Trentham, D. R. (1968), *Biochem. J. 109,* 603.
Twu, J. S., Chin, C. C. Q., and Wold, F. (1973), *Biochemistry 12,* 2856.
Vallee, B. L., and Hoch, F. L. (1955), *Proc. Natl. Acad. Sci. U.S.A. 41,* 327.
Vanaman, T. C., and Stark, G. R. (1970), *J. Biol. Chem. 245,* 3565.
Velick, S. F. (1953), *J. Biol. Chem. 203,* 563.
Virtanen, P. O. I., and Korhonen, R. (1973), *Acta Chem. Scand. 27,* 2650.
Viscidi, E., Consiglio, E., and Roche, J. (1966), *Biochim. Biophys. Acta 121,* 424.

Two Human Trypsinogens. Purification, Molecular Properties, and N-Terminal Sequences†

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ABSTRACT: The two human trypsinogens have been isolated from human pancreatic juice in a sufficient amount to study molecular and structural properties. The purification procedure included filtration on Sephadex G-100 followed by ion-exchange chromatography on DEAE-cellulose. The two trypsinogens represent 19% of total proteins of pancreatic juice. Trypsinogen 1, the major form, is present in a quantity twice that of trypsinogen 2, which is the most anionic protein in human pancreatic juice. The two proteins have partial immunological identity, close molecular weights (23 438 and 25 006 for trypsinogens 1 and 2, respectively) and similar amino acid compositions. The N-terminal sequences are the same for the first 9 residues: Ala-Pro-Phe-Asp_4-Lys-Ile. The two proteins differ in the activation peptides released during the transformation to trypsins. Trypsinogen 2 liberates one octapeptide Ala-Pro-Phe-Asp_4-Lys while trypsinogen 1 liberates two peptides, the same octapeptide and the pentapeptide $(Asp)_4$-Lys.

The molecular properties of some human pancreatic proteolytic enzymes—chymotrypsins (Coan et al., 1971; Coan & Travis, 1972), trypsins (Travis & Roberts, 1969; Mallory & Travis, 1973; Feinstein et al., 1974), protease E (Mallory & Travis, 1975), elastases (Largman et al., 1976), and carboxypeptidases A (Peterson et al., 1976) and B (Geokas et al., 1975)—have recently been studied. However, very few zymogens of these enzymes have been isolated because of the difficulty in obtaining nonactivated starting material. We have previously reported the purification of the two human chymotrypsinogens (De Caro et al., 1975) and demonstrated that chymotrypsinogen A, the major form, is the only precursor of the three chymotrypsins isolated from autolyzed pancreas. Human pancreatic juice contains two anionic trypsinogens (Figarella et al., 1969; Clemente et al., 1972). The human trypsins have been extensively studied for their molecular properties and their interactions with proteinase inhibitors (Travis & Roberts, 1969; Mallory & Travis, 1973; Feinstein et al., 1974; Feeney et al., 1969). However, our studies of the inhibition spectra of these enzymes derived from the purified trypsinogens (Figarella et al., 1975) have pointed out some differences in the properties of trypsins obtained by controlled activation and trypsins prepared from activated material. These results demonstrate the importance of the isolation of zymogens. In addition, since trypsinogen activation seems to play an important part in pancreatitis (Ohlsson & Tegner, 1973), the study of human trypsinogens may be of clinical significance.

We have previously characterized the activation peptide of trypsinogen 2, the most anionic trypsinogen (Guy et al., 1976). In this paper, we report the purification and some molecular properties of the two human trypsinogens and we discuss the activation peptides of trypsinogen 1.

Materials and Methods

Materials

Human pancreatic juice was collected by catheterization of the pancreatic duct from patients with normal pancreatic function. Samples devoid of free proteolytic activity were lyophilized and stored at −20 °C.

Porcine enterokinase was kindly supplied by Dr. S. Maroux and pancreatic trypsin inhibitor (Kunitz) was a gift of Dr. E. Sache (Laboratoire Choay).

Trypsin inhibitor–Sepharose was prepared by activating Sepharose 4B (Pharmacia) with cyanogen bromide and coupling activated Sepharose with Kunitz pancreatic trypsin inhibitor according to the procedure of Cuatrecasas et al. (1968).

Methods

Column Chromatography. All chromatographic procedures of enzyme purification were carried out at 4 °C and performed in buffers containing 1 mM benzamidine to prevent the activation.

Protein concentration was determined by measuring the absorbance at 280 nm, using a mean extinction coefficient $E_{1cm}^{1\%} = 20.0$.

