrypsin were removed, mixed with 0.7 volume of 0.2 N HCl and frozen in an ethanol–dry ice bath. When the experiment was completed, all samples were heated at 80 °C for 2 min and the protein was precipitated with sulfosalicylic acid. After 4 h the precipitates were removed by centrifugation and the supernatants were passed through a Millipore filter (No. HAWP 01300). The results of amino acid analysis of the filtrates are shown in Figure 5. These data show that the carboxy-terminal sequence is Ser-Lys-Phe-Leu. The first and second residues agree with the carboxyl-terminal dipeptide determined by Horng and Gan (1974) and by Chan and his colleagues (1976). While the explanation for the differences between the carboxyl-terminal dipeptide found on α-1-antitrypsin by Johnson and Travis (1976) and those established by the present data is not clear, it is possible that the use of outdated plasma may permit the loss of the carboxyl-terminal dipeptide by a dipeptidase such as angiotensin I converting enzyme (Erdos, 1977). Alternatively, the differences may reflect phenotypic variants.

Proof of New Carboxyl-Terminal Residue Produced by Trypsin. Since potassium hydroxide was shown to split the enzyme–inhibitor complex and since the α-1-antitrypsin component (50 000-dalton band, Figure 3) liberated was lower in molecular weight than native α-1-antitrypsin, it seemed possible that the α-1-antitrypsin–trypsin complex is an acyl or tetrahedral intermediate of the enzyme involving linkage of trypsin to α-1-antitrypsin through an arginyl or lysyl residue, which, in either case, would become a new carboxyl-terminal arginyl or lysyl residue of the amino-terminal peptide of α-1-antitrypsin after base-catalyzed hydrolysis. To test this possibility, α-1-antitrypsin, 50 mg, was combined in 0.1 M ammonium acetate buffer, pH 6.5, with 5.0 mg of active trypsin in 1 mM HCl. The solutions were thereafter monitored in the pH stat to assess the absence of trypsin esterolytic activity. A control sample was prepared similarly. $PhCH_2SO_2F$ was added to treated and control samples to prevent extraneous cleavages by freed trypsin. In the treated sample the pH was raised to 10 with 1% ammonium hydroxide, and the resultant solution was incubated at 37 °C for 4 h. Distilled water was added to the control sample instead of ammonium hydroxide. Otherwise, the two aliquots were treated in the same manner. The pH was returned to 6.5 in the treated sample and the volume of the control sample was equalized with distilled water. Carboxypeptidase B, 40 μg, was added to both treated and control samples, followed by incubation at 37 °C for 1 h. The samples were deproteinated with sulfosalicylic acid and

TABLE III: Basic Amino Acids Removed with Carboxypeptidase B[a] from Intact (Control) and Alkali-Split OH^- Treated α-1-Antitrypsin–Trypsin Complex.

	Lysine (nmol)[b]	Arginine (nmol)
OH^- treated	233[c]	4.0
(SE)	(15.59)	(0.248)
Control	103[c]	4.3
(SE)	(6.2)	(0.505)

[a] $N = 4$. [b]Nanomoles released in each experiment. [c] $p < 0.001$ by Student's t test (unpaired). Lysine (treated) − lysine (control) = 130 ± 4.7 (SE). Therefore, 65% of the 200 nmol of complex was split. The background lysines and arginines are derived from autolytic cleavages in trypsin (see Experimental Procedure).

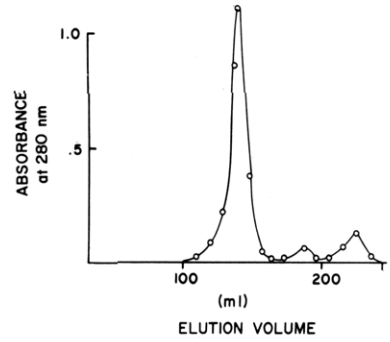


FIGURE 6: Chromatograph of α-1-antitrypsin–trypsin reaction mixture separated on a column of Sephadex G-100 with 8 M urea. (See text for details.)

