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REVIEW

Recent Developments in the Activation Process of Bovine Chymotrypsinogen A

SATISH K. SHARMA[1] AND THOMAS R. HOPKINS

Department of Chemistry, Texas Christian University, Fort Worth, Texas 76129 and Chemicals Branch, Research and Development, Phillips Petroleum Company, Bartlesville, Oklahoma 74004

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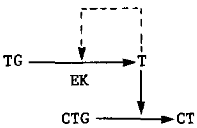
A brief account is given of the history, distribution, and activation events of proteins of the bovine chymotrypsinogen family. Recent developments in the investigation of the activation process of bovine chymotrypsinogen A are discussed, and a revised scheme for the overall activation process is presented.

Many important physiologically active proteins, enzymes, and hormones are synthesized as inactive precursors (zymogens) that are subsequently converted to the active form by the selective enzymatic cleavage of a limited number of peptide bonds in the zymogen structure. The ultimate physiologically expression of a zymogen system is achieved by limited proteolysis, either by a single proteolytic enzyme or by a consecutive series of proteolytic enzymes (cascade). The zymogens of the pancreatic serine proteases, in particular, have served as models for detailed studies of the nature of the molecular changes that are involved in the dramatic increase in enzyme activity that ensues upon limited proteolysis. This phenomenon of induction of biological activity by limited hydrolysis of an inactive precursor is fairly widespread in nature, having been observed in bacterial, yeast, plant, and invertebrate systems (*1–8*). In a way it represents a kind of biochemical amplification and control mechanism of which conversion of bovine chymotrypsinogen A to α-chymotrypsin, under the influence of the enzyme trypsin, is by far the best understood example.

The key to activation of the pancreatic chymotrypsinogen is enterokinase (EK), which is attached to the external side of the brush border membrane (*9*). The initial stage in the two-step cascade process is the conversion of pancreatic trypsinogen (TG) to trypsin (T). The product of the second stage, active trypsin, in turn catalyzes the conversion of chymotrypsinogens (CTG) to chymotrypsins (CT) (*10*), as shown below.



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[1] Author to whom correspondence and reprint requests should be addressed.

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The dotted arrow indicates that trypsin itself can activate trypsinogen autocatalytically. However, whereas trypsin will potentially cleave any lysyl or arginyl bond in the protein substrate, enterokinase seems to be completely specific for that lysyl bond which is preceded by a series of aspartyl residues (*11*). This restricted specificity renders enterokinase resistant to self-digestion during storage in the intestinal cell, in contrast to the case of trypsin, which for its own protection needs to be stored as a zymogen.

BOVINE CHYMOTRYPSINOGENS

Bovine pancreatic juice appears to contain three different chymotrypsinogens: large and nearly equal quantities of two chymotrypsinogens (*12*)—chymotrypsinogen A (CTG A) which is cationic at pH 8, and chymotrypsinogen B (CTG B) which is anionic. Each protein represents about 16% of the total proteins of the juice. Chymotrypsinogen C (CTG C) has been proposed as a more suitable name for the protein originally designated subunit II of bovine procarboxypeptidase (*13*). The amino acid compositions of these chymotrypsinogens is shown in Table 1. Interestingly, the peptide regions involved in proteolytic activation of chymotrypsinogens A, B, and C are highly homologous (*13–17*), as shown below. The vertical arrow denotes the site of proteolysis during zymogen activation.

CTG A	(7 res) . . .	Pro–Val–Leu–Ser–Gly–Leu–Ser–Arg – Ile . . .
CTG B	(7 res) . . .	Pro–Val–Leu–Ser–Gly–Leu–Ala–Arg – Ile . . .
CTG C	(7 res) . . .	Pro–Ser–Tyr–Pro–Pro–Thr–Ala–Arg – Ile . . .

The bovine chymotrypsinogens are briefly reviewed as follows.