Trypsin Activation and Trypsin Activity. (a) Routine Activation in Chromatographic Fractions. Fractions containing trypsinogen were activated with porcine enterokinase (1% by

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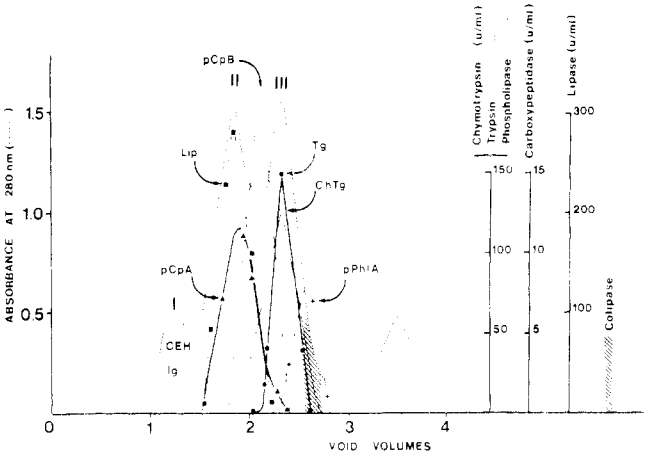

FIGURE 1: Gel filtration of human pancreatic juice on Sephadex G-100. The column (2 × 120 cm) was equilibrated and developed with 500 mM NaCl, 1 mM benzamidine, 10 mM Tris-HCl buffer (pH 8.0). Sample: 120 mg of protein dissolved in 15 mL of buffer. Ig, immunoglobulin; CEH, carboxylic ester hydrolase; pCp, procarboxypeptidase; Lip, lipase; pPhl, prophospholipase; Tg, trypsinogen; ChTg, chymotrypsinogen.

weight) in the column buffer 1 mM in benzamidine in the presence of 20 mM $CaCl_2$ at 0 °C for 18 h.

(b) Activation for the Isolation of the Activation Peptides. Trypsinogen (0.5 μmol) was activated autocatalytically by dissolving the protein (3 mg/mL) in a 5 mM Tris-HCl buffer (pH 7.8) containing 20 mM $CaCl_2$ and 40 mM NaCl. In some experiments the activation was performed at pH 5.6 in presence of enterokinase (0.5 to 1%) and Ca^{2+} (20 mM). The activation was carried out until a plateau level was reached.

(c) Trypsin activity was measured potentiometrically in a pH-stat at pH 7.8 at 25 °C in a 5 mM Tris-HCl buffer containing 100 mM KCl, 20 mM $CaCl_2$ using 10 mM TosArg-OMe[1] as substrate.

Other Enzyme Assays. The activation of zymogens present in the chromatographic fractions at pH 8.0 and containing benzamidine was performed by addition of bovine trypsin (50% by weight). Full activation was reached after 3 h at 0 °C for chymotrypsinogen and prophospholipase A and after 30 min at 35 °C for procarboxypeptidases A and B.

Chymotrypsin, carboxypeptidases A and B, lipase and phospholipase activities were assayed as reported before (De Caro et al., 1975; Figarella et al., 1975). Colipase was measured by the increase in enzyme activity of a sample of human lipase devoid of colipase (Rathelot et al., 1975). Carboxylic ester hydrolase activity was tested spectrophotometrically at 400 nm and 25 °C using as substrate *p*-nitrophenyl acetate (Erlanson, 1970).

Molecular weight determinations were performed by poly(acrylamide) gel electrophoresis in sodium dodecyl sulfate according to the technique of Weber & Osborn (1969) with the modifications described elsewhere (De Caro et al., 1975). The reference proteins were horse cytochrome *c* (molecular weight, 12 400), soybean trypsin inhibitor (21 500), bovine trypsinogen (24 000), bovine deoxyribonuclease (31 000), yeast alcohol dehydrogenase (38 000), and human albumin (68 000).

Amino Acid Analysis of Proteins and Peptides. Samples containing 10 to 20 nmol of protein were hydrolyzed in evacuated sealed tubes with 1 mL of 6 M HCl at 100 °C for 24, 48, 72 h. Amino acid analyses were performed on duplicate hydrolysates using a JEOL amino acid analyzer. Half-cystine and methionine were determined as cysteic acid and methionine sulfone after performic acid oxidation (Moore, 1963). Tryptophan was determined by the method of Matsubara & Sasaki (1969). Calculated serine and threonine values are extrapolations to zero time of hydrolysis. The glutamine and asparagine contents were determined by amino acid analysis of enzymatic hydrolysates of S-2-aminoethylated protein according to the method of Pubols et al. (1974) on an automatic instrument as modified by Benson et al. (1967). Amino acid compositions of peptides were determined after 18 h hydrolysis at 100 °C with 6 M HCl.

Determination of N-Terminal Sequences. The determination of the N-terminal sequence of protein was made by the phenyl isothiocyanate method in an automatic protein sequenator Socosi Ps100 using the Quadrol protein program. Phenylthiohydantoin amino acids were identified by thin-layer chromatography on silica gel plates according to Edman (1975).

The determination of the N-terminal sequence of peptides was made by the manual method of Edman degradation using a subtractive procedure (Bartelt & Greene, 1971).

The amino terminal residues of proteins were determined by the dansyl technique of Gray (1972) and the amino-terminal residues of peptides by the dansyl technique of Hartley (1970).

Reduction and Carboxymethylation of Trypsinogen. The protein was reduced with mercaptoethanol in 8 M urea and subsequently alkylated with iodoacetate according to Crestfield et al. (1963).

Detection of Peptides. Following chromatography, peptides were detected by reaction of aliquots of column effluent with ninhydrin after alkaline hydrolysis by measuring the absorbance at 570 nm. Peptides were detected on paper with a solution of ninhydrin (1% in acetone).