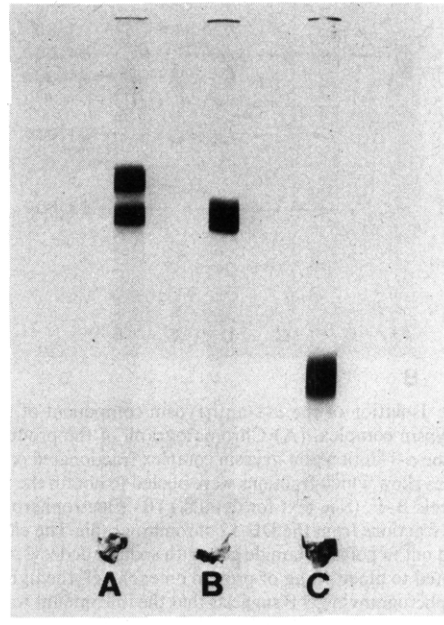


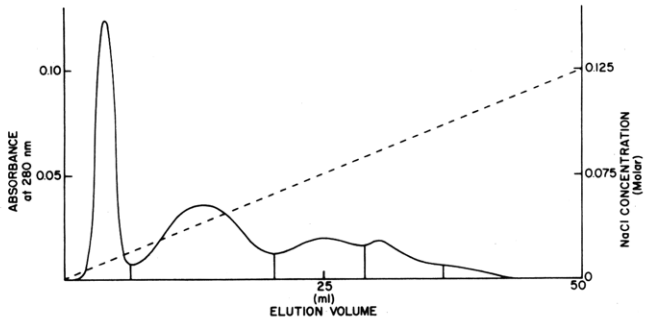
FIGURE 7: Electropherograms of control polyacrylamide gels with sodium dodecyl sulfate for determination of amino-terminal residues. Gel A contained α-1-antitrypsin, 50 μg, and trypsin, 14 μg; gel B contained α-1-antitrypsin, 50 μg; and gel C contained trypsin, 34 μg. Bands were identified by comparing them with stained gels and trypsin-containing bands were further identified by examining gel slices for the presence of the ^{125}I.

transferred to the amino acid analyzer. The results are shown in Table III. In four experiments there were significantly more lysyl residues removed from the split complex than from the intact complex. In addition, if the lysine residues from the control sample are subtracted from those of the treated samples, an average of 130 ± 9.4 (SD) additional residues of lysine were released from the split complex. If 1 nmol of lysine were released for every nmol of trypsin added, then 200 nmol of lysine would have been measured. Therefore, an average of 65% of the predicted number of lysines was recovered. It can be seen in Figure 3 (gels B and C) that not all of the complex is split in 4 h and that the predicted number of lysines is therefore even closer to the observed values. These data show that one new carboxyl-terminal lysine is formed per mole of complex split at high pH.

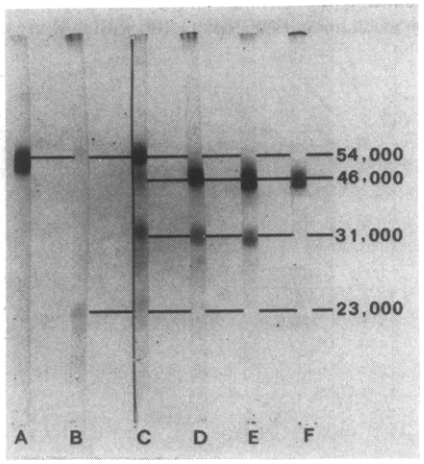
Amino-Terminal Residues on the α-1-Antitrypsin–Trypsin Complex. The amino-terminal amino acids were identified on complexes in two different systems. In the first experiment, 7.0 mg of trypsin in 1 mM HCl was added to α-1-antitrypsin, 22.76 mg, in 0.1 M Tris-HCl buffer, pH 7.6, at 26 °C. After 10 min, diisopropyl phosphofluoridate (5.6×10^{-7} M final concentration) was added and the reaction was held in an ice bath until application to the column. The solution was chromatographed on a column of Sephadex G-100, 1.6 × 167 cm. The buffer employed was 0.1 M Tris-HCl, pH 7.6, containing 8 M urea. Elution was carried out at 6 mL/h. The elution profile is shown in Figure 6. Immunologic and isotopic analysis of the solutions showed that the first peak contained the complex, the second peak contained α-1-antitrypsin and the third peak contained inactive trypsin. No smaller peptides were eluted from the column. Amino-terminal analysis of peak I showed isoleucine, serine, valine, and glutamic acid. Addition of dansylated threonine to the polyamide sheets resulted in a new spot, proving that no amino-terminal threonine occurred in the complex.

