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Reevaluation of the Activation of Bovine Chymotrypsinogen A*

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ABSTRACT: α-Chymotrypsin is the only activated form of bovine chymotrypsinogen A which has been found to self-associate to a significant extent at low pH, in agreement with early studies by Schwert (Schwert, G. W. (1949), *J. Biol. Chem. 179*, 655). Variability in the dimerization constants of this enzyme has been shown to be due to the presence of small amounts of contaminating materials in the preparations, removable by active-site affinity chromatography. The dimerization assay, amino-terminal amino acid residue determinations, and disc gel electrophoresis have been used to investigate some of the intermediates in the activation of chymotrypsinogen A. A new species of chymotrypsin has been isolated and partially characterized. It is called κ-chymotrypsin and it has cystine (assumed), isoleucine (determined), and threonine (determined) as amino-terminal residues. It is produced from trypsin-free δ-chymotrypsin at pH 3.1 and room temperature in 48 hr and does not reversibly dimerize at low pH. κ-Chymotrypsin can be converted to both γ-chymotrypsin and α-chymotrypsin by variation of the crystallization conditions. Under conditions of classical slow activation (pH 7.5, 5°, 48 hr) trypsin-free δ-chymotrypsin is relatively stable and shows only about 50% conversion to electrophoretically different species. These results support the activation scheme of Wright *et al.* (Wright, H. T., Kraut, and Wilcox, P. E. (1968), *J. Mol. Biol. 37*, 363) that α-chymotrypsin is formed from neochymotrypsinogens rather than from δ-chymotrypsin. It is desirable, however, to insert κ-chymotrypsin into this scheme between δ- and γ-chymotrypsin.

Bovine chymotrypsinogen A can be activated to yield several active chymotrypsins A. α-Chymotrypsin, the first to be discovered (Kunitz and Northrup, 1935), has been well studied. β- and γ-chymotrypsins (Kunitz, 1938) can also be crystallized from "slow" (0.01% trypsin) activation mixtures. π- and δ-chymotrypsins (Jacobsen, 1947) result from the "rapid" (3% trypsin) activation of chymotrypsinogen.

The peptide-bond cleavages associated with the activation of chymotrypsinogen are well established (Dreyer and Neurath, 1955; Rovery *et al.*, 1955, 1957; Hartley, 1964). The essential step in the activation is a tryptic cleavage at the Arg_{15}–Ile_{16} bond (Oppenheimer *et al.*, 1966). Limited tryptic digestion of chymotrypsinogen results in the formation of π-chymotrypsin (Jacobsen, 1947) which is rapidly converted to δ-chymotrypsin by hydrolysis of the bond Leu_{12}–Ser_{13} (Rovery *et al.*, 1955; Hartley, 1964).

In the formation of α- or γ-chymotrypsin, four peptide bonds are split with the release of two dipeptides: Ser_{13}–Arg_{14} and Thr_{147}–Asn_{148} (Hartley, 1964). Recent crystallographic examinations (Matthews *et al.*, 1967; Cohen *et al.*, 1970; Davies *et al.*, 1969) have not revealed differences in amino acid sequence or any major structural difference, although the crystal habits of the two forms are quite different.

Recently, Wright *et al.* (1968) published a modification of Desnuelle's (1960) scheme for the genesis of the various forms of chymotrypsins (Figure 1). In the scheme of Wright *et al.* (1968), α-chymotrypsin is not derived directly from δ-chymotrypsin by autocatalytic degradation, but only by activation of neochymotrypsinogens (Rovery *et al.*, 1957). The immediate product of autocatalytic splitting of Thr_{147}–Asn_{148} from δ-chymotrypsin is proposed to be γ-chymotrypsin.

This investigation was initiated for several reasons. First, we wished to determine which, if any, of the activation schemes is correct by a characterization of the product formed after limited autolysis of δ-chymotrypsin. Second, the results of Bettelheim and Neurath (1955) showed that when a rapid activation mixture was allowed to autolyze at 5° for 56 hr, the product had the same carboxyl-terminal residues as α-chymotrypsin, but had only 0.21 residue of amino-terminal alanine/mole. We were interested in this enzyme since it might have been an intermediate between δ- and γ-chymotrypsins in the Wright *et al.* (1968) scheme. Third, if the activation mechanism of Wright *et al.* (Figure 1) is correct, α-chymotrypsin produced from δ-chymotrypsin might yield a more homogeneous α-chymotrypsin preparation than is available commercially.

We have been successful in isolating a new form of chymotrypsin, κ-chymotrypsin; and we propose that it is an intermediate in the scheme of Wright *et al.* (1968, Figure 1). In

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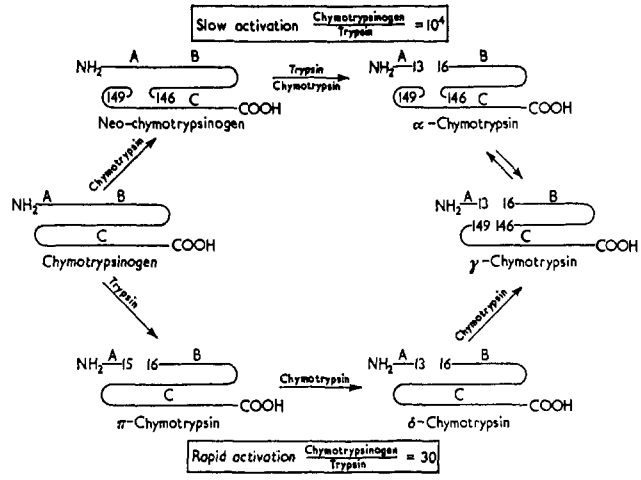

FIGURE 1: Schemes for the slow and rapid activations of chymotrypsinogen A (from Wright *et al.*, 1968).