High Voltage Paper Electrophoresis. Electrophoresis was performed on Whatman 3 MM paper at 3000 V for 35 min in a buffer pH 5.3 (pyridine/acetic acid/H_2O,10:4:986). Peptides were eluted from paper by the mixture pyridine/water (1:1 by volume).

Double Diffusion for Immunological Studies. Double diffusion was performed on agar plates (1.5% in agarose) in a 0.05 M veronal–acetate buffer (pH 8.6) according to Ouchterlony (1958). Benzamidine (5 mM) was added to the buffer to prevent zymogen activation. Two antisera were used for the precipitin reactions: an antisera to the proteins of unactivated pancreatic juice and an antisera to the proteins of activated pancreatic juice.

Affinity Chromatography of Human Trypsins on Trypsin Inhibitor–Sepharose. The column (0.9 × 10 cm) was equilibrated with 10 mM Tris-HCl buffer (pH 7.8) containing 20 mM $CaCl_2$ and 200 mM NaCl. One thousand to three thousand trypsin units was applied to the column. After the elution of inactive material, trypsin was eluted by a solution of 20 mM $CaCl_2$, 200 mM NaCl adjusted to pH 2.2.

Results

Purification of Human Trypsinogens

(a) *Gel Filtration.* Proteins of human pancreatic juice were submitted to a gel filtration on Sephadex G-100. Figure 1 shows a typical elution diagram with most of the potential and direct enzymatic activities of pancreatic juice. Peak I contains a carboxylic ester hydrolase and immunoglobulins that we have previously characterized in human pancreatic juice (Clemente et al., 1971). Peak II is composed of lipase and procarboxy-

[1] Abbreviations used: TosArgOMe, tosyl-L-arginine methyl ester; BzArgOEt, benzoyl-L-arginine ethyl ester; Dns, dansyl.

TABLE I: Purification of Human Trypsinogens.

Steps of purification	Protein (mg)	Potential units (μmol of TAME hydrolyzed/min)	Unit recovery (%)	Specific act.[a]	
				a	*b*
Pancreatic juice	1000	40 000	100	40	
Sephadex G-100	215	28 000	70	130	
DEAE-cellulose					
Tg 1	24.6	6 160	22	250	190
Tg 2	10.4	3 640	13	350	265

[a] Specific activities have been calculated using the extinction coefficient $E_{1cm}^{1\%} = 20.0$ except for *b*. Coefficients used were determined experimentally (15.2 for trypsinogen 1 and 15.1 for trypsinogen 2).

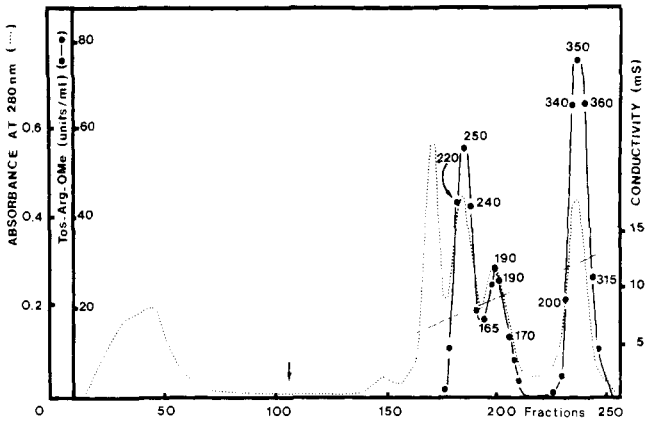

FIGURE 2: Chromatography of trypsinogens on DEAE-cellulose. The column (0.9 × 100 cm) was equilibrated with 25 mM NaCl, 1 mM benzamidine, 5 mM Tris-HCl buffer (pH 8.0). At the fraction indicated by the arrow a linear concentration gradient (25 mM to 350 mM) was applied to the column. Volume of each gradient chamber: 300 mL. Volume of fractions: 3 mL. The numbers on the unit profile indicate potential specific activities of trypsinogen. Sample: 60 mg of protein eluted from Sephadex G-100.

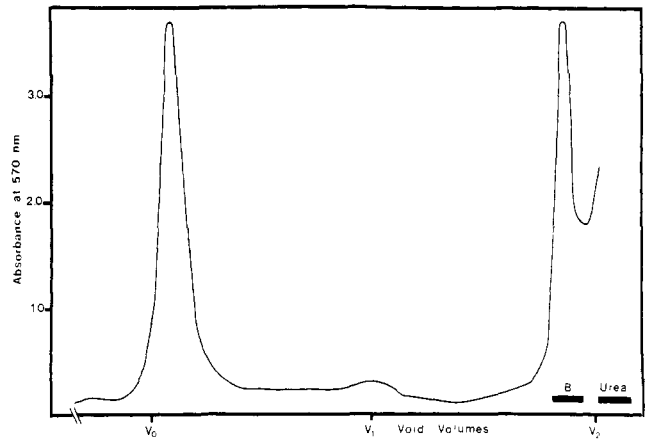

FIGURE 3: Chromatography of reduced and carboxymethylated trypsinogen 1 on Sephadex G-100. The column (1.4 × 176 cm) was equilibrated and developed with 50% acetic acid at 25 °C. Protein and peptides were located by ninhydrin analysis after alkaline hydrolysis. Volume of fractions: 1.9 mL.

peptidase A and peak III contains trypsinogen, chymotrypsinogen, and prophospholipase A. Colipase has been found in the last fractions of peak III and procarboxypeptidase B is located between peaks II and III.