In the second experiment polyacrylamide gel electrophoresis was performed in sodium dodecyl sulfate. The first six gels each contained α-1-antitrypsin, 50 μg, and trypsin, 14 μg; the second set of six gels contained α-1-antitrypsin, 50 μg; and the last six gels contained trypsin, 34 μg. The first six samples were incubated for 10 min at room temperature in 0.1 M Tris-HCl buffer, pH 7.6, before application to the gels. The gels were fixed with trichloroacetic acid and one gel from each set was stained with Coomassie Blue (Figure 7). An identical gel was frozen. The segments containing the complex, α-1-antitrypsin alone, and trypsin alone were cut from the gels and crushed, then dialyzed intensively with distilled water, and lyophilized, and the resulting material was dansylated for amino-terminal analysis. The results showed that the complex (top band, gel A, Figure 7) contained isoleucine, serine, valine, and glutamic acid as the amino-terminal residues. No threonine was present. α-1-Antitrypsin (gel B) had an amino-terminal glutamic acid, and trypsin (gel C) exhibited isoleucine, serine, and valine.

Isolation of the α-1-Antitrypsin Component of the α-1-Antitrypsin–Trypsin Complex. Trypsin, 2.2 mg, was slowly added with stirring to 6 mg of α-1-antitrypsin in a Tris-HCl buffer, 0.05 M, pH 7.6, containing sodium chloride, 0.3 M. The solution was incubated for 10 min at room temperature, and then sufficient diisopropyl phosphofluoridate was added to make a final concentration of 1.5×10^{-7} M. α-1-Antitrypsin has been shown to have a high affinity for thiol groups (Laurell et al., 1975). In order to remove any unreactive trypsin which might complicate the subsequent analysis of the isolated degradation products, the sample was fractionated on thiol–Sepharose (Pharmacia Fine Chemicals). Dithioerythritol was added to the sample to bring the concentration of this reagent to 0.025 mM. After 1 h at 26 °C, the solution was mixed with thiol–Sepharose equilibrated with the same buffer and mixed end-over-end. The unbound fraction was washed from the gel with 40 mL of starting buffer and the bound complex and unreacted α-1-antitrypsin were eluted with 50 mL of the



A



B

FIGURE 8: Isolation of the α-1-antitrypsin component of the α-1-antitrypsin–trypsin complex. (A) Chromatograph of the products of dissociation of the α-1-antitrypsin–trypsin complex fractionated on DE-52. The vertical lines show which fractions were pooled to obtain the five fractions shown in gels B–F. (See text for details.) (B) Electropherograms of the proteins in fractions from the DE-52 chromatograph. The electrophoresis was carried out in polyacrylamide gels with sodium dodecyl sulfate. While we attempted to place 40 μg of protein on each gel, the light staining of the electropherogram in gel B suggests that the full amount was not applied to this gel. Gel A contained native α-1-antitrypsin alone. Gels B–F contained samples of the proteins in the peaks eluted from the DE-52 column from left to right respectively. (See text for details.)