Chymotrypsinogen A (CTG A)

Chymotrypsinogen A (CTG A) was first isolated by Kunitz and Northrop (*18*) in 1935. Composed of 245 amino acids, the complete amino acid sequence (*19–22*) and the crystal structure have been determined for the zymogen (*23*). Studies of the primary structure of chymotrypsinogen A got underway in the 1950s with reports (*14, 24, 25*), that the zymogen contained one N-terminal (half-cystine) and one C-terminal (asparagine) and that the protein was therefore a single polypeptide chain. Performic acid oxidation of the ultimate active enzyme derived from chymotrypsinogen A cleaved the disulfide bonds and produced three distinct peptide chains. Called the A, B, and C chains (*26*) the respective amino acid residues were 1–13, 16–146, and 149–245. The 13-residue A chain was sequenced in 1956 by Meedom (*26*), but the B and C chains, containing 131 and 97 residues, respectively, were not elucidated until 1969 (*22*). The two dipeptides released during the conversion of the zymogen to the α form of the enzyme were identified as serine–arginine (*27, 28*) and threonine–asparagine (*29*).

Chymotrypsinogen B (CTG B)

Although difficult to obtain in pure form (*30*), chymotrysinogen B (CTG B) is now commercially available. The amino acid sequence of CTG B (*15*) which, like

TABLE 1

BOVINE CHYMOTRYPSINOGENS: AMINO ACID COMPOSITIONS[a]

	Chymotrypsinogen		
Residue	A	B	C
Ala	22	23	15–16
Arg	4	5	8–9
Asn + Asp	23	20	24–25
Cys	10	10	8
Gln + Glu	15	18	22
Gly	23	23	22
His	2	2	5
Ile	10	9	13
Leu	19	19	20–21
Lys	14	11	7
Met	2	4	1
Phe	6	7	7
Pro	9	13	11
Ser	28	22	14
Thr	23	23	16
Try	8	8	13
Tyr	4	3	7
Val	23	25	19
Amide	23	16	—
Method[b]	s	s	H[c]
Reference	*19, 20, 22*	*15*	*13, 32–35*

[a] Number of amino acid residues per mole protein.

[b] s, From sequence determination; H, by hydrolysis and chromatography.

[c] Rounded to integers by original researcher.

chymotrypsinogen A, contains 245 amino acid residues, is also similar in the sequence (*22*). It appears to coincide at 194 of the 245 amino acid residues (*22*). Trypsin activates CTG B at nearly the same rate as it does CTG A (*31*).

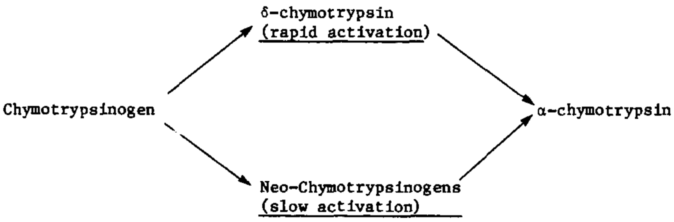
Chymotrypsinogen C

The amino acid composition of this zymogen has been determined and closely resembles that of porcine chymotrypsinogen C (*13, 32–34*). The complete amino acid sequence has not been reported, but the 14 amino acid residues at the amino terminus are highly homologous with those in the same region of the porcine zymogen (*13*). The purification and properties of this protein and its activation product have been reviewed elsewhere (*35*).

The bulk of research on chymotrypsins has been done on members of the enzyme family derived from bovine pancreas chymotrypsinogen A (*36*), probably because of the ready availability of crystalline preparations. The other bovine chymotrypsins B and C and the chymotrypsins of the other species have received much less attention. For a survey of chymotrypsinogens from other species the interested reader is referred to a review by Bender and Killheffer (*37*).

THE ACTIVATION PROCESS OF CHYMOTRYPSINOGEN A

Bovine chymotrypsinogen apparently is activated *in vivo* by trypsin. However, thrombokinase (*38*), papain (*39*), subtilisin (*40, 41*), and aspergillopeptidase (*42*) are also capable of activating bovine chymotrypsinogen A. Initially, the chemical events of activation were identified by end group analysis (*24, 29, 43–50*), by isolation of the liberated peptides (*24, 27–29, 50*) and by electrophoretic studies (*24, 44, 49*). Based on these findings, an integrated activation scheme was proposed for the first time by Rovery *et al.* (*29*). Three years later, Desnuelle (*51*) proposed that the two pathways called "rapid" and "slow" activation both yield α-chymotrypsin as the final end product but that the two pathways proceed through different intermediates:



The complexity of chymotrypsinogen activation was apparent in the very first papers published on the subject (*18, 52*). Three crystalline forms, α-, β-, and γ-chymotrypsins were described by Kunitz and Northrup (*19, 52*). Then later, two more forms (π- and δ-chymotrypsin) were added to these from the activation studies of Jacobsen (*53*). This advance was followed by systematic studies on the limited proteolytic attack of chymotrypsin on chymotrypsinogen, performed by Rovery and co-workers (*29, 54*), which pinpointed the four peptide bonds involved in the entire activation mechanism. These four peptide bonds are depicted in Fig. 1. Absolutely essential to the activation process is the hydrolytic cleavage of the $-Arg_{15}-Ile_{16}-$ bond. This step frees the H_2N-terminal isoleucine which is involved in the formation of a functional binding site in the enzyme (*55*). Calculations show that the number of species having combinations of zero to all four possible bonds split is 16. Eight of these are zymogens, and the other eight are active chymotryptsins. A somewhat more conservative analysis of possible members of the chymotrypsinogen family is shown in Fig. 2.

As a result of many years of work, the activation pathways and various intermediates are now fairly well characterized, as will be described later in this article. Since the first activation scheme of Rovery *et al.* (*29*), there have been numerous modifications in the activation scheme (*37, 51, 56–61*). Table 2 summarizes those activation schemes relevant to the *in vivo* process, which appeared during the last two decades.

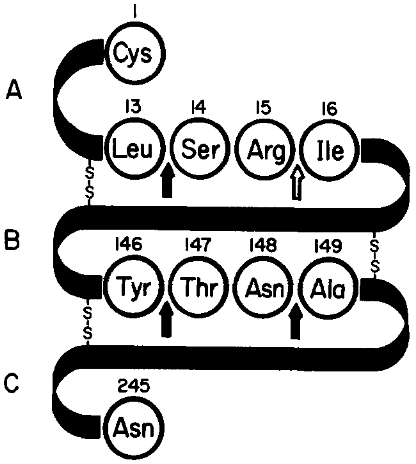


FIG. 1. Bonds cleaved during the activation of chymotryptinogen A by trypsin ($-Arg_{15}-Ile_{16}-$) and chymotrypsin ($-Leu_{13}-Ser_{14}-$, $-Tyr_{146}-Thr_{147}-$, $-Asn_{148}-Ala_{149}-$). The number corresponds to the position of various residues in the protein chain. Two- and three-chain proteins are covalently linked together by disulfide bonds. Reproduced from Sharma and Hopkins (*61*) with permission of the publisher.

CLASSICAL SLOW ACTIVATION

The classical slow activation process developed by Kunitz and Northrop (*18, 62*) produced α-chymotrypsin. In their method, a 2.5% solution of chymotrypsinogen in 0.1 *M* phosphate buffer, pH 7.6, was activated with trypsin at 5°C for 48 hr with a trypsin to chymotrypsinogen ratio of 1: 10,000 (w/w). Two other active forms, β- and γ-chymotrypsin (*52*), are also prepared by the classical "slow activation" of chymotrypsinogen (*18, 63*). The enzymes α-, β-, and γ-chymotrypsin are chemically indistinguishable from each other (*46, 64*); and, despite their

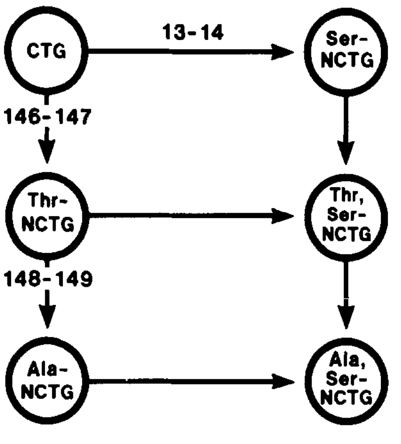


FIG. 2. Genesis of the various possible inactive members of the chymotrypsinogen family.