(b) *Chromatography on DEAE-Cellulose.* Effluents corresponding to peak III and devoid of procarboxypeptidase A were combined. This pool contained 60 to 80% of the total potential trypsin activity with a specific activity on Tos-ArgOMe of approximately 130. Proteins in the high ionic strength buffer of the Sephadex G-100 column were equilibrated in the buffer of the next chromatography (DEAE-cellulose) by filtration on Sephadex G-25. The elution diagram of the DEAE-cellulose chromatography is given in Figure 2. Three peaks with potential TosArgOMe activity were separated by the concentration gradient. The first two peaks of activity correspond to two chromatographic forms of trypsinogen 1. The specific activity of trypsinogen 1 reaches 250 in the major form A and 190 in the minor form B. The proportions of forms A and B were variable from one chromatography to another, even when the same sample of pancreatic juice was used. Moreover, form B submitted to the same chromatography on DEAE-cellulose was separated again into two components. This demonstrates a heterogeneity at this level of the purification, heterogeneity which did not appear when pancreatic juice was submitted to DEAE-cellulose chromatography before gel filtration (Figarella et al., 1969). Trypsinogen 2 was eluted at the end of the gradient in one peak with a maximal potential specific activity of about 350. The different steps of purification are summarized in Table I. The two proteins displayed a single band by poly(acrylamide) gel electrophoresis in the presence of sodium dodecyl sulfate.

(c) *Chromatography of Reduced and Carboxymethylated Trypsinogen 1 on Sephadex G-100.* Eight milligrams of reduced and carboxymethylated trypsinogen 1 was submitted to a filtration on Sephadex G-100 in 50% acetic acid. The diagram (Figure 3) shows one main peak of protein eluted with the void volume without any shorter polypeptide chain eluted between proteins and urea. Pool B submitted to acid hydrolysis did not contain any specific protein or free amino acids released during reduction and carboxymethylation.

Molecular Properties

(a) *Amino Acid Composition and Extinction Coefficient.* The amino acid compositions of trypsinogens 1 and 2 are not very different as shown in Table II. The most striking differences are the number of methionine residues since trypsinogen 1 contains two residues while trypsinogen 2 has only one, and the hydrophobic residue content (valine, isoleucine, and leucine) with a total of 9 added residues in trypsinogen 2. However, some minor differences appear in the number of lysine (3), serine (6), glutamic acid (3), and proline (2). When compared with the other trypsinogens the two human zymogens are characterized by the presence of only 5 disulfide bridges instead of 6 as found in the trypsinogens of most species. The determinations of glutamine and asparagine contents of trypsinogen 2 were performed by enzymatic digestion of the reduced S-2-aminoethylated protein with 100% yield of free amino acids. This yield demonstrates that this method used for smaller proteins is feasible for protein of 25 000 molecular weight. The extinction coefficients of human

TABLE II: Amino Acid Compositions of Human, Bovine, and Porcine Trypsinogens.

Amino acid	Human		Bovine		Porcine	
	1	2	Anionic[b]	Cationic[c]	Anionic[b]	Cationic[d]
Lys	13.3 (13)	10.0 (10)	9	15	9	11
His	3.9 (4)	4.2 (4)	5	3	4	4
Arg	6.2 (6)	5.9 (6)	4	2	6	4
Asp	29.2 (29)	27.8 (28)	30	26	30	28
Thr	8.8 (9)	6.9 (7)	10	10	9	11
Ser	16.0 (16)	21.4 (22)	18	33	20	25
Glu	20.0 (20)	22.7 (23)	24	14	23	17
Pro	9.7 (10)	12.3 (12)	10	9	13	11
Gly	23.7 (24)	24.2 (24)	25	25	24	25
Ala	14.8 (15)	16.1 (16)	19	14	20	15
Cys/2	10.3 (10)	9.6 (10)	12	12	12	12
Val	16.6 (17)	17.8 (18)	16	18	16	16
Met	1.9 (2)	0.9 (1)	2	2	2	2
Ile	13.1 (13)	16.7 (17)	17	15	14	15
Leu	12.3 (12)	16.3 (16)	21	14	22	16
Tyr	9.3 (10)	11.1 (11)	12	10	11	8
Phe	4.2 (4)	4.9 (5)	4	3	5	5
Trp	3.6 (4)	2.6 (3)	7–8	4	7–8	4
Amides		26[e]	18	29	22	26
Total	217	233	245–246	229	247–248	229
Mol wt	23 438	25 006	26 735	23 990	26 917	24 210

[a] Calculated according to the method developed by Delaage (1968) and expressed as residues/mole. [b] From Louvard & Puigserver (1974). [c] From Walsh & Neurath (1964). [d] From Charles et al. (1963). [e] Determined as 18 Asn and 8 Gln.