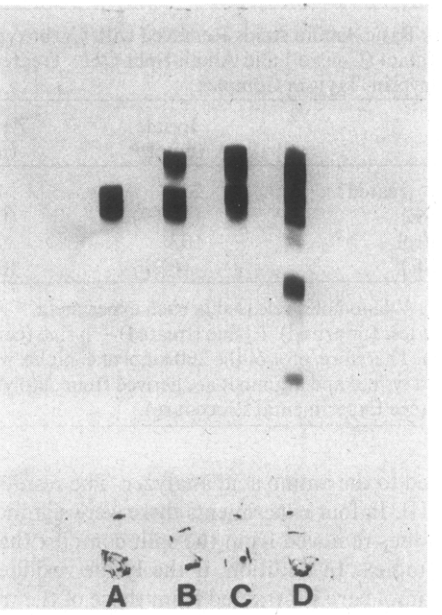


FIGURE 9: Effect of 8 M urea on the stability of the α-1-antitrypsin–trypsin complex. Gel A contained 50 μg of α-1-antitrypsin. Gels B–D contained α-1-antitrypsin, 50 μg, and trypsin, 20 μg. Samples were treated as follows: (B) incubated for 10 min at 26 °C before electrophoresis; (C) sufficient urea added to make the solution 8 M and then incubated for 72 h at 37 °C before electrophoresis; and (D) treated as for C, except that no urea was added.

same buffer containing 5 mM cysteine hydrochloride. Electropherograms on polyacrylamide gels with sodium dodecyl sulfate (not shown) confirmed the removal of unreactive trypsin. The pH of the fraction eluted with cysteine hydrochloride was raised to 10 with 1.0 M potassium hydroxide and urea was added to bring the final concentration to 8 M. The sample was then incubated for 24 h at 37 °C. The sample was dialyzed against a 0.005 M sodium phosphate buffer, pH 6.5, containing sodium chloride, 0.025 M, and urea, 8 M, and chromatographed on a 2.5 × 10 cm DE-52 column equilibrated with the same buffer. The chromatogram was developed with a linear gradient of 0.025 M to 0.125 M sodium chloride. The elution profile is shown in Figure 8A, and the molecular weights of the proteins in the fractions designated by the vertical lines on the chromatogram are shown in the sodium dodecyl sulfate–polyacrylamide gel electropherograms shown in Figure 8B. The 46 000-dalton peptide shown in gel F and eluted in the last peak of the chromatogram reacted with antiserum against α-1-antitrypsin and not to antiserum against trypsin and corresponds within the limits of this method with previous estimations of the molecular weight of the fragment of α-1-antitrypsin removed from the complex at high pH (see Figure 3). Threonine was the only amino-terminal residue demonstrated in this fraction. The first peak was eluted at the position where trypsin has been eluted in other experiments and the presence of trypsin is confirmed by the 23 000-dalton fragment shown in the electropherograms in Figure 8B, gel B. Two other proteins are shown in the electropherograms in Figure 8B, gels B–F. One of these proteins seen in gel C is the excess native α-1-antitrypsin as shown by the control α-1-antitrypsin in gel A. The origin of the 30 000-dalton band shown in gels C, D, and E has not been determined but is close to the molecular weight predicted for the acyl intermediate and may, therefore, represent complexes which have undergone only partial degradation. The addition of 8 M urea before raising the pH seems to favor the stability of this band which was not seen after dissolution of the complex in the absence of urea (Figure 3). We are currently attempting to isolate and further characterize the protein in this band.