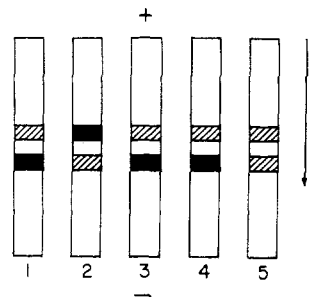

FIGURE 2: Relative mobilities of the various forms of chymotrypsin. (1) δ preparation + zymogen; (2) δ preparation + PMS-δ; (3) δ preparation + γ; (4) δ preparation + PMS-α; (5) δ preparation alone.

addition, our results support that scheme since we have been able to prepare both γ-chymotrypsin and α-chymotrypsin from trypsin-free δ-chymotrypsin and found that δ-chymotrypsin is stable under slow activation conditions. However, the yield and homogeneity of the α-chymotrypsin is not sufficient to warrant its routine production by this pathway. Finally, the results indicate that δ-chymotrypsin undergoes limited autolysis at pH 3.1 at room temperature.

Materials and Methods

Chemical Methods. Protein concentrations were determined from optical density measurements of protein solutions with a Zeiss spectrophotometer. The extinction coefficient, $E^{1\%}_{280\,nm}$, is 20.0 (Wilcox *et al.*, 1957).

The assay for esterase activity (BTEE)[1] was modified from that of Hummel (1959) by omitting the methanol in the assay mixture. Since the rates depend markedly on the temperature, an enzyme standard was used each time (Worthington α-chymotrypsin lot CDI-6LD, nominal activity 46 units/mg). Activities are expressed as percent of this α-chymotrypsin activity.

The number of active sites per mole of the enzyme was determined by titration with *N-trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961). In this assay, one active site per molecule equals 100% activity.

Gel chromatography was carried out at 5° with a 1.5 × 90 cm. G-75 Sephadex column pumped at 0.23 ml/min with 0.2 M KCl–0.01 M acetate (pH 4.40) as the solvent. Fraction size was approximately 1 ml.

The pH values were measured at room temperature.

The analytical disc gel procedure used was based on the pH 4.5 buffer system originated by Reisfeld *et al.* (1962). The solution polymerized in forming the "running gel" contained 5.25% acrylamide and the "stacking gel" solution contained 1.6% acrylamide. The gels were formed and run in 0.6 × 10 cm glass tubes. The gel apparatus was a Buckler analytical type (Fort Lee, N. J.). The upper and lower buffer chambers were filled with a solution containing 3.12 g of β-alanine and 0.8 ml of glacial acetic acid per l. The protein samples (which had previously been dialyzed *vs.* 1 mM HCl) were diluted with cold (about 5°) 30% sucrose in 0.001 N HCl to a final protein concentration of 0.02–0.04 mg/ml. (The final sucrose concentration was never less than about 20%.) Sample volume was either 0.025 or 0.050 ml and was applied to the top of the stacking gel with a micropipet. The gels were run at 2–6° for 3 hr at 3 mA/tube, using a Heathkit Model IP-32 regulated voltage supply. The gels were stained and fixed by immersion overnight in 5% trichloroacetic acid, 5% sulfosalicyclic acid, 0.012% coomassie blue, and background stain was then removed by immersion in 5% trichloroacetic acid, 5% sulfosalicyclic acid. The protein ran toward the cathode in this system.

Preliminary experiments showed that δ-chymotrypsin was the slowest moving product of chymotrypsinogen A activation. Figure 2 illustrates the result of mixture experiments which helped establish this. Comparison of gel 5 (δ-chymotrypsin alone) with gels 1 (δ + zymogen), 2 (δ + PMS-δ), 3 (δ + γ), and 4 (δ + PMS-α) revealed that chymotrypsinogen, γ-, and PMS-α-chymotrypsins migrated with the lower band of the δ preparation while the PMS-δ preparation migrated with the upper band of the δ preparation. Thus, this system is capable of separating δ-chymotrypsin from the zymogen, γ-, and α-chymotrypsin.

Amino-terminal residues were determined according to the method of Stark (1967) after first inhibiting the enzyme with a fourfold molar excess of PMSF (Gold and Fahrney, 1964) at pH 7.5, 0° for 45 min. Autolysis produced spurious end groups if the PMSF inhibition step was omitted. The amino-terminal residues are reported as fractions relative to the observed amount of isoleucine, since all active forms of chymotrypsin have one amino-terminal isoleucine per mole.

In order to obtain trypsin-free δ-chymotrypsin a procedure developed by E. Surbeck and P. E. Wilcox (unpublished data) was employed. It involves mixing acetylated trypsin with the zymogen in a 1:25 ratio and allowing the reaction to proceed for 1 hr at pH 7.5. A typical preparation started by dissolving 2.5 g of chymotrypsinogen in 100 ml of ice-cold water. $CaCl_2$ (0.5 ml of 1 M) and acetylated trypsin (10 ml of 10 mg/ml dissolved in water) were added and the solution was brought to and maintained at pH 7.5 for 60 min using a pH-Stat to add 0.5 N NaOH. Temperature was kept between 0 and 4° with ice water circulated through the hollow walled reaction vessel. The pH was lowered to 3 with 0.6 N HCl at the end of 60 min. This material was placed on a CM-cellulose ("CM-52 microgranular cellulose," Whatman) column, previously equilibrated with 0.05 M potassium phosphate (pH 6.2) to remove the trypsin. However, this column pro-

[1] Abbreviations used are: BTEE, benzoyl-L-tyrosine ethyl ester; PMSF, phenylmethanesulfonyl fluoride; CM-cellulose, carboxymethyl-cellulose.