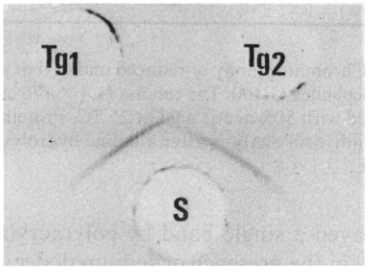

FIGURE 4: Immunological cross-reaction between trypsinogens 1 and 2. S = antiserum to human pancreatic juice.

trypsinogens are very close, 15.2 for trypsinogen 1 and 15.1 for trypsinogen 2.

(b) *Molecular Weight.* The molecular weights calculated on the basis of the amino acid analysis are 23 438 for trypsinogen 1 and 25 006 for trypsinogen 2. Both values are close to the value determined by gel electrophoresis in the presence of sodium dodecyl sulfate (26 000 ± 1000 for the two trypsinogens).

(c) *Immunological Studies.* Immunological cross-reaction between the two trypsinogens has been determined. Figure 4 shows partial identity between the two proteins with additional antigenic determinants for trypsinogen 1 when both proteins were submitted to immunodiffusion against antiserum to whole human pancreatic juice. Experiments performed with antiserum against either unactivated or activated pancreatic juice gave the same results.

Isolation and Characterization of the Activation Peptides of Trypsinogen 1

After the activation, trypsinogen 1 (a mixture of forms A and B) was submitted to an affinity chromatography on a column of trypsin inhibitor–Sepharose. Trypsin 1 was separated from the inactive material which contains peptides and benzamidine associated with the zymogen during purification. This inactive material was fractionated by gel filtration on Sephadex G-25. The diagram of Figure 5 shows a major peak

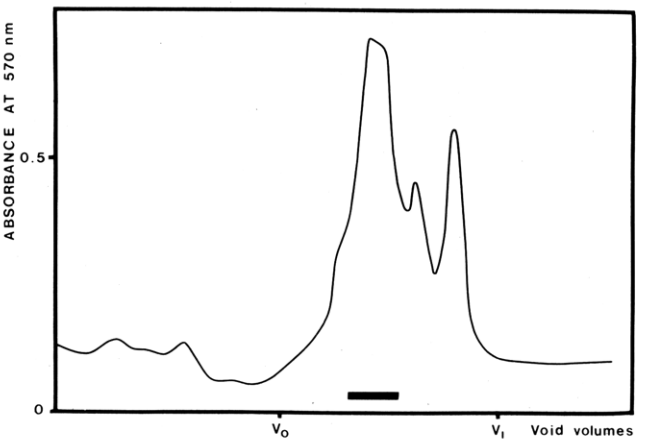

FIGURE 5: Gel filtration of peptides liberated by activation of trypsinogen 1 on Sephadex G-25. The column (0.9 × 200 cm) was equilibrated with 10% acetic acid.

of absorbance at 570 nm. The fractions indicated by the solid bar were pooled, concentrated, and submitted to a high voltage paper electrophoresis. Figure 6 represents the acidic part of the fingerprint obtained from two different activation mixtures of trypsinogen 1 (Tg1-X and Tg1-Y). Tg1-X gave only one spot (peptide 2) and Tg1-Y gave two spots (peptide 1 which migrates like the activation peptide of trypsinogen 2 and peptide 2). The three peptides were eluted from paper and analyzed. The results of analysis and of one step of Edman degradation are given in Table III. The two peptides 2 with the same electrophoretic mobility have the same amino acid composition. Moreover the electrophoretic mobility of peptide 2 which is more acidic than peptide Val-Asp_4-Lys permits us to assume that peptide 2 does not contain amide. Peptide 1 from trypsinogen 1-Y which migrates like the activation peptide of trypsinogen 2 has the same amino acid composition and the same N-terminal residue as this octapeptide (Guy et al., 1976). These results added to the unambiguous specificity of trypsin led to the conclusion that the sequences of the activation

TABLE III: Amino Acid Compositions of the Activation Peptides of Trypsinogen 1.

	Trypsinogen 1-X[a]		Trypsinogen 1-Y[a]		
	Peptide 2		Peptide 1		
Amino acid	Anal.	Edman degradation	Anal.	Edman degradation	Peptide 2 Anal.
Lys	0.88	0.72	0.62	0.54	0.83
Asp	14.10	*3.2*	4.50	4.25	4.17
Pro			0.99	1.00	
Ala			1.19	*0.26*	0.23
Phe			1.01	1.00	

[a] X and Y are two different samples of trypsinogen 1.

peptides of trypsinogen 1 are:

peptide 1: Ala-(Pro,Phe,Asp_4)-Lys

peptide 2: (Asp_4)-Lys

In Tg1-Y, the molar ratio of peptide 2 to peptide 1 is 0.6.