TABLE 2

A SUMMARY OF ACTIVATION SCHEMES FOR CHYMOTRYPSINOGEN

Author(s)	Concluding remarks	Reference(s)
Desnuelle (1960)	Both rapid and slow activation give rise to α-CT as the final end product	*51*
Corey *et al.* (1965)	α and γ-CT are conformational isomers of the same protein	*56*
Wright *et al.* (1968)	γ-CT to be the final autolytic product of δ-CT	*57*
Miller *et al.* (1971)	κ-CT to be intermediate species between δ-CT and γ-CT	*58*
Avery and Hopkins (1973)	γ-CT is not the final autolytic product of δ-CT	*59*
Sharma and Hopkins (1978)	α_1- and κ-CT are conformational isomers and the pathway CTG $\rightarrow$ Thr-neoCTG $\rightarrow$ Ala-neoCTG $\rightarrow$ α-CT was established	*60, 69*
Sharma and Hopkins (1979)	Two new enzyme species, μ- and ω-CT, were shown to be the intermediate species in the activation process	*61*

different crystal morphologies, no major differences in molecular structure of these enzymes have been observed by high-resolution X-ray crystallography (*65–67*). γ-Chymotrypsin is distinguished operationally by the tetragonal form of the crystals (*52*) and by its unique rate of denaturation in 8 *M* urea (*59*). While β-chymotrypsin has not been studied much, it has been reported to have needle-like crystal morphology similar to α-chymotrypsin (*52*) and to have a rate constant of denaturation in 8 *M* urea and a dimerization constant at low pH like that of γ-chymotrypsin (*58, 59*).

NEOCHYMOTRYPSINOGENS

In the early stages of the slow activation procedure, trypsin cleavage rapidly produces concentrations of active chymotrypsin exceeding the initial trypsin concentration. As a result, some of the chymotrypsinogen remaining in the reactive mixture undergoes limited hydrolysis by chymotrypsin to several altered, inactive forms of chymotrypsinogen, which are collectively called neochymotrypsinogens (*29*). In 1957, Rovery *et al.* (*29*) first prepared stable preparations of neochymotrypsinogen[2] by incubating mixtures of α-chymotrypsin with chymotrypsinogen in a ratio of 1 : 10 (w/w). One-hour digestion at 25°C and pH 7.6 with no added ammonium sulfate yielded mostly threonine-neochymotrypsinogen (The-neoCTG), so called because the neochymotrypsinogen formed had threonine

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[2] The terminology used in naming these inactive derivatives of chymotrypsinogens (neochymotrypsinogens) is based on the N-terminal(s) formed after the proteolytic cleavage at any or all of the following three bonds in the protein chain: $-\text{Leu}_{13}-\text{Ser}_{14}-$, $-\text{Tyr}_{146}-\text{Thr}_{147}-$, and $-\text{Asn}_{148}-\text{Ala}_{149}-$. For example, if the cleavage occurs only between the $-\text{Tyr}_{146}-\text{Thr}_{147}-$ bond, the resulting two-chain inactive molecule is called threonine-neochymotrypsinogen abbreviated as Thr-neoCTG (*69*).

as an N-terminal amino acid. Much longer incubation periods at 4°C, especially in the presence of 0.3 *M* ammonium sulfate, yielded mostly alanine-neochymotrypsinogen (Ala-neoCTG) with alanine as an N-terminal amino acid. In addition, smaller amounts of other N-terminal amino acids were detected in each of these preparations.

Since then two other laboratories have reported on the preparation of neochymotrypsinogen species. Valenzuela and Bender (*68*) have prepared threonine-neochymotrypsinogen from chymotrypsinogen, and Sharma and Hopkins (*60*) have detailed an extensive study on the purification and characterization of this neochymotrypsinogen. Likewise, in another paper by Sharma and Hopkins (*69*), optimal conditions for the preparation, purification, and characterization of alanine-neochymotrypsinogen (Ala-neoCTG) are described.