Complicating Side Reaction. During isolation of the α-1-antitrypsin–trypsin complex and the search for the new carboxyl-terminal residue, two observations were made which indicated a complicating side reaction of the interaction of the enzyme and inhibitor. When a large amount of the inhibitor (greater than 50 mg) was added to trypsin (5 mg), a small residual catalytic activity toward TAME was observed. In four experiments an average of 0.72% (SD = 0.02) of the initial trypsin hydrolytic activity remained after α-1-antitrypsin was added. The activity was unaffected by adding three- to tenfold molar excess of α-1-antitrypsin, but was inhibited by $PhCH_2SO_2F$ or diisopropyl phosphofluoridate. When ^{35}S-labeled $PhCH_2SO_2F$ was added to α-1-antitrypsin and trypsin in solution (1.5 molar ratio of inhibitor to enzyme) and the reactants were electrophoresed in polyacrylamide gels with or without sodium dodecyl sulfate, subsequent scintillation counting of the solubilized slices of gels showed that the label migrated with the buffer front or with the trypsin and not with the complex. This suggested the presence of a form of trypsin not inhibited by α-1-antitrypsin, rather than a form of α-1-antitrypsin–trypsin complex in which the catalytic site of the enzyme remained available. The second observation which suggested a complicating side reaction was that when α-1-

antitrypsin was incubated with trypsin at pH 7.6 for 72 h or more, multiple new bands, as visualized by gel electrophoresis with sodium dodecyl sulfate, were formed (Figure 9). Most of the deterioration of the complex after 72 h of incubation could be prevented by treating the solution with $PhCH_2SO_2F$, diisopropyl phosphofluoridate or 8 M urea (Figure 9).

In order to exclude the possibility that the trypsin was contaminated, several rabbits were immunized with the trypsin. Each antisera produced a single precipitin arc against the antigen and did not react with porcine elastase or chymotrypsin in double immunodiffusion analysis. The inhibition with $PhCH_2SO_2F$ and diisopropyl phosphofluoridate indicates that the enzyme which is not inhibited by α-1-antitrypsin is a serine esterase. The enzymes which could most likely contaminate such a preparation of trypsin are elastase and chymotrypsin. Both of these enzymes are inhibited by α-1-antitrypsin. These data suggest the possibility that α-1-antitrypsin fails to inhibit a form of trypsin. Pseudotrypsin, a known autolytic product of bovine pancreatic trypsin with the Lys-176–Asp-177 bond hydrolyzed (Smith and Shaw, 1969), is the most likely possibility, since it has lost most of its reactivity toward positively charged residues and evidence presented herein suggests that trypsin attacks α-1-antitrypsin at a lysyl residue. However, it is not known whether pseudotrypsin is present in porcine pancreatic trypsin preparations and a firm conclusion in this regard would require the definitive isolation of an enzyme which fails to interact with α-1-antitrypsin.

Discussion

Stoichiometry of the α-1-Antitrypsin–Trypsin Complex. Several investigators have found a 1:1 molar combination of α-1-antitrypsin with trypsin by kinetic measurements (Moroi and Yamasaki, 1974; Cohen, 1973; Crawford, 1973; Kress and Laskowski, 1973). Johnson and co-workers (1974) reported a 1:2 molar combination. In the present experiments the amino acid composition of the isolated complexes of α-1-antitrypsin and trypsin corresponds to predicted values for a 1:1 molar combination.

Mechanism of Action of α-1-Antitrypsin. These experiments have shown that there is one complex which forms between α-1-antitrypsin and trypsin. The complex was calculated to have a slightly lower molecular weight than that predicted for a 1:1 molar ratio of enzyme and inhibitor, but the molecular weight may equal a 1:1 combination complex since the sodium dodecyl sulfate–polyacrylamide gels are only accurate for molecular weight determination to ±10% (Weber and Osborn, 1969). Other estimates of the molecular weight of the complex range from 74 000 (Moroi and Yamasaki, 1974) to 76 000 (Oda et al., 1977). The complex is split into a large fragment of α-1-antitrypsin (46 000–50 000) and active trypsin by treatment with alkali. The α-1-antitrypsin released from the complex has lost a fragment of about 4000–8000. These estimates correspond within the limits of the methods to values in published reports (Moroi and Yamasaki, 1974; Johnson and Travis, 1976). The amino-terminal residues on the intact complex are identical with those on trypsin and α-1-antitrypsin. However, the large peptide of α-1-antitrypsin released from the complex has an amino-terminal threonine, proving that a peptide bond was broken during alkaline hydrolysis. The small peptide released has not yet been isolated and characterized. An additional protein of about 30 000 was observed after partial degradation of the complex and may be the acyl intermediate.