N-Terminal Sequences of Trypsinogens 1 and 2

Thin-layer chromatography of the dansylated trypsinogens showed one main spot of Dns-alanine for trypsinogen 2. The determination of the N-terminal residue of trypsinogen 1 gave different results. Most samples showed two N-terminal residues, alanine and aspartic acid, and in one sample we have characterized one N-terminal residue of alanine.

The N-terminal residues of trypsins 1 and 2 purified by affinity chromatography have been identified as isoleucine. The sequence of the first 6 residues of the polypeptide chain of trypsinogen 1 has been determined by automated phenyl isothiocyanate degradation of a sample of reduced and S-carboxymethylated protein which gave only one N-terminal residue of alanine by dansylation. These results added to the structural data on the activation peptides are consistent with the following N-terminal sequence of trypsinogen 1:

Ala-Pro-Phe-Asp-Asp-Asp-Asp-Lys-Ile

This result confirms the same sequence of trypsinogen 2 previously determined on 8 residues by an indirect method (Guy et al., 1976). Therefore, the N-terminal sequences of the two trypsinogens are identical for the first 9 residues. However, in some samples of trypsinogen 1 the polypeptide chain has been shortened by three residues, Ala, Pro, Phe.

Discussion

The two human trypsinogens have been purified from pancreatic juice. Trypsinogens represent 19% of the total proteins of pancreatic juice. Trypsinogen 1 is present in approximately twice the concentration of trypsinogen 2. The proportions of these two zymogens are quite different from that of the two bovine and the two porcine trypsinogens where the anionic forms do not exceed 10% of the cationic forms (Louvard & Puigserver, 1974). On TosArgOMe (10 mM), the potential specific activity of trypsinogens 2 is 1.4 times higher than that of trypsinogen 1, while on BzArgOEt (10 mM), the potential specific activities of the two trypsinogens are the same (Figarella et al., 1969). This difference in the behavior of the two enzymes toward substrates agrees with the differences previously observed in the interactions of the two trypsins with proteinase inhibitors (Figarella et al., 1975).

The separation of two forms A and B of trypsinogen 1 by DEAE-cellulose chromatography after gel filtration on Sephadex G-100 demonstrates a heterogeneity at this level of purification. A similar chromatographic behavior has been observed with other human pancreatic proteins such as chymotrypsinogens (De Caro et al., 1975) and secretory trypsin inhibitor (Pubols et al., 1974). As demonstrated for trypsin inhibitor, the multiple chromatographic forms are probably due to deamidation. Therefore all studies of trypsinogen 1 have been performed on the mixture of forms A and B.

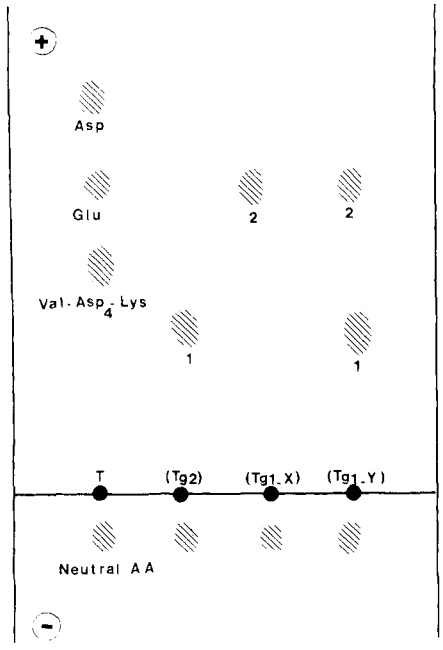

FIGURE 6: High voltage paper electrophoresis of the activation peptides of trypsinogen 1 at pH 5.3. The activation peptides of bovine trypsinogen (Val-Asp_4-Lys) and trypsinogen 2 (spot 1 of Tg 2) are given as reference.

Trypsinogens 1 and 2 have similar molecular weights (close to 25 000). No striking difference appears between the amino acid compositions of the two proteins. Comparison of the amino acid compositions of human trypsinogens with amino acid compositions of human trypsins reported in the literature was inconclusive. Table IV gives the results of our analysis compared to those of Mallory & Travis (1973) and Feinstein et al. (1974). It was not possible to relate each enzyme to its zymogen on the basis of amino acid composition. The assignment has been made on the basis of the inhibition spectra (Figarella et al., 1975). Trypsins isolated from autolyzed pancreas were probably partly degraded since we have noticed that the activation of human trypsinogens liberates in addition to the activation peptide other small peptides whose importance is not yet known.

The N-terminal sequences of human trypsinogens are identical on 9 residues. This sequence contains the charac-

TABLE IV: Amino Acid Compositions of Human Trypsins.