TABLE I: Dimerization Constants for Various Forms of Chymotrypsins in 0.01 M Acetate–0.2 M KCl (pH 4.4).

Row	Material	Supplier	Lot No.	Treatment	Temp (°C)	K_2 (× 10^3), l./mole	% α-Chymotrypsin
1	α-Chymotrypsin	N.B.C.	6397	None	5	11.1	82
2	α-Chymotrypsin	N.B.C.	6397	Recrystallization	5	9.4	78
3	α-Chymotrypsin	Worthington	CDI-6144-5	None	20	44.4	100
4	α-Chymotrypsin	Worthington	CDI-6LD	None	20	35.7	98
5	α-Chymotrypsin	Worthington	CDS-7EA	None	5	14.9	89
6	α-Chymotrypsin	Worthington	CDS-7EA	None	20	22.4	90
7	α-Chymotrypsin	Worthington	CDS-7EA	Sepharose chromatography	20	43.8	100
8	α-Chymotrypsin	Worthington	CDI-8VS	None	20	17.2	85
9	α-Chymotrypsin	Worthington	CDI-8VS	Sepharose chromatography	20	37.6	99
10	Chymotrypsinogen	Worthington	CG	None	20	2.0	0
11	Chymotrypsinogen	Princeton		Purified by P. Wilcox	5	0.4	0
12	δ-Chymotrypsin	Worthington	CDD6032	None	5	0.2	0
13	δ-Chymotrypsin	Row 11 zymogen		Activated zymogen purified by K. Hapner	5	2.4	<24
14	γ-Chymotrypsin	Worthington	CDG6204-5	None	5	2.4	<28
15	β-Chymotrypsin	Worthington	CDB7FA	None	20	1.4	0

cedure resulted in a partial conversion of δ-chymotrypsin to a form with a greater mobility on disc gel electrophoresis (see Figure 2). If a batchwise technique was employed instead of the column, trypsin-free δ-chymotrypsin could be prepared in a relatively pure form. The procedure involved stirring the acetylated-trypsin–chymotrypsin mixture with CM-cellulose equilibrated at pH 6.2 with 0.05 M potassium phosphate and centrifuging the mixture. Typically, the ratio of activated chymotrypsinogen to cellulose was 1.25 g of zymogen to 15 g of dry cellulose. The supernatant contained the trypsin while the chymotrypsin remained with the CM-cellulose. The chymotrypsin was then removed by changing the buffer to 0.08 M potassium phosphate (pH 6.2) and centrifuging the CM-cellulose as a precipitate. Usually three washes with 0.05 M phosphate were done first and then four washes with 0.08 M phosphate.

The procedure for the slow activation of chymotrypsinogen A was that of Kunitz and Northrop as described by Laskowski (1955). The only modification used was the omission of 5 N H_2SO_4 to dissolve the zymogen.

Preparation of Affinity Columns. Cuatrecasas *et al.* (1968) have described an affinity column for chymotrypsin of ε-amino-*n*-caproyl-D-trytophan methyl ester linked to Sepharose 4B. No details of the methods used to synthesize the tryptophan peptide were given in that paper. The procedure which we used is described in the Appendix.

Affinity Column Characteristics. The peptide was linked to Sepharose 4B (Pharmacia Fine Chemicals AB) as described by Cuatrecasas *et al.* (1968). Although the resultant column did separate an inactive fraction from α-chymotrypsin when developed with 0.05 M Tris-HCl (pH 8.0) at room temperature, the specific activity of the bound material was not increased relative to the starting material. If the bound chymotrypsin was allowed to remain on the column for longer than 1 hr, the specific activity actually decreased. These results are undoubtedly due to autolysis of α-chymotrypsin under these conditions (Kumar and Hein, 1970).

The specific enzymatic activity could be increased by adding 0.05 M $CaCl_2$ to the buffer, running the column at low temperature (2–6°), applying the α-chymotrypsin in a small volume of 0.001 N HCl, and removing the bound material from the column with 0.1 M acetate (pH 3.0) as soon as possible commensurate with base-line separation of the inactive and active components. A typical example of the chromatograms obtained under these conditions is shown in Figure 3A. Figure 3B shows the results of the rechromatography on the same column. The breakthrough material comprised 8.5 and 0.85%, respectively, of the eluted optical densities in the initial and rechromatography experiments.

Ultracentrifugation. High-speed sedimentation equilibrium experiments using Rayleigh interference optics were performed as described by Teller *et al.* (1969). The photographic plates were read on a modified Nikon microcomparator (Teller, 1967).

Equilibrium constants for dimerization were calculated by the method of Hoagland and Teller (1969) assuming a molecular weight for monomeric chymotrypsin of 25,000 g/mole and a partial specific volume of 0.736 ml/g (Schwert, 1951). The centrifuge runs were done at 32,000 rpm and 5°, except as otherwise stated. Equilibrium was reached in about 12 hr. In all reported experiments, the solvent was 0.01 M acetate–0.2 M KCl (pH 4.40).

Results and Discussion

Dimerization of Chymotrypsins. Table I summarizes the dimerization of chymotrypsinogen A and its activated forms. The equilibrium constant for self-association of α-chymotrypsin is maximum at pH 4.4 (Egan *et al.*, 1957; T. A. Horbett and D. C. Teller, unpublished data). Further, the self-association does not extend beyond dimer at this pH (Steiner, 1954; T. A. Horbett and D. C. Teller, unpublished data). Several characteristics of the dimerization are noteworthy as seen in Table I. First, the data verify that α-chymotrypsin is the only species which forms significant amounts of dimer in reversible equilibrium. This fact was not clear until now because Schwert had ascribed the much lower maximum sedimentation rate he observed for γ-chymotrypsin compared

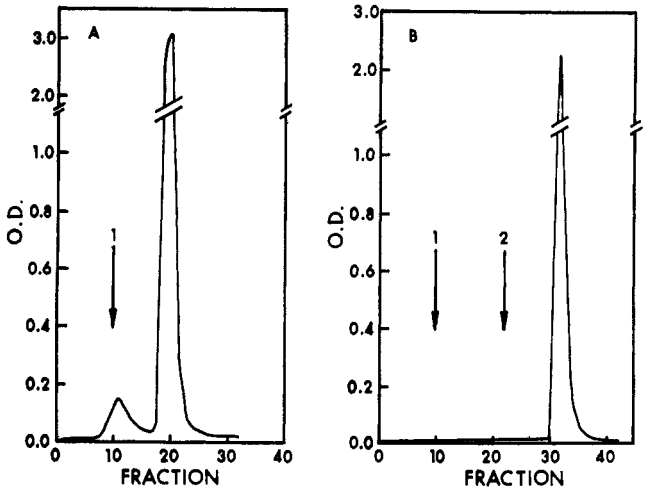

FIGURE 3: Chromatography of α-chymotrypsin CDS-7EA on peptide-Sepharose in 0.05 M $CaCl_2$–0.05 M Tris-HCl (pH 8.0) at 2–6°. Column size was 0.9 × 25.6 cm. Flow rate 48 ml/hr. The fraction size was 2.4 ml. (A) 0.6 ml of a 42.1-mg/ml solution of α-chymotrypsin in 0.001 N HCl was applied to the column. The arrow marks the last fraction collected before 0.1 M acetate (pH 3.0) was applied to the column. (B) 0.2 ml of peak 2 of Figure 3A at 49.1 mg/ml in 0.1 M acetate was applied to the column. The arrow numbered 1 indicates the expected breakthrough peak position while the arrow labeled 2 indicates the last fraction collected before 0.1 M acetate (pH 3.0) was applied to the column.

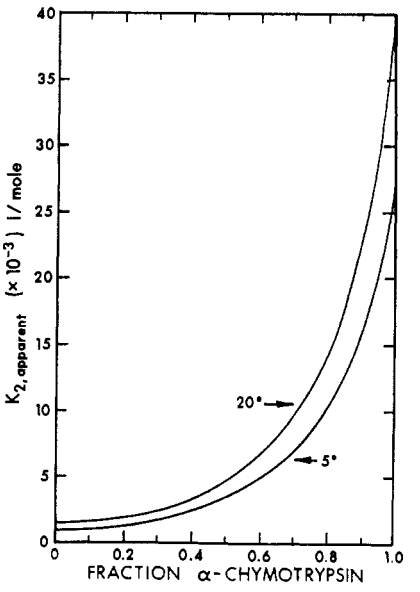

FIGURE 4: Calibration graph for the determination of the fraction of α-chymotrypsin present in mixtures of chymotrypsins according to the calculation procedure of Teller *et al.* (1969). These graphs are used to estimate the amount of α-chymotrypsin present in activation mixtures (*cf.* Table I).

to α-chymotrypsin to the supposedly lower molecular weight of γ-chymotrypsin (Schwert, 1949). However, if molecular weights of 25,000 are assigned to both α- and γ-chymotrypsin and if further we apply Gilbert's (1963) theory to Schwert's data for γ-chymotrypsin at pH 3.86, 25° in 0.2 M ionic strength buffer, we calculate a dimerization constant of 1100 l./mole which compares well with 2400 l./mole we obtained for γ-chymotrypsin (Table I, line 14). It is not possible to make strict comparisons since the experimental conditions were not exactly the same, but it is nonetheless clear that the much lower dimerization of γ-chymotrypsin observed in our studies is confirmed by proper interpretation of Schwert's studies. Furthermore, while our data were obtained under a single set of experimental conditions (pH 4.4 at 5°), Schwert's (1949) experiments were done at several different pH values and at about 25°. The two sets of results taken together therefore strongly confirm the conclusion that γ-chymotrypsin and the other activated forms of chymotrypsinogen A all self-associate much less strongly than α-chymotrypsin, and that this difference is not restricted to the experimental conditions we used.

Secondly, as has been pointed out by Teller *et al.* (1969), the variability of the dimerization constant between various lots of α-chymotrypsin seen in Table I is not due to experimental error in the measurement of equilibrium constants but is due to the preparations themselves. For example, we have measured the equilibrium constant of lot CDI 6144-5 (Table I, row 3) three times over a period of 4 years under identical conditions and obtained values of 42.8 × 10^3, 44.4 × 10^3, and 39.5 × 10^3 l. per mole.

One possible cause of variability in the dimerization constants between various lots of α-chymotrypsin is the presence of monomeric chymotrypsins which do not dimerize (Teller *et al.*, 1969). Figure 4 demonstrates that minor contamination of a dimerizing preparation by such incompetent monomeric molecules can cause large decreases in equilibrium constants. An experimental example of this is the preparation CDI-8VS from Worthington Biochemicals Corp. (Table I, row 8 and 9). Removal of 9% of ultraviolet-absorbing material using affinity chromatography (see Figure 3A) resulted in a 7 ± 4% increase of BTEE activity, a 4 ± 1% increase in titratable active sites, and a twofold increase in dimerizability. From values of the equilibrium constant measured for the sample before and after treatment with the affinity column, and assuming that the final value is pure α-chymotrypsin, then (using Figure 4) 15% of the original preparation was calculated to be incapable of dimerizing in the initial preparation. Since all of these estimates (affinity chromatography, BTEE assay, active-site titration, and dimerization constant assay) are in agreement within experimental error, we conclude that the original preparation contained about 10% of a material which was enzymatically inert *and* did not self-associate. The purified-preparation may contain nondimerizing material which co-chromatographs with α-chymotrypsin. However, this material cannot exceed 10–20% of the protein since the range of initial concentrations we used in the sedimentation equilibrium experiments would have allowed detection of a larger fraction of incompetent molecules (Harris *et al.*, 1969).

Use of the dimerization constant to detect α-chymotrypsin is reliable only if greater than 50% of the material is α-chymotrypsin (Figure 4). Due to this low sensitivity to small amounts (<50%) of α-chymotrypsin and the uncertainty in the equilibrium constants, in Table I and subsequent tables, we have stated these uncertainties in amount of α-chymotrypsin by indicating that the estimated amount of α-chymotrypsin is less than or equal to the percentage stated.

Slow Activation. Chymotrypsinogen was treated with 0.01% trypsin for 48 hr as described in the Materials and Methods section. Table II summarizes the characteristics of the product. The disc gel results illustrated in Figure 5 showed very little material at the δ-chymotrypsin position above the main band. A gel filtration experiment with 2 ml of a 37-mg/ml protein solution applied to the column described in

TABLE II: Characteristics of Slow Activation Products.[a]

	K_2	% α-Chymotrypsin
a, Product before crystallization conditions	5,925	65
b, Crystal suspension (crystals were stirred and a sample was taken)	5,950	65
c, Crystal wash and supernatant (crystals were washed with 50% saturated ammonium sulfate)	3,290	50
d, Crystals	10,500	81

[a] (1) Disk gels show one band in the α position, a trace of material in the δ position. (2) BTEE activity = 105% α-chymotrypsin. (3) Centrifuge runs were done on four samples.

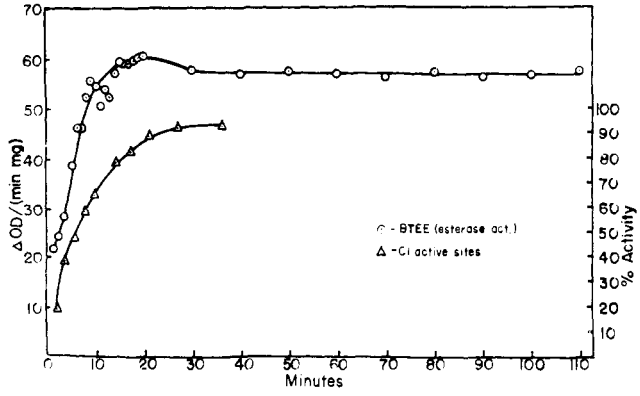

FIGURE 6: Change in activity with time during the rapid activation of chymotrypsinogen. Upper curve (○, left ordinate) is the change in absorbancy/(min mg) for BTEE at 256 nm. The lower curve (△, right ordinate) is the per cent activity determined by cinnamoylimidazole titrations (Schonbaum *et al.*, 1961).

Materials and Methods was performed. Very little size heterogeneity was detected since the main peak, which was quite symmetrical, contained more than 95% of the eluted optical density. Thus, the observed dimerization constant of 5925 l./mole (Table II) could be safely interpreted to mean that about 65% of the slow activation product was capable of dimerization. Crystallization of the product did not change the dimerization constant of the entire preparation since a value of 5950 l./mole was observed from the crystal suspension. However, the dimerization constant observed for the crystalline material (10,500 l./mole) and for the noncrystalline supernatant (3290 l./mole) clearly show that the product was a mixture of dimerizing and nondimerizing forms. Furthermore, this result indicates that only molecules in the α form crystallize at pH 4.0 in 50% saturated ammonium sulfate.

Fast Activation. Active-site titrations and esterase activity measurements were used to examine the time required for complete conversion of zymogen to δ-chymotrypsin. Figure 6 shows these results. The greater initial slope of the esterase activity curve relative to the active sites curve and the subsequent decline of the esterase activity curve is in agreement with Jacobsen's (1947) observation that π-chymotrypsin is more active than δ-chymotrypsin. Activation appears to be complete in about 40 min.

δ-Chymotrypsin was prepared as described in the Methods section. Table III summarizes the characteristics of this product. Figure 7 demonstrates that the batchwise cellulose detrypsinization (see Materials and Methods) did not alter the electrophoretic band pattern unlike column chromatographic detrypsinization (see Methods). This experiment also demonstrates that "δ-chymotrypsin" as usually prepared is not electrophoretically homogeneous (see also Figure 2, gel 5). The amino-terminal residue data (Table III) indicate that about 25% of the δ-chymotrypsin has been converted to another form having a threonine amino terminal. The 75:25 ratio shown was the maximum obtainable under the conditions used despite many attempts to improve it.

To study conversion of δ-chymotrypsin to α- or γ-chymotrypsin, several experimental conditions were examined.

First, a trypsin-free δ-chymotrypsin preparation was brought to pH 7.5 with 0.5 N NaOH. The protein concentration was 10 mg/ml in this experiment. The temperature was kept at 5°. The conversion was followed on disc gels and the results shown in Figure 8 (pattern 1) demonstrate that even after 48 hours at this high pH about 50% of the molecules were still in the δ form.

Second, a trypsin-free δ preparation (27 mg/ml) was brought to pH 7.5 and allowed to reach room temperature. Under these conditions three hours was apparently sufficient for essentially complete conversion, as shown in Figure 8 (pattern 2) and Table III, column 3. A gel filtration experiment with 2 ml of an 18-mg/ml solution of this material applied

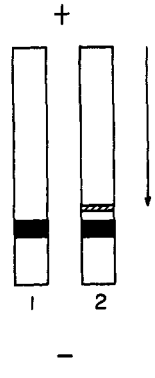

FIGURE 5: Disc gel patterns of zymogen and slow activation product. (1) Zymogen and (2) slow activation product.

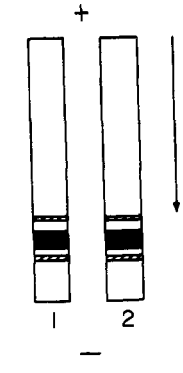

FIGURE 7: Disc gel pattern of a δ preparation before and after batch wise method of acetyl-trypsin removal. (1) δ preparation before trypsin removal; (2) δ preparation after trypsin removal.

TABLE III: Characteristics of Chymotrypsin Preparations.

Preparation	δ-Chymotrypsin	pH 7.5, 20° Conversion Product	pH 3.1, 20° Conversion Product	α-Crystals from pH 3.1 Conversion	Supernatant of α-Crystals
Number of bands on disc gel electrophoresis	2	2	2		
Major band on disc gel electrophoresis	Upper	Lower	Lower		
BTEE activity (%)	130	81	87		
Association constant K_2 (l./mole)	2500	1254	2195	15,680	2521
α-Chymotrypsin (%)	<40	<20	<37	90	<40
N-Terminal residues[a]					
Ala	0.05	0.35	0.10	0.85	0.34
Thr	0.25	0.56	1.0	0.24	0.44
Ser	0.08	0.49	0.26	0.32	0.14
Gly	0.14	0.24	0.26	0.18	0.22
Asp	0.10	0.22	0.13	0.14	0.15

[a] Fractional values relative to the observed amount of isoleucine. Glutamic acid, leucine, and valine levels were also determined but fractional values were 0.11 or less in all preparations.

to the column (see Material and Methods) showed a trailing peak in the chromatogram containing approximately 12% of the eluted optical density, so that some nonspecific autolysis occurred during this treatment. This experiment compared to the previous one indicates that temperature is a very important factor in this conversion.

A third set of conditions for the conversion of δ-chymotrypsin involved pH 3.1 and room temperature and an enzyme concentration of 34 mg/ml. Under these conditions the conversion appeared to be essentially complete in 48 hr, as shown in Figure 8 (pattern 3). Table III, column 4 shows further characterizations of this material. A gel filtration experiment, in which 2 ml of a 34-mg/ml solution of this material was applied to the column described in Material and Methods, revealed no trailing peak in the chromatogram. These conditions thus appeared to be better than those involving a higher pH since less nonspecific autolysis took place.

When chymotrypsin prepared at pH 3.1 at room temperature was chromatographed on CM-cellulose at pH 6.2 with a linear phosphate gradient, the chromatogram of Figure 9 was obtained. The major peak of chromatographed material (fractions 115–125 of Figure 9) was lowered to pH 3.1 with HCl and concentrated to 50 mg/ml. This material was crystallized under two different conditions. One involved α conditions (50% saturated ammonium sulfate and pH 4.2). The crystallization solutions were set up by adding dropwise, with stirring, an equal volume of saturated ammonium sulfate to the protein solutions. With the α-crystallization conditions, crystals were obtained in about 24 hr. These crystals were diamond shaped and flat, characteristic α crystals. The yield was about 27%. The dimerization constant for this material was 15,689 l./mole, very close to that of α-chymotrypsin. Table III, column 5, shows amino-terminal residue characterization of these crystals as well as the supernatant solution (Table III, column 6). The results of the amino-terminal analysis indicate that the material with 1.0 residue of threonine was converted to materials containing predominantly alanine either during the CM-cellulose chromatography or during the crystallization.

The other conditions for crystallization were those required for the formation of γ crystals (50% saturated ammonium sulfate and pH 5.6). The crystals obtained under these conditions differed markedly from those obtained at pH 4.2.

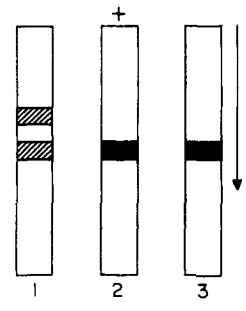

FIGURE 8: Disc gel patterns obtained after exposure of δ-chymotrypsin to various conditions. (1) 48 hr at pH 7.5 and 5°; (2) 3.0 hr at pH 7.5 at room temperature; (3) 48 hr at pH 3.1 at room temperature.

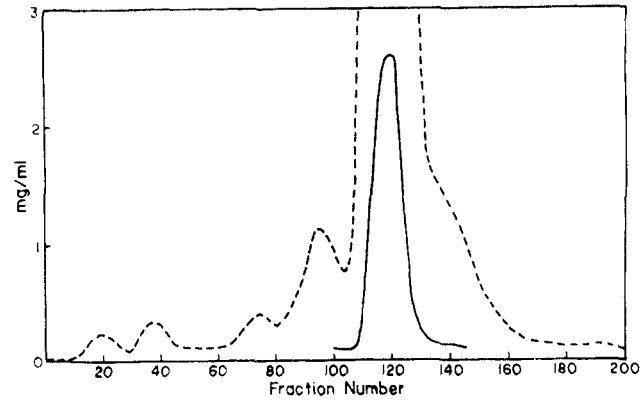

FIGURE 9: CM-cellulose chromatogram of κ-chymotrypsin. Dashed line depicts ten times the ordinate concentration. 2.2 ml of a 27 mg/ml of protein solution was applied to a 100 × 2.5 cm column. A linear elution gradient was applied, beginning with 0.05 M phosphate, pH 6.2 (2 l.), and ending with 0.15 M phosphate, pH 6.2 (2 l.). Flow rate was 90 ml/hr, fraction size was 15 ml, and temperature was 5°.

They resembled poorly formed γ crystals, having a tetragonal shape, similar to the type Corey *et al.* (1965) obtained in their first crystallization of γ-chymotrypsin.

The disc gel and amino-terminal residue results obtained from the low pH room temperature experiment (Table III, column 4, Figure 8) suggest that yet another form of chymotrypsin should be distinguished. This form appears in high yield in the low pH conversion of δ-chymotrypsin. Although disc gels show that δ-chymotrypsin is converted almost completely to another form after 48 hr at pH 3.1 and room temperature, the amino-terminal residue data indicate that only about 10% of the molecules have end groups corresponding to those of α and γ. The 1.0 residue of threonine indicates that the Thr-Asn dipeptide has not been split out. This new form, which will be called κ-chymotrypsin, has amino-terminal isoleucine and threonine. Its association, at pH 4.4, is low, nearly the same as that observed for δ-chymotrypsin (Table III, column 2) and it has the same electrophoretic mobility as α- and γ-chymotrypsins.

While it has been known for a long time (Bettleheim and Neurath, 1955) that κ-chymotrypsin exists, it was believed to be a transient intermediate. The results presented in this work indicate that it is quite stable at low pH and low temperature and is readily formed from δ-chymotrypsin at low pH and room temperature. At high pH it appears to convert to γ-chymotrypsin.

The modified activation scheme of Wright *et al.* (1968) differs notably from the earlier scheme of Desnuelle (1960) in proposing that δ-chymotrypsin is not directly involved in the formation of α-chymotrypsin by the slow activation process, but that it does precede α-chymotrypsin in the rapid activation. Our data support this idea in the following way. In the slow activation (for 48 hr) little or no material appeared at the δ-chymotrypsin position on disc gel electrophoresis. However, when δ-chymotrypsin was treated with these slow activation conditions for 48 hr, 50% of the material remained as δ-chymotrypsin. This stability of δ-chymotrypsin under the slow activation conditions, and the absence of δ in the slow activation product, strongly imply that δ-chymotrypsin is not a direct intermediate in the slow activation. Secondly, both the slow activation and rapid activation material can be converted to material having α-chymotrypsin end groups, crystal habit, and dimerization behavior, showing that there exists a pathway for forming α-chymotrypsin from δ-chymotrypsin. Whether this pathway includes γ-chymotrypsin is not clear from our data because our best method for distinguishing α and γ, their differing dimerization, is subject to severe limitations when dealing with mixed systems containing low amounts of α-chymotrypsin. Nevertheless, from the results of these experiments, it seems clear that the modified scheme of Wright *et al.* (1968) is essentially correct. It is desirable, however, to include κ-chymotrypsin between δ- and γ-chymotrypsin in the scheme.

The apparent conversion of γ to α observed in the crystallization experiment is in partial accord with the results of Corey *et al.* (1965). They observed that crystalline γ could be converted to crystalline α by recrystallizing γ at pH 4.0, but the requirement of a 2-month time period for this conversion does not agree with the 24-hr conversion noted here. This difference could be due to the concentration of the enzyme in the crystallization mixture. The concentration in our experiments was about 25 mg/ml, probably higher than in the Corey *et al.* (1965) experiment. Further, Corey *et al.* (1965) started from crystalline γ and we started from a solution of γ that had never been crystallized.

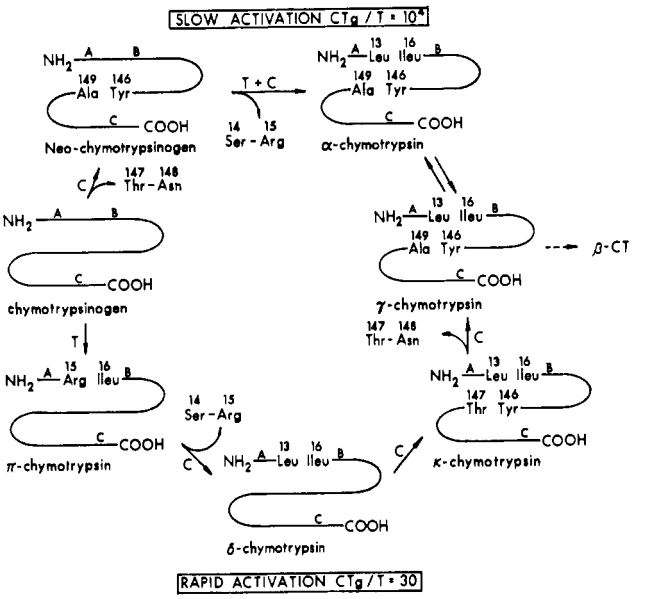

FIGURE 10: New scheme for the activation of chymotrypsinogen. CC denotes a chymotryptic cleavage; TC denotes a tryptic cleavage. The letters A, B, and C refer to the three chains which are formed when the single chain of chymotrypsinogen A undergoes these proteolytic hydrolyses. The three chains are held together by disulfide bridges.

Conclusions

We have shown that α-chymotrypsin is the only one of the activated forms of chymotrypsinogen A which self-associates to a significant extent at low pH. Variability in the dimerization behavior of this enzyme at pH 4.4, 0.01 M acetate and 0.2 M KCl is due to variability in the enzyme preparations rather than experimental errors in measurement of the equilibrium constants. By means of ϵ-amino-*n*-caproyl-D-tryptophan methyl ester affinity columns (Cuatrecasas *et al.*, 1968) used at low temperature, it has been possible to remove nonbinding impurities and increase dimerization constants of impure α-chymotrypsin preparations.

Since the degree of dimerizability of a preparation can be related to nondimerizing impurities by calibration curves, it has been shown that dimerizability can be utilized as an assay for the amount of α-chymotrypsin in an activation mixture; but only if α-chymotrypsin represents more than 50% of the molecules present. This type of assay system, together with more conventional amino-terminal determinations have been used to elucidate some of the characteristics of the mechanism of activation of chymotrypsinogen A.

A new species of chymotrypsin has been isolated and characterized. It is called κ-chymotrypsin and it has *cystine* (assumed), isoleucine (measured), and threonine (measured) amino-terminal residues. It follows δ-chymotrypsin in the rapid activation scheme and it does not form dimers at pH 4.4, 0.2 M ionic strength. Remarkably, this species is produced almost quantitatively from δ-chymotrypsin at pH 3.1 and room temperature in 48 hr. These results indicate that δ-chymotrypsin is not a stable intermediate in the rapid activation of chymotrypsinogen, and, it may be speculated that many of the enzymatic properties attributed to δ-chymotrypsin are those of κ-chymotrypsin.

As a result of this study we propose a new scheme for the activation of chymotrypsinogen A as shown in Figure 10. The scheme differs from that of Wright *et al.* (1968) only by

the inclusion of κ-chymotrypsin. Since κ-chymotrypsin can be converted to α-chymotrypsin and γ-chymotrypsin simply by variation of the crystallization conditions (this work and Corey *et al.*, 1965), we feel that the $\alpha \rightleftarrows \gamma$ isomerization of Figures 1 and 10 has been established. Finally, the proposal that α-chymotrypsin arises chiefly from neochymotrypsinogen in the slow activation (Wright *et al.*, 1968) has been supported since trypsin-free δ-chymotrypsin is relatively stable under slow activation conditions, whereas, under classical (Kunitz and Northrop, 1936) slow activation conditions, containing low amounts of trypsin, almost no δ-chymotrypsin exists. Nevertheless, many details of the slow activation pathway from chymotrypsinogen A to α-chymotrypsin remain obscure.

Appendix

Peptide Synthesis. D-Tryptophan methyl ester hydrochloride (15 g, 0.059 mole) (Nutritional Biochemicals) was mixed with 120 ml of dichloromethane (Eastman) and 8.25 ml (0.059 mole) of triethylamine (Eastman). Carbobenzoxy-ε-amino-*n*-caproic acid (20.8 g, 0.075 mole, 1.3 equiv) (Cyclo Chemicals) was dissolved in 120 ml of dichloromethane. The two solutions were mixed whereupon a precipitate formed (probably the salt discussed by Schröder and Lübke, 1965) but was not removed. Melted dicyclohexylcarbodiimide (15.4 g, 0.075 mole, 1.3 equiv) (Eastman) was added, the solution was deaerated by bubbling N_2 through it for 10 min, and the flask was sealed and allowed to stand with stirring for 15 hr at room temperature. Then, 10 ml (0.175 mole, 3 equiv) of glacial acetic acid was added and the solution was stirred for another hour. Ethyl acetate (750 ml, 3 volumes) was added and the resultant solution cooled to −10° and allowed to stand 1 hr at −10°. The precipitate was filtered and discarded. The filtrate was extracted twice with 600 ml of 1 N HCl, twice with 600 ml of 10% $NaHCO_3$, and twice with 600 ml of 10% KCl. The organic phase was then dried twice by mixing it with 200 g of anhydrous Na_2SO_4 and filtering. The solvent was evaporated at 60° or less (rotary evaporation) to give an oil which was further dried *in vacuo* over "Tel Tale" desiccant to yield a yellow-brown carmel. Thin-layer chromatography on 5% methanol in chloroform showed one major I_2 staining spot with an R_F of 0.7 and a faint spot trailing this main one. The yield of 280-nm-absorbing material was 70%.

Deblocking the Peptide. The carbobenzoxy-ε-amino-*n*-caproyl-D-tryptophan methyl ester (0.0412 mole) was dissolved in anhydrous methanol to a final volume of 50 ml; 2.84 ml (0.050 mole, 1.2 equiv) of glacial acetic acid and 2.06 g of methanol-rinsed palladium oxide catalyst (Mattheson, Coleman & Bell) were added and the solution subjected to 30 psi of hydrogen for 16 hr on a Parr pressure reaction apparatus. The reaction mixture was filtered through Celite on a sintered-glass funnel and the filtrate was evaporated at 60° or less. The resultant oil was suspended in 200 ml of water and extracted twice with 60 ml of ethyl acetate to remove unreacted carbobenzoxy peptide. The ethyl acetate remaining in the aqueous fraction was removed by rotary evaporation and the remaining solution was lyophilized. The brownish carmel was dissolved in butanol–acetic acid–water (4:1:1, v/v) and applied to a 5 × 48 cm silica gel column (0.05–0.2 mm mesh, E. M. Reagents Division, Brinkman Instruments) equilibrated with the same solvent. The 700–1100-ml effluent fraction, containing 84% of the applied 280-nm absorbance, was lyophilized, dissolved in 200 ml of water, and extracted three times with 300 ml of ethyl acetate to remove the butanol. The aqueous portion was lyophilized to give 9.2 g (0.028 mol) of a tan powder corresponding to an overall yield of 47%.

The purity of the final product was estimated to be greater than 90% by thin-layer chromatography (butanol–acetic acid–water, 4:1:1, v/v), high-voltage paper electrophoresis at pH 6.5, ultraviolet spectrum, and elemental analysis.

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