Other possible proteolytic reactions where the $-\mathrm{Leu}_{13}-\mathrm{Ser}_{14}-$ bond is hydrolyzed by α-chymotrypsin in CTG, Thr-neoCTG, or Ala-neoCTG, to the respective Ser-neoCTG, Thr, Ser-neoCTG, or Ala,Ser-neoCTG, are outlined in Fig. 2. These are considered unlikely reactions in the slow activation of chymotrypsinogen because the $-\mathrm{Leu}_{13}-\mathrm{Ser}_{14}-$ bond in CTG is not readily attacked at 4°C (*54*). This limitation in the genesis of neochymotrypsinogens has received direct support from Sharma and Hopkins (*60, 69*), who were unable to detect even low concentrations of neochymotrypsinogens containing the amino-terminal serine during preparation of Thr-neoCTG or Ala-NeoCTG by limited chymotryptic proteolysis of CTG at 4°C.

CLASSICAL RAPID ACTIVATION

Studies of the rapid activation of chymotrypsinogen, by relatively high concentrations of trypsin (CTG to trypsin ratio of 70:1, w/w) led Jacobsen (*53*) to postulate the existence of a chymotrypsin species more active than crystalline α-chymotrypsin. This enzyme was called δ-chymotrypsin. Soon afterward, Bettelheim and Neurath (*24*) prepared another new species of chymotrypsin by rapidly activating CTG in the presence of 0.1 *M* β-phenylpropionate, a competitive inhibitor of chymotrypsin. The properties of this species differed from that of δ-chymotrypsin, and the new enzyme species was named π-chymotrypsin. In the absence of inhibitor π-chymotrypsin rapidly autolyzed to δ-chymotrypsin by autolytic cleavage at the $-\mathrm{Leu}_{13}-\mathrm{Ser}_{14}-$ bond with elimination of the dipeptide, serine–arginine. These findings were later confirmed in other laboratories by N- and C-terminal end group analysis (*24, 29, 42–49*). Thus the chemical events in the rapid activation can be summarized as a two-step reaction:

$$\mathrm{CTG} \rightarrow \pi\text{-CT} \rightarrow \delta\text{-CT},$$

where trypsin catalyzes the splitting of the $-\mathrm{Arg}_{15}-\mathrm{Ile}_{16}-$ bond which in turn is followed by rapid autolytic cleavage of the $-\mathrm{Leu}_{13}-\mathrm{Ser}_{14}-$ bond. Under conditions of rapid activation, the first step is rate limiting and appreciable levels of the π-CT intermediate are not observed.

AUTOLYSIS PRODUCT OF δ-CHYMOTRYPSIN

A very slow autolytic sequence following the rapid activation of chymotrypsinogen to δ-chymotrypsin has been a subject of study since 1955. Bettelheim and Neurath (*24*) found that prolonged incubation (56 hr) of rapid activation system give rise to a new enzyme which possessed an additional C-terminal, tyrosine. However, N-terminal analysis indicated two additional amino-terminal amino acids, threonine and alanine, suggesting that the product was a mixture, of which one component was either α- or γ-chymotrypsin while the other was a new enzyme species in which the amino-terminal was threonine.

In 1962, Chervenka (*70*) showed that the autolytic product(s) of δ-chymotrypsin unfolded in 8 *M* urea at a rate similar to that of α-chymotrypsin but unlike that of δ-chymotrypsin or chymotrypsinogen. Whether the product was α-chymotrypsin, or other related active forms such as γ-chymotrypsin or α_1-chymotrypsin (*29*), a form in which $-\mathrm{Thr}_{147}-\mathrm{Asn}_{148}-$ dipeptide is still attached at the Ala_{149} residue of the C chain, was not clear from this study.

Up to 1965, it had been assumed that each different crystalline form of chymotrypsin was a chemically unique species. It was then of considerable surprise when Corey *et al.* (*56*) clearly demonstrated that α-chymotrypsin (α-CT) and γ-chymotrypsin (γ-CT) were conformational isomers of the same protein species. Furthermore, the two forms could be reversibly interconverted merely by changing the pH of the medium in which they were dissolved. The α form of the enzyme was stable at pH 3 and the γ form at pH 5.6. The rate of conformational isomerization of one form to the other at the appropriate pH was extremely slow, taking several weeks to several months for even partial conversion to occur (*56*).