Amino acid	Trypsin 1			Trypsin 2		
	Our data[a]	Cationic trypsin[b]	Trypsin$_2$[c,d]	Our data[a]	Anionic trypsin[b]	Trypsin 1[c,d]
Lys	12	12	12	9	12	8
His	4	3	4	4	5	4
Arg	6	6	6	6	7	5
Asp	25	22	27	24	25	22
Thr	9	11	8	7	11	6
Ser	16	25	19	22	20	17
Glu	20	22	23	23	24	21
Pro	9	10	9	11	12	10
Gly	24	21	23	24	23	21
Ala	14	14	16	15	15	13
½-Cys	10	8	8	10	8	10
Val	17	17	16	18	15	15
Met	2	1	2	1	2	1
Ile	13	13	12	17	13	14
Leu	12	13	12	16	18	14
Tyr	10	7	7	11	8	7
Phe	3	4	4	4	7	4
Trp	4	3	3	3	3	3
Total	209	212	211	225	228	195

[a] Our data are the amino acids of trypsinogens 1 and 2 minus the amino acids of the activation peptides. [b] From Mallory & Travis (1973). [c] From Feinstein et al. (1974). [d] The assignments of trypsins were made on the basis of the inhibition spectra.

teristic poly(aspartyllysine) structure of all trypsinogens but the sequence of the first three residues Ala-Pro-Phe is markedly different from that of all other known trypsinogens, as discussed before for trypsinogen 2 (Guy et al., 1976).

Human trypsinogen 2 gives by activation a single peptide, the octapeptide Ala-Pro-Phe-(Asp)$_4$-Lys (Guy et al., 1976). By contrast trypsinogen 1 yields two activation peptides, one octapeptide identical with the activation peptide of trypsinogen 2, and the pentapeptide (Asp)$_4$-Lys. Five samples gave only the pentapeptide, two samples gave the two peptides and we never obtained the octapeptide alone. The liberation of two activation peptides (one octapeptide and one hexapeptide) has been already observed in a variety of species (Louvard & Puigserver, 1974; Bricteux-Gregoire et al., 1976, 1971; Bricteux-Gregoire, 1970). As discussed by Louvard & Puigserver, this duality can be the result of a proteolysis during the purification and/or the activation of the zymogens or the result of the existence of two genes yielding two distinct proteins. In human trypsinogen 1 the transformation octapeptide–pentapeptide would require the hydrolysis of a Phe-Asp bond which is a chymotrypsin-like cleavage. This cleavage could be due to a specificity of trypsin 1 similar to bovine trypsin (Maroux et al., 1966) and different from trypsin 2. However, during prolonged activation of trypsinogen 1 we never observed a quantitative decrease of the octapeptide and a concomitant increase of the pentapeptide, excluding the possibility of a chymotrypsin-like activity due to trypsin 1 or to a contaminating enzyme. The possibility of a peptide cleavage during the purification of the protein cannot be completely rejected but it is surprising to notice that this same cleavage never appeared in trypsinogen 2 which migrates with trypsinogen 1 and chymotrypsinogens during the filtration of pancreatic juice on Sephadex G-100. The possibility of an extra-splitting in the molecule of trypsinogen 1 would be therefore only due to a peculiar exposition of the amino-terminal sequence since the molecule behaves like one polypeptide chain during the chromatography of reduced and carboxymethylated trypsinogen 1 on Sephadex G-100.

For the trypsinogens of the other species so far studied the presence of two activation peptides has been explained by the existence of two distinct genes. This hypothesis cannot be excluded for human trypsinogen 1. However, in any case this duality may be also related to the recent "signal hypothesis" proposed by Blobel & Dobberstein (1975) and documented by the experiments on the dog pancreatic secretory proteins (Devillers-Thiery et al., 1975). The mRNAs for these secretory proteins are translated in vitro into larger precursor proteins extended at the amino-terminal sequence. The proteolytic removal of this sequence during the conversion to authentic secretory proteins would be achieved by an endoproteolytic event occurring in vivo by a membrane-bound enzyme. The attractive possibility of an additional and/or different cleavage during the transport of trypsinogens from ribosomes to zymogen granules must be evoked.

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References

Bartelt, D. C., & Greene, L. J. (1971) *J. Biol. Chem. 246,* 2218.

Benson, J. V., Gordon, M. J., & Patterson, J. A. (1967) *Anal. Biochem. 18,* 228.

Blobel, G., & Dobberstein, B. (1975) *J. Cell Biol. 67,* 835.

Bricteux-Gregoire, S. (1970) *Arch. Int. Physiol. Biochim. 78,* 399.

Bricteux-Gregoire, S., Schyns, R., & Florkin, M. (1966) *Biochim. Biophys. Acta 127,* 277.

Bricteux-Gregoire, S., Schyns, R., & Florkin, M. (1971) *Biochim. Biophys. Acta 229,* 123.

Charles, M., Rovery, M., Guidoni, A., & Desnuelle, P. (1963)