When the α-1-antitrypsin and trypsin in the complex are separated at high pH under conditions which block the freed trypsin, a new carboxyl-terminal lysine becomes available for removal by carboxypeptidase B. These observations indicate that a Lys-Thr bond is broken during base-catalyzed disruption of the complex.

In our earlier studies (Cohen, 1973) it was suggested that enzymes might combine with α-1-antitrypsin, as with other substrates; however, one of the intermediate complexes known to occur during the interaction of trypsin with its substrates is stable. Investigators subsequently have proposed two opposing hypotheses. Moroi and Yamasaki (1974) found that the α-1-antitrypsin–trypsin complex had the same amino-terminal residues as the constituent proteins, that the complex could be separated with hydrazine at high pH, and that the α-1-antitrypsin released from the complex had a lower molecular weight than native α-1-antitrypsin. They concluded that the α-1-antitrypsin–trypsin complex was an acyl intermediate of trypsin, bound through a new carboxyl-terminal residue which would probably be an arginine or lysine in the inhibitor.

Johnson and Travis (1976) have found that, when the α-1-antitrypsin was released from the complex with the nucleophile, benzamidine hydrochloride, the α-1-antitrypsin released from the complex had an amino-terminal threonine residue and a carboxyl-terminal residue which was the same as that of the α-1-antitrypsin in the native inhibitor preparation utilized. It was concluded that the trypsin cleaves α-1-antitrypsin at a threonine bond near the amino-terminal end to activate the inhibitor and then becomes bound by an unknown mechanism to a carboxyl group at a different site on α-1-antitrypsin.

We have reexamined these apparently contradictory data and have found that the basic findings from both investigations are correct. In addition, we have obtained quantitative and qualitative evidence that a bond is cleaved during base-catalyzed hydrolysis of the complex and that a new carboxyl-terminal lysine residue is formed during the splitting of the complex with high pH. Furthermore, by using Sephadex columns with 8 M urea in the buffers, we have been able to prevent the breakdown of the complex during its separation from free α-1-antitrypsin and inactive trypsin. There is no evidence of a peptide with less than the molecular weight of trypsin in the eluate from the column. However, a small peptide is formed during breakdown of the complex at high pH. In addition, in recent investigations we have shown, when the splitting of the complex is catalyzed by $^{18}OH^-$ labeled base instead of hydrazine, that the new carboxyl-terminal lysyl residue becomes labeled with ^{18}O (Cohen et al., 1978). These studies conform to the known distribution of oxygens which occurs during the base-catalyzed hydrolysis of acyl esters (Polanyi and Szabo, 1934; Bender, 1951; Bender and Thomas, 1961) and which would be likely to occur if the α-1-antitrypsin–trypsin complex were an acyl ester or if an acyl intermediate formed during the base-catalyzed hydrolysis of a tetrahedral adduct.

Based on our investigations, we conclude that there is a hypothesis which is strongly supported by all of the available evidence. Trypsin binds to a lysyl group in α-1-antitrypsin at a Lys-Thr bond near the amino-terminal end of the inhibitor. The bond is probably analogous to that usually formed transiently between trypsin and its substrates (Bender, 1964); however, as with other inhibitors in nature, the bond is stable (Tschesche, 1974). Since the amino-terminal peptide of α-1-antitrypsin is still bound in the complex and there are apparently no intrachain disulfide bonds in α-1-antitrypsin, a tetrahedral complex may be more likely.

Acknowledgments

We wish to express our appreciation for the expert technical assistance of Mr. Neil Munderloh.

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