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Activation of Bovine Chymotrypsinogen A. Isolation and Characterization of μ- and ω-Chymotrypsin†

Satish K. Sharma‡ and Thomas R. Hopkins*

ABSTRACT: Threonine-neochymotrypsinogen and alanine-neochymotrypsinogen were prepared by limited hydrolysis of bovine chymotrypsinogen A with α-chymotrypsin. The two neochymotrypsinogens were then activated under conditions designed to trap the immediate protein species arising from the cleavage of the Arg^{15}-Ile^{16} bond. Two trapping procedures were used. In one procedure the neochymotrypsinogen was activated with trypsin under classical rapid activation conditions but in the presence of a competitive inhibitor of chymotryptic action, β-phenylpropionate. In the other procedure, the neochymotrypsinogen was activated with an acid proteinase isolated from *Aspergillus oryzae* using pH conditions which inhibit autolytic activity. Both were successful in preventing autolytic attack of the initial protein and both yielded identical results. The immediate protein species obtained from activated threonine-neochymotrypsinogen, called μ-chymotrypsin, had Thr, Ile, and half-cystine as amino-terminal amino acids. The specific esterase activity of μ-chymotrypsin toward *N*-acetyl-L-tyrosine ethyl ester was two times greater than its stable autolytic product, α_1-chymotrypsin. Similarly, the immediate active species of alanine-neochymotrypsinogen, called ω-chymotrypsin, had Ala, Ile, and half-cystine as amino-terminal amino acids and also displayed an esterase activity which was two times greater than its stable autolytic product, α-chymotrypsin. The first-order rate constants of denaturation in 8 M urea for these two new enzyme species were 0.8 min^{-1} for μ-chymotrypsin and 1.4 min^{-1} for ω-chymotrypsin, respectively. These rate constants differ from all previously known species of chymotrypsin. The genesis of μ- and ω-chymotrypsin and their relationship to current schemes for the activation of bovine chymotrypsinogen A are discussed.

The classical rapid activation of bovine chymotrypsinogen A with trypsin occurs in two sequential steps: the mandatory tryptic hydrolysis of the Arg^{15}-Ile^{16} bond (open arrow in Figure 1) yielding an active but unstable intermediate chymotrypsin called π-chymotrypsin, followed by the rapid autolytic cleavage of the Leu^{13}-Ser^{14} bond (solid arrow in Figure 1) to produce δ-chymotrypsin (Jacobsen, 1947; Bettelheim & Neurath, 1955). Two other bonds in chymotrypsinogen are also subject to chymotryptic hydrolysis and are involved in the slow activation processes of bovine chymotrypsinogen to α-chymotrypsin (Rovery et al., 1957; Desnuelle, 1960), the conversion of δ-chymotrypsin to κ-chymotrypsin and to α-chymotrypsin (Miller et al., 1971; Avery & Hopkins, 1973; Sharma & Hopkins, 1978a), and the production of threonine-neochymotrypsinogen and alanine-neochymotrypsinogen from chy-

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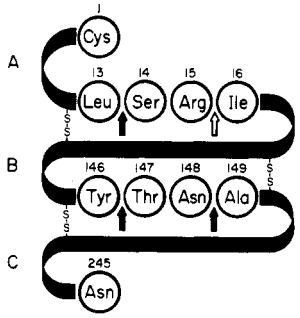

FIGURE 1: Bonds cleaved during the activation of bovine chymotrypsinogen A by trypsin (15–16) and chymotrypsin (13–14, 146–147, 148–149). The number corresponds to the position of various residues in the protein chain. Cleavage of bonds 15–16; 15–16 and 13–14; 15–16, 13–14, and 146–147; or 15–16, 13–14, 146–147, and 148–149 give rise to π-, δ-, α_1-(κ-), or α-(γ-)chymotrypsin, respectively. Two- and three-chain proteins are covalently linked together by disulfide bonds.

motrypsinogen A (Rovery et al., 1955; Valenzuela & Bender, 1973; Sharma & Hopkins, 1978b).[1]

The transient intermediate, π-chymotrypsin, can be trapped by carrying out rapid activation of chymotrypsinogen A with trypsin in the presence of a potent chymotrypsin inhibitor such as β-phenylpropionate (Bettelheim & Neurath, 1955). The same intermediate can also be obtained by activating chymotrypsinogen A with an acid proteinase isolated from *Aspergillus oryzae* (Davidson et al., 1975). Under acid conditions and 4 °C, the autolysis of π-chymotrypsin to δ-chymotrypsin is prevented.

Analogous to the two-step, rapid activation of chymotrypsinogen A to δ-chymotrypsin, others have speculated that π-chymotrypsin-like transient intermediates should also be produced during the rapid activation of threonine-neochymotrypsinogen to α_1-chymotrypsinogen (Bender & Killheffer, 1973) and alanine-neochymotrypsinogen to α-chymotrypsin (Sharma & Hopkins, 1978b). In this report threonine-neochymotrypsinogen and alanine-neochymotrypsinogen are activated under conditions designed to trap transient, active intermediates. The intermediates are purified and partially characterized.

Methods

Crystalline preparations of bovine pancreas chymotrypsinogen A, α-chymotrypsin, γ-chymotrypsin, bovine pancreas trypsin, and soybean trypsin inhibitor were purchased from Mann Biochemicals and Sigma Chemical Co. Because preparations of δ-chymotrypsin are impure (Avery & Hopkins, 1973), δ-chymotrypsin was prepared fresh by rapid activation of bovine chymotrypsinogen A with trypsin (Bettelheim & Neurath, 1955). Threonine-neochymotrypsinogen and α_1-chymotrypsin were prepared by a method described by Valenzuela & Bender (1973) with minor modifications (Sharma & Hopkins, 1978a). Alanine-neochymotrypsinogen was prepared as described earlier (Sharma & Hopkins, 1978b). A crude preparation of acid proteinase from *Aspergillus oryzae* was obtained from Sigma Chemical Co. and purified according to the procedure of Davidson et al. (1975).

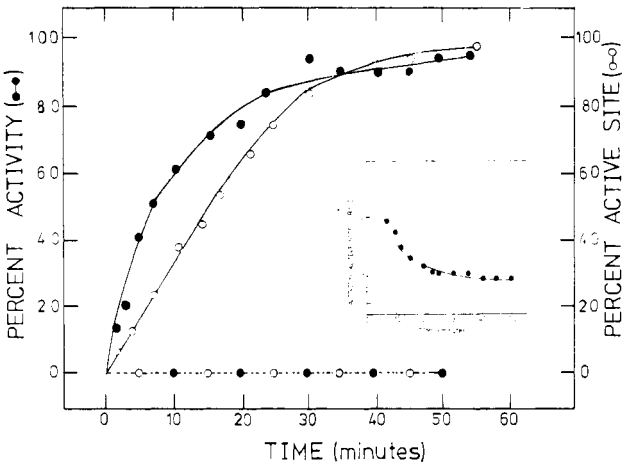

FIGURE 2: Change in activity with time during trypsin activation of threonine-neochymotrypsinogen under classical rapid activation conditions. The left ordinate (●—●) is the percent esterase activity determined with *N*-acetyl-L-tyrosine ethyl ester. The right ordinate (○—○) is the percent active sites determined by *N-trans*-cinnamoylimidazole titrations. Lower symbols (○----●----○----●) show the effect of incubating threonine-neochymotrypsinogen in the presence of trypsin inhibitor. (Insert) The change in ratio of esterase activity to active sites during the trypsin activation of threonine-neochymotrypsinogen.

Protein concentrations of members of the chymotrypsinogen family were determined spectrophotometrically at 280 nm using a molar extinction coefficient of 5×10^4 M^{-1} cm^{-1} (Dixon & Neurath, 1957; Wilcox et al., 1957). Enzyme activities were measured spectrophotometrically using *N*-acetyl-L-tyrosine ethyl ester and benzoyl-L-tyrosine ethyl ester as substrates (Schwert & Takenaka, 1955; Hummel, 1959). The moles of active sites per mole of enzyme was determined by titration with *N-trans*-cinnamoylimidazole (Schonbaum et al., 1961). Polyacrylamide gel electrophoresis in the presence of sodium lauryl sulfate was performed by the general procedure of Weber et al. (1972). Quantitation of NH_2-terminal amino acid residues was done using a fluorometric procedure described by Zanetta et al. (1970). The NH_2-terminal half-cystine, present in all chymotrypsinogen derivatives, is refractory to the above method of NH_2-terminal analysis. It is assumed to be present in all NH_2-terminal analysis results given below. The kinetics of denaturation in 8 M urea was determined using the procedure of Hopkins & Spikes (1967, 1968). Denaturation conditions were 8 M urea, 0.25 M sodium phosphate buffer, pH 7.3 at 30 °C.

Results and Discussion

Activation of Threonine-neochymotrypsinogen

A. Activation with Trypsin. Threonine-neochymotrypsinogen was activated in the same manner as the rapid activation of chymotrypsinogen A described by Jacobsen (1947) and others (Bettelheim & Neurath, 1955). The zymogen was dissolved in ice cold 0.1 M sodium phosphate buffer, pH 7.6, at concentrations of 30 to 40 mg/mL. Activation was initiated by adding trypsin to give a 30:1 (w/w) ratio of chymotrypsinogen A to trypsin. The time course of activation at 4 °C was followed by assaying for chymotrypsin activity using *N*-acetyl-L-tyrosine ethyl ester for the kinetic assay and *N-trans*-cinnamoylimidazole for active site titration. The results are shown in Figure 2.

In agreement with an earlier study by Valenzuela & Bender (1973), activation of threonine-neochymotrypsinogen to α_1-

[1] The activation scheme of bovine chymotrypsinogen is further complicated by the fact that α-chymotrypsin is a conformational isomer of γ-chymotrypsin (Corey et al., 1965). Similarly, κ-chymotrypsin is a conformer of α_1-chymotrypsin (Sharma & Hopkins, 1978a). One form can isomerize to the other depending upon the pH of the medium. In the acid pH range, α- and κ-chymotrypsin are the stable forms and, at neutral pH values, γ- and α_1-chymotrypsin are favored. In solution both of these two pairs of conformers can be distinguished from one another by their individual rates of denaturation in 8 M urea (Avery & Hopkins, 1973; Sharma & Hopkins, 1978a).

Table I: Activation of Threonine-neochymotrypsinogen and Alanine-neochymotrypsinogen in the Presence and Absence of β-Phenylpropionate

zymogen	activating enzyme	β-phenyl-propionate (2 mM)	k_u[c] (min^{-1})	NH_2 terminals (mol/mol of protein)		
				Ile	Thr	Ala
Thr-neochymotrypsinogen	trypsin[a]	−	1.6	0.88	0.80	0.00
	trypsin[a]	+	0.8	0.86	0.82	0.00
	acid proteinase[b]	−	0.8	0.90	0.86	0.00
Ala-neochymotrypsinogen	trypsin[a]	−	1.8	0.90	0.00	0.88
	trypsin[a]	+	1.4	0.86	0.00	0.82
	acid proteinase[b]	−	1.4	0.92	0.00	0.90

[a] Activation was carried out for 90 min in 0.1 M phosphate buffer, pH 7.6 at 4 °C. [b] Activation was carried out for 4 h in 0.1 M sodium formate buffer, pH 3.6 at 4 °C. [c] k_u value for threonine-neochymotrypsinogen is 0.6 min^{-1} (Sharma & Hopkins, 1978b), for alanine-neochymotrypsinogen is 1.0 min^{-1} (Sharma & Hopkins, 1978b), for α-chymotrypsin is 1.6 min^{-1} (Sharma & Hopkins, 1978a), and for α-chymotrypsin is 1.8 min^{-1} (Avery & Hopkins, 1973).

chymotrypsin was completed in 60 min. Similar times for maximum activation have also been found in the rapid activation of chymotrypsinogen A and chymotrypsinogen B (Guy et al., 1966). When the activation was attempted in the presence of soybean trypsin inhibitor, no activation was observed.

There are significant differences in the details of the activation curve as measured by kinetic assay and active site titration. The time course of this difference is brought out in the insert of Figure 2. The greater initial increase in activity measured by kinetic assay relative to production of active sites can be explained by assuming that an intermediate enzyme species having two times greater specific activity than α_1-chymotrypsin was formed during the early stages of the activation process. Unlike kinetic enzyme assays using good substrates, active site titrations are insensitive to the turnover number of the enzyme. This interpretation is not without precedence. It has been known for some time that, during the rapid activation of chymotrypsinogen A to δ-chymotrypsin, a transient enzyme species, π-chymotrypsin, is produced and that it has a greater specific activity than its stable autolysis product (Jacobsen, 1947; Bettelheim & Neurath, 1955).

The NH_2-terminal amino acids and a first-order rate constant of denaturation in 8 M urea, k_u, of the stable product of trypsin-catalyzed activation of threonine-neochymotrypsinogen are given in Table I. These properties are characteristic of α_1-chymotrypsin, as was also concluded in earlier work (Valenzuela & Bender, 1973; Sharma & Hopkins, 1978a).

B. Activation with Trypsin in the Presence of β-Phenylpropionate. The competitive inhibitor of chymotrypsin, β-phenylpropionate, suppresses autolytic cleavage in chymotrypsin and has been used to trap π-chymotrypsin during the two-step conversion of chymotrypsinogen A to δ-chymotrypsin (Bettelheim & Neurath, 1955). Using the same strategy, an intermediate chymotrypsin species formed during the two-step activation of threonine-neochymotrypsinogen to α_1-chymotrypsin was isolated.

The results of the activation in the presence of 2 mM β-phenylpropionate are summarized in Table I. The active protein species trapped in the presence of inhibitor displayed a twofold lower rate of denaturation in 8 M urea than α_1-chymotrypsin. Its NH_2-terminal amino acids were Ile, Thr, and half-cystine (assumed). The new chymotrypsin species is assigned the name μ-chymotrypsin. Other properties of μ-chymotrypsin are examined in a subsequent section.

C. Activation with Acid Proteinase. Davidson et al. (1975) used an acid proteinase purified from *Aspergillus oryzae* to activate bovine chymotrypsinogen A by a single step hydrolysis of the Arg^{15}-Ile^{16} bond, thus producing π-chymotrypsin. This transient enzyme species remained stable as long as the pH of the medium was kept low enough to prevent autolysis. This approach was used to activate threonine-neochymotrypsinogen.

Purified neochymotrypsinogen was dissolved in ice-cold 0.1 M sodium formate buffer, pH 3.6 at a concentration of 10 mg/mL. Activation was initiated by adding purified *Aspergillus oryzae* acid proteinase to a final concentration of 1 mg/mL. Aliquots were taken from the activation mixture during the incubation at 4 °C and assayed for enzyme activity and active site content. Maximum activity was reached in 3-h incubation. Unlike the two-step tryptic activation of threonine-neochymotrypsinogen to α_1-chymotrypsin above, both kinetic enzymatic activity and active sites increased in a parallel manner (data not shown). As seen in Table I, the NH_2-terminal amino acids and the first-order rate constant of denaturation in 8 M urea were indistinguishable from those of μ-chymotrypsin produced by tryptic activation of threonine-neochymotrypsinogen in the presence of β-phenylpropionate. As was concluded in an analogous study of the activation of chymotrypsinogen A with acid proteinase (Davidson et al., 1975), the data in Table I are consistent with an activation process involving cleavage of a single Arg^{15}-Ile^{16} bond.

Activation of Alanine-neochymotrypsinogen

A. Activation with Trypsin. Using experimental conditions identical with those used to activate threonine-neochymotrypsinogen to α_1-chymotrypsin, the kinetics of activation of alanine-neochymotrypsinogen were studied. As shown in Figure 3, maximum activity was reached in about 60 min. No activation was observed when soybean trypsin inhibitor was included in the activation mixture. Like the activation of threonine-neochymotrypsinogen to α_1-chymotrypsin, there was a significant difference in the activation kinetics as measured by kinetic activity measurements and by active site titration. The time course of the difference is emphasized in the figure insert. The apparent faster rate of activation as measured by the kinetic assay indicates that an active intermediate enzyme species having a specific activity about two times greater than α-chymotrypsin was produced in the early stages of the activation process. The rate of tryptic activation of alanine-neochymotrypsinogen was comparable to rates of activation of threonine-neochymotrypsinogen and chymotrypsinogen A (Bettelheim & Neurath, 1955; Valenzuela & Bender, 1971).

The activation of alanine-neochymotrypsinogen by trypsin has been reported earlier and the active product has been identified as α-chymotrypsin (Sharma & Hopkins, 1978b). The properties of the active product examined in this study (see Table I) are consistent with their conclusions.

B. Activation with Trypsin in the Presence of β-Phenyl-

Table II: Autolysis of μ-Chymotrypsin and ω-Chymotrypsin

reaction	β-phenyl-propionate (2 mM)	k_u (min^{-1})[a] before incuba-tion	k_u (min^{-1})[a] after incuba-tion	NH_2 terminals[b] (mol/mol of protein) Ile	Thr	Ala	sp act.[c] (BTEE)
μ-CT $\xrightarrow{\text{pH 7.1}}$ α_1-CT[d]	−	0.80	1.60	0.82	0.86	0.00	81
μ-CT (at pH 3, control)	−	0.80	0.76	0.88	0.90	0.00	94
μ-CT (at pH 7.1, inhibitor control)	+	0.80	0.78	0.87	0.83	0.00	nd
μ-CT $\xrightarrow{\text{pH 7.1}}$ α-CT	−	1.40	1.80	0.82	0.00	0.86	52
ω-CT (at pH 3, control)	−	1.40	1.30	0.90	0.00	0.81	70
ω-CT (at pH 7.1, inhibitor control)	+	1.40	1.40	0.84	0.00	0.85	nd

[a] Denaturation conditions: 8 M urea, 0.25 M sodium phosphate, pH 7.3 at 30 °C. [b] Determined after the incubation period. [c] The activity is expressed in μmol of benzoyl-L-tyrosine ethyl ester (BTEE) hydrolyzed per min per mg of protein. All assays were done at the end of incubation period. nd, not determined. [d] In this table the several chymotrypsin species have been abbreviated as CT.

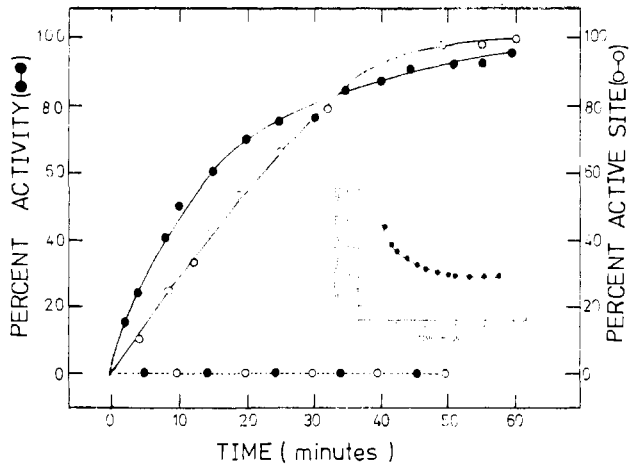

FIGURE 3: Change in activity with time during trypsin activation of alanine-neochymotrypsinogen under classical rapid activation conditions. The left ordinate (●—●) is the percent esterase activity determined with *N*-acetyl-L-tyrosine ethyl ester. The right ordinate (o—o) is the percent active site determined by *N-trans*-cinnamoylimidazole titrations. Lower symbols (●----o----●----o) show the effect of incubating alanine-neochymotrypsinogen in the presence of trypsin inhibitor. (Insert) The change in ratio of esterase activity to active sites during trypsin activation of alanine-neochymotrypsinogen.

propionate. The above results suggested that an active but transient enzyme intermediate was produced during the conversion of alanine-neochymotrypsinogen to α-chymotrypsin. Therefore the activation of alanine-neochymotrypsinogen with trypsin was done in the presence of a chymotrypsin inhibitor in order to trap the intermediate.

Table I lists some properties of the active product obtained from tryptic attack of alanine-neochymotrypsinogen in the presence of 2 mM β-phenylpropionate. The product had a unique rate constant of denaturation in 8 M urea of 1.4 min^{-1} and the NH_2-terminal amino acids were Ala, Ile, and half-cystine (assumed). This new species of chymotrypsin is assigned the name ω-chymotrypsin. Other properties of ω-chymotrypsin are examined in a subsequent section.

C. Activation with Acid Proteinase. Using experimental conditions used to activate threonine-neochymotrypsinogen with *Aspergillus oryzae* acid proteinase, alanine-neochymotrypsinogen was fully activated in 3 h. No activation was observed when alanine-neochymotrypsinogen was incubated at 4 °C in the absence of acid proteinase. The new enzyme had a rate constant of denaturation in 8 M urea and NH_2-terminal amino acids like that of the ω-chymotrypsin produced by activation of alanine-chymotrypsinogen with trypsin in the presence of β-phenylpropionate.

Preparation and Autolysis of μ- and ω-Chymotrypsin

On the basis of NH_2-terminal analysis and rates of denaturation in 8 M urea, it appeared that the chymotrypsin produced by tryptic activation of the appropriate neochymotrypsinogen in the presence of β-phenylpropionate and the active chymotrypsin produced by activation with *Aspergillus oryzae* acid proteinase were the same transient species. Additional support for this conclusion came from the demonstration that α_1- and α-chymotrypsin were the respective autolytic products of μ- and ω-chymotrypsin. It will be recalled that α_1- and α-chymotrypsin are the products of the two-step, rapid tryptic activation of threonine-neochymotrypsin and alanine-neochymotrypsinogen, respectively (Sharma & Hopkins, 1978b).

The two new chymotrypsins were produced by the acid proteinase procedure and purified by chromatography on DEAE-cellulose. Sixty milligrams of each neochymotrypsinogen was activated with acid proteinase as described above except that the buffer solution was 0.05 M sodium formate buffer, pH 3.1. After 4-h incubation at 4 °C, the mixture was applied to a DEAE-cellulose column (32 × 2.5 cm) previously equilibrated with 0.05 M sodium acetate buffer, pH 3.5. The column was developed with the same buffer at a flow rate of 50 mL/h. The chymotrypsin was eluted in the breakthrough volume, whereas acid proteinase was retained on the resin and could be eluted only by a tenfold increase in buffer concentration. Pooled fractions containing the chymotryptic activity were desalted by passing through a column of Sephadex G-25 (35 × 2.6 cm) equilibrated with 1 mM HCl. The purified protein was stored in lyophilized form. Each of the two new enzymes appeared to be homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and filtration through a column of Sephadex G-25. Titration of the active site of purified μ-chymotrypsin gave 0.92 mol of active sites/mol and for purified ω-chymotrypsin gave 0.86 mol of active sites/mol.

Autolyses of these two chymotrypsins were initiated by incubating each enzyme at a concentration of 25 mg/mL in 0.1 M phosphate buffer, pH 7.1 at 4 °C for 2 h. As seen in Table II, autolysis of μ-chymotrypsin was accompanied by an increase in the rate constant for denaturation in 8 M urea, k_u, from 0.80 to 1.6 min^{-1}. The k_u value of the autolytic product was indistinguishable from that of α_1-chymotrypsin. As expected for a conversion of μ-chymotrypsin to α_1-chymotrypsin (Sharma & Hopkins, 1978b), there was no change in the NH_2-terminal amino acid residues. The autolytic product of μ-chymotrypsin also had a lower specific esterase activity than that of its parent compound. The change in properties observed during the incubation at pH 7.1 was due to autolytic

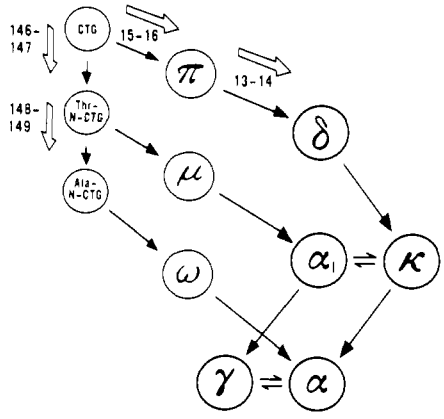

FIGURE 4: A scheme of activation of bovine chymotrypsinogen A. Peptide bonds cleaved are indicated by residue numbers drawn by the top and left side of the activation scheme. Also shown are two reversible reactions (↔) not involving peptide bond cleavage. These later reactions are pH-dependent conformational isomerizations of chemically identical enzyme species (Sharma & Hopkins, 1978a).

action because a control reaction mixture which contained β-phenylpropionate remained unchanged. Taken together, the evidence supports the conclusion that α_1-chymotrypsin is the stable autolytic product of μ-chymotrypsin.

In a manner similar to μ-chymotrypsin, autolysis of ω-chymotrypsin was accompanied by an increase in k_u from 1.4 to 1.8 min^{-1}, no change in the NH_2-terminal amino acids, and a decrease in specific esterase activity (see Table II). In the presence of the inhibitor, β-phenylpropionate, no changes in the above properties were observed. The properties of the autolysis product of ω-chymotrypsin closely resembled those of authentic α-chymotrypsin.

Revised Activation Scheme of Chymotrypsinogen A

The above activation studies support the activation schemes: threonine-neochymotrypsinogen → μ-chymotrypsin → α_1-chymotrypsin and alanine-neochymotrypsinogen → ω-chymotrypsin → α-chymotrypsin. These pathways have been incorporated into the activation scheme of bovine chymotrypsinogen A shown in Figure 4. In this scheme, based upon an earlier, partially hypothetical scheme proposed by Sharma & Hopkins (1978a), only *proven* pathways and chymotrypsinogen family members have been incorporated.

The transient nature of π-, μ-, and ω-chymotrypsin in neutral solutions indicates that these chymotrypsins have a high affinity for cleavage of their own Leu^{13}-Ser^{14} bond. Once the Ser^{14}-Arg^{15} dipeptide is removed, the evolving enzymes (δ-, α_1-, and α-chymotrypsin) display decreased specific activities and relatively good resistance to further autolysis. The autolytic cleavage of peptide bonds in the bovine chymotrypsins is not random. The preferred order of autolytic cleavage is the Leu^{13}-Ser^{14} bond followed by the Tyr^{146}-Thr^{147} bond and then the Asn^{148}-Ala^{149} bond. This is best illustrated in the known pathway: π → δ → κ → α (Bettelheim & Neurath, 1955; Miller et al., 1971; Sharma & Hopkins, 1978a). If special conditions are used to create an active chymotrypsin having one or two of the above peptide bonds already cleaved, then the remaining bonds are autolyzed in the preferred order, hence the pathways μ → α_1 → γ and ω → α (Sharma & Hopkins, 1978a, present studies).

Some previously proposed pathways of activation can now be rejected using kinetic data and pH considerations. Activation of threonine-neochymotrypsinogen to α_1-chymotrypsin cannot proceed via the route, threonine-neochymotrypsinogen → μ → κ → α_1, because the rate of isomerization of κ-chymotrypsin to α_1-chymotrypsin, although favored at neutral pH, is far too slow to account for the complete conversion of threonine-neochymotrypsinogen to α_1-chymotrypsin within 90 min (Valenzuela & Bender, 1973; Sharma & Hopkins, 1978a). A similar argument can be applied in rejecting the activation sequence: alanine-neochymotrypsinogen → ω → γ → α. Again, the rate of isomerization of γ-chymotrypsin to α-chymotrypsin is orders of magnitude too slow and, in this case, the isomerization is not favored at neutral pH (Corey et al., 1965).

The exact sequence of bond cleavage during the classical slow activation of bovine chymotrypsinogen A to α-chymotrypsin is not known. However, it is generally believed that one or more neochymotrypsinogen species are intermediates in the activation scheme (Desnuelle, 1960; Wright et al., 1968; Miller et al., 1971; Bender & Killheffer, 1973; Sharma & Hopkins, 1978a,b). Further refinements in the activation scheme for the conversion of chymotrypsinogen A to α-chymotrypsin by slow activation with trypsin can be made. Hypothetical pathways which incorporate α_1-chymotrypsin as an intermediate should be rejected because α_1-chymotrypsin is neither autolyzed nor isomerized to any significant extent in the 24–48-h period of the slow activation process (Sharma & Hopkins, 1978a). Likewise all hypothetical pathways which include γ-chymotrypsin are unsuitable because of the extremely slow isomerization rate of γ-chymotrypsin to α-chymotrypsin (Corey et al., 1965).

Reactions where the Leu^{13}-Ser^{14} bond is hydrolyzed in chymotrypsinogen, threonine-neochymotrypsinogen, or alanine-neochymotrypsin, to the respective serine-neochymotrypsinogen, threonine,serine-neochymotrypsinogen or alanine,serine-neochymotrypsinogen (see activation scheme in Sharma & Hopkins, 1978a), are not likely because the Leu^{13}-Ser^{14} bond in chymotrypsinogen is resistant to proteolysis at 4 °C (Rovery et al., 1957; Rovery & Bianchetta, 1972). Furthermore, Sharma & Hopkins (1978a,b) did not detect any neochymotrypsinogens containing an NH_2-terminal serine during the preparation of threonine- and alanine-neochymotrypsinogen by limited chymotryptic proteolysis of chymotrypsinogen at 4 °C.

This leaves the following description of the classical slow activation of chymotrypsinogen. The initial activation step is the tryptic cleavage of some of the chymotrypsinogen to π-chymotrypsin. The π-chymotrypsin then rapidly autolyzes to δ-chymotrypsin. As δ-chymotrypsin accumulates in the reaction mixture, the main reaction shifts to the formation of threonine-neochymotrypsinogen from the remaining chymotrypsinogen. Further proteolysis by δ-chymotrypsin leads to alanine-neochymotrypsinogen. Finally, tryptic attack forms ω-chymotrypsin from alanine-neochymotrypsinogen and rapid autolysis gives α-chymotrypsin. It is likely that there is also a minor pathway during slow activation of chymotrypsinogen. The δ-chymotrypsin produced during the early stages of the slow activation process undergoes autolytic hydrolysis to α-chymotrypsin (Avery & Hopkins, 1973) with the intermediate in this two-step process being κ-chymotrypsin (Miller et al., 1971; Sharma & Hopkins, 1978a).

The above scheme for the activation of chymotrypsinogen only applies to special activation conditions created in the laboratory. Obviously, in vivo activation of the zymogen does not occur at 4 °C nor are the concentrations of chymotrypsinogen in the gut likely to be 1–5%. Furthermore, food protein in the gut probably interferes with several of the lytic reactions presented in the scheme shown in Figure 4. The initial active species of chymotrypsin produced from chymotrypsinogen may

bind tightly to protein particles in the masticated food (Archer et al., 1973; Bhumiratana et al., 1977), thus suppressing the autolytic conversion of π- and δ-chymotrypsin to other active chymotrypsin species or the hydrolysis of chymotrypsinogen to neochymotrypsinogen.

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Stability of Polysome-Associated Polyadenylated RNA from Soybean Suspension Culture Cells†

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ABSTRACT: The half-life of polysome-associated, poly(A)-RNA in exponentially growing soybean (*Glycine max*) suspension culture cells was determined with pulse-chase experiments. Based on a best fit from a computer analysis of the data, two decay components for poly(A)-RNA were found. One component had a half-life of approximately 0.6 h, while the other had a half-life of about 30 h, similar to the doubling time of the cultures. At the beginning of the chase period, the short-lived component represented approximately 90% of the total poly(A)-RNA in the polysomes. This percentage decreased with time so that, under steady-state conditions, the long-lived component probably represented the majority of poly(A)-RNA.

The relatively long half-life of messenger RNA in most higher eukaryotic cells allows for the temporal and spatial separation of mRNA and protein synthesis. Regulation of the stability of mRNA through differential turnover rates is a potential mechanism for post-transcriptional control of the steady-state abundance of various turnover species in the cytoplasm and, in turn, the rate of synthesis of various proteins (Kafatos, 1972). In several developmental systems, cell specialization is associated with unusual stability of the mRNA coding for the major protein synthesized by that cell type (Kafatos, 1972). Studies using various mammalian and insect cell cultures have indicated the existence of at least two major half-life components in the polyadenylated messenger RNA population (Singer & Penman, 1973; Pucket et al., 1975; Perry et al., 1975; Spradling et al., 1975).

Plants are known to contain polyadenylated, polysome-associated, heterodisperse RNA (Key & Silflow, 1975; Hammett & Katterman, 1975; Covey & Grierson, 1976), and this RNA has been shown to have mRNA activity in cell-free translation systems (Gray & Cashmore, 1976; Larkins et al., 1976). Direct investigations of the half-life of presumptive

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