Biochim. Biophys. Acta 69, 115.
Clemente, F., Ribeiro, T., Figarella, C., & Sarles, H. (1971) *Clin. Chim. Acta 33,* 317.
Clemente, F., De Caro, A., & Figarella, C. (1972) *Eur. J. Biochem. 31,* 186.
Coan, M. H., & Travis, J. (1972) *Biochim. Biophys. Acta 268,* 207.
Coan, M. H., Roberts, R. C., & Travis, J. (1971) *Biochemistry 10,* 2711.
Cresfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238,* 622.
Cuatrecasas, P., Wilchek, M., & Anfinsen, C. B. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61,* 636.
De Caro, A., Figarella, C., & Guy, O. (1975) *Biochim. Biophys. Acta 379,* 431.
Delaage, M. (1968) *Biochim. Biophys. Acta 168,* 573.
Devillers-Thiery, A., Kindt, T., Scheele, G., & Blobel, G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 5016.
Edman, P. (1975) in *Protein Sequence Determination* (Needleman, S. B., Ed.) pp 232–279.
Erlanson, C. (1970) *Scand. J. Gastroent. 5,* 333.
Feeney, R. E., Means, G. E., & Bigler, J. C. (1969) *J. Biol. Chem. 244,* 1957.
Feinstein, G., Hofstein, R., Koifmann, J., & Sokolovsky, M. (1974) *Eur. J. Biochem. 43,* 569.
Figarella, C., Clemente, F., & Guy, O. (1969) *FEBS Lett. 3,* 351.
Figarella, C., Negri, G. A., & Guy, O. (1975) *Eur. J. Biochem. 53,* 457.
Geokas, M. C., Largman, C., Brodrick, J. W., Raeburn, S., & Rinderknecht, H. (1975) *Biochim. Biophys. Acta 391,* 396.
Gray, W. R. (1972) *Methods Enzymol. 25,* 121.
Guy, O., Bartelt, D. C., Amic, J., Colomb, E., & Figarella, C. (1976) *FEBS Lett. 62,* 150.
Hartley, B. S. (1970) *Biochem. J. 119,* 805.
Largman, C., Broderick, J. W., & Geokas, M. C. (1976) *Biochemistry 15,* 2491.
Louvard, M. N., & Puigserver, A. (1974) *Biochim. Biophys. Acta 371,* 177.
Mallory, P. A., & Travis, J. (1973) *Biochemistry 12,* 2847.
Mallory, P. A., & Travis, J. (1975) *Biochemistry 14,* 722.
Maroux, S., Rovery, M., & Desnuelle, P. (1966) *Biochim. Biophys. Acta 122,* 147.
Matsubara, H., & Sasaki, R. M. (1969) *Biochem. Biophys. Res. Commun. 35,* 175.
Moore, S. (1963) *J. Biol. Chem. 238,* 235.
Ohlsson, K., & Tegner, H. (1973) *Scand. J. Gastroent. 8,* 129.
Ouchterlony, O. (1958) *Prog. Allergy 5,* 1.
Peterson, L. M., Sokolovsky, M., & Vallee, B. L. (1976) *Biochemistry 15,* 2501.
Pubols, M. H., Bartelt, D. C., & Greene, L. J. (1974) *J. Biol. Chem. 249,* 2235.
Rathelot, J., Julien, R., Canioni, P., Coeroli, C., & Sarda, L. (1975) *Biochimie 57,* 1117.
Travis, J., & Roberts, R. C. (1969) *Biochemistry 8,* 2884.
Walsh, K. A., & Neurath, H. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52,* 884.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244,* 4406.

Stopped-Flow Kinetics of the Resynthesis of the Reactive Site Peptide Bond in Kallikrein Inhibitor (Kunitz) by β-Trypsin†

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ABSTRACT: Modified kallikrein inhibitor (I*), in which the reactive site peptide bond Lys-15–Ala-16 is split, reacts with β-trypsin (E) according to E + I* ($k_x = 10^5\ M^{-1}\ s^{-1}$) $\rightleftharpoons$ ($k_{-x} = 0.35\ s^{-1}$) X ($k_c = 10^{-2}\ s$) $\rightleftharpoons$ ($k_{-c} = 3 \times 10^{-10}\ s^{-1}$) C (all rate constants at pH 7.5 and 23 °C). In the stable complex C the peptide bond is reestablished and the carboxyl carbon of Lys-15 forms a tetrahedral adduct with Ser-195 of the enzyme. The precomplex X is characterized by an equilibrium constant $K_x = k_{-x}/k_x = 3.5 \times 10^{-6}$ M and an enthalpy of formation of 1.6 kcal/mol. Activation energies of k_x and k_c are 10 kcal/mol. The pH dependencies of K_x, k_x, k_c and of the overall rate constant $k_{on}^* = k_c/K_x$ were measured. For k_{on}^* a bell-shaped pH profile with a maximum at pH 6.8 was observed. With I*-OMe in which the carboxyl group of Lys-15 is esterified by methyl alcohol, the intermediate X was not detected and the rate of C formation was 100-fold faster than in the case of I*. The complex formation with I*-OMe was very similar with that of virgin inhibitor in which the reactive peptide bond is intact. In both cases the pH profile exhibited a plateau in the range of 8–10 and a steep decrease at low pH. The comparison of the data for I*-OMe and I* suggests that in the intermediate complex X the peptide bond is still open and neither the tetrahedral nor the acyl adducts with Ser-195 are formed. For fast formation of the first tetrahedral intermediate, the carboxyl group of Lys-15 must be either protonated or esterified.

Because of the detailed x-ray crystallographic knowledge the interaction of protein inhibitors with serine proteases constitutes a well-defined example of protein–protein interactions. Furthermore, the mechanism of interaction resembles the catalytic process in which a susceptible peptide bond in the reactive site of the inhibitor is split. The normally very stable

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