Noting that the crystal morphology of δ-chymotrypsin (δ-CT) was similar to γ-chymotrypsin (γ-CT), but unlike that of α-chymotrypsin (α-CT), Wright *et al.* (*57*) hypothesized that γ-CT, rather than α-CT, was the final autolytic product of δ-CT. Consequently, they proposed a modification of Desnuelle's scheme (*51*) as follows:

$$\mathrm{CTG} \rightarrow \pi\text{-CT} \rightarrow \delta\text{-CT} \rightarrow \gamma\text{-CT} \rightleftarrows \alpha\text{-CT}.$$

Wright *et al.* (1968) did not consider the isomerization of γ-CT to α-CT a significant pathway for the production of α-CT since this conversion took several weeks to occur at acid pH and was not even favored under neutral conditions of autolysis. Therefore according to their scheme, α-CT was not derived from δ-CT by autocatalytic degradation, but only by activation of neochymotrypsinogens (*57*).

κ- AND α_1-CHYMOTRYPSINS

In 1971, another new active species of chymotrypsin was discovered (*58*). It was called κ-chymotrypsin and had cystine, isoleucine, and threonine as amino-terminal residues. Produced from the autolysis of δ-chymotrypsin incubated at pH 3.1 and room temperature for 58 hr, it differed from α-CT in having Thr_{147} as the amino-terminal of the C chain instead of Ala_{149}. When attempts were made to crystallize the enzyme by salting out from 50% saturated ammonium sulfate at pH 4.2, the enzyme converted to crystalline α-CT, and at pH 5.6, poorly formed crystals of γ-CT were formed.

Miller *et al.* (*58*) also demonstrated that the product of the rapid activation of chymotrypsinogen could be converted into material having amino end groups, crystal structure, and dimerization behavior like that of α-CT. These results supported the idea that there exists a pathway for the formation of α-CT from δ-CT. Whether this pathway also included γ-CT, as proposed in the Wright scheme (*57*), was not clear from their experimental evidence. Their best method for distinguishing α-CT from γ-CT, i.e., the difference in dimerization behavior, was subject to severe limitations when dealing with mixed systems containing low amounts of α-CT. Nevertheless, they incorporated the proposed scheme of Wright *et al.* (*57*) and suggested the following autolytic sequence: δ-CT → κ-CT → γ-CT ⇄ α-CT.

Two years later, Valenzuela and Bender (*68*) prepared and further studied α_1-chymotrypsin, the enzyme species identified by Rovery *et al.* in 1955 (*29*). Its structure was compared with a preparation of κ-chymotrypsin and, based on quantitative amino-terminal analysis, the two species appeared to be chemically identical. Their preparative procedure for α_1-CT, however, differed from that of Miller *et al.* (*58*). The method involved rapid activation of Thr-neoCTG to α_1-CT in a two-step reaction analogous to that for the classical rapid activation of CTG to δ-CT, Viz., tryptic hydrolysis of the $-Arg_{15}-Ile_{16}-$ peptide bond followed by rapid autolytic cleavage of the $-Leu_{13}-Ser_{14}-$ bond. It was suggested that the pathway CTG → Thr-NeoCTG → α_1-CT should be included in future activation schemes as an alternative route to α-CT and related chymotrypsins.

About this time, Bender and Killeheffer (*37*) reviewed the work on the activation of bovine chymotrypsinogen A. The presence of a new, unidentified active intermediate in the scheme Thr-neoCTG → α_1-CT was predicted. The apparent chemical identity of α_1-CT and κ-CT was noted. While there was some doubt whether α_1- and κ-CT were conformationally identical enzymes or were two isomers differing in conformation, a hypothetical scheme was presented where the autolytic product of α_1-CT was α-CT and γ-CT was the autolytic product of κ-CT.

RECENT DEVELOPMENTS

In 1973, a technique was developed for the differentiation of α-CT from γ-CT based on denaturation kinetics (*59*). Avery and Hopkins found that α-CT unfolded twice as fast in 8 *M* urea as γ-CT. Using this kinetic marker, they showed that the first-order rate of denaturation in 8 *M* urea (k_u) of the final autolytic product of δ-CT was distinctly different from γ-CT. Thus, the scheme proposed by Wright *et al.* (*57*), δ-CT → γ-CT, was no longer valid. While the urea denaturation kinetics suggested that α-CT was the autolytic product of δ-CT, it was not possible to conclude this with certainty until the k_u values of two other candidates, α_1-CT and κ-CT, were known.

Thus there remained several questions that needed to be answered before the various pathways of activation of chymotrypsinogen could be integrated and unified. Therefore, in 1974 work was started on this problem by attempting to isolate and characterize several hypothetical intermediates and to establish their place in the above activation pathways. The remainder of this article presents recent findings from our laboratory and the implications of these findings in the light of the overall activation process.

REINVESTIGATION OF AUTOLYSIS OF δ-CHYMOTRYPSIN

The autolysis product of δ-chymotrypsin (δ-CT) was reinvestigated in greater detail (*60*). Quantitative amino-terminal analysis and first-order denaturation rates (k_u) were used to characterize the final, stable autolytic product. The final autolytic product was indistinguishable from α-CT in terms of amino-terminal analysis and k_u measurements. Moreover, the specific activity of the autolytic product toward N-acetyl-L-tyrosine ethyl ester was similar to that found for crystalline α-CT. Notably, α_I-CT differs from α-CT in its amino-terminal composition and has a higher specific activity than α-CT (*68*). Thus, the stable autolytic product of δ-chymotrypsin at neutral pH was indeed α-chymotrypsin, in agreement with the original scheme of Desnuelle (*51*).

CONFORMATIONAL ISOMERIZATION OF α_I- AND κ-CHYMOTRYPSIN

It was initially assumed, based on the work of others (*58, 68*), that α_I-CT and κ-CT were identical in all respects. However, despite the fact that these two enzymes, derived from bovine chymotrypsinogen by quite different routes (*58, 68*), are chemically indistinguishable they were found to unfold in 8 *M* urea at different rates (*60*). Thus, as in the case with α- and γ-chymotrypsin (*56*), the evidence suggested that these two enzymes were conformational isomers of the same protein. This hypothesis was tested and found to be correct (*60*). The reversible interconversion ($\alpha_I \rightleftarrows \kappa$) of the α_I form with κ form was demonstrated utilizing denaturation rates in 8 *M* urea as kinetic markers. In order to prevent undesirable autolytic side reactions, the phenylmethanesulfonyl (PMS) derivatives of the enzymes were employed in the study. The stability of each protein conformer was pH dependent, with the α_I form being favored at pH 7 and the κ form being favored at pH 3. At pH 3 and 4°C, the isomerization of PMS-α_I-CT to PMS-κ-CT was completed in a few days, while the reverse transition at pH 7 and 4°C occurred in a few hours.

The reversible transformation of α_I- and κ-chymotrypsin (*60*) and the pH-dependent isomerization of α- and γ-chymotrypsin conformers studied by Corey *et al.* (*56*) have both interesting similarities and differences. Like α-chymotrypsin, κ-chymotrypsin is most stable at acid pH and, like γ-chymotrypsin, α_I-chymotrypsin is stabilized in a more neutral pH region. An important distinction between the results of Sharma and Hopkins ($\alpha_I \rightleftarrows \kappa$) and those of Corey *et al.* ($\alpha \rightleftarrows \gamma$) is the difference in rates of the conformational transition. The isomerization rates of the PMS-α_1-CT (PMS-κ-CT) pair appeared to be one to two orders of magnitude faster (*60*) than those observed for the DIP-α-CT (DIP-γ-CT) conformers. This discrepancy has been explained (*60*) in terms of the difference in solution conditions and the acyl derivatives used to study these isomerizations.

The above findings were not in accord with some of the conclusions of Miller *et al.* (*58*), who reasoned that α-chymotrypsin crystals formed from their κ-chymotrypsin solutions incubated at pH 4.2 must have evolved through the isomerization of a γ-chymotrypsin intermediate. But such a reaction sequence requires that γ-chymotrypsin be converted to α-chymotrypsin within 24 hr, a rate that is not supported by our own observations (*59*) nor that of Corey *et al.* (*56*).

AUTOLYSIS OF α_1 AND κ-CHYMOTRYPSIN

Enzymatically active autolytic products of the conformational isomer pair α_1- and κ-CT were characterized by quantative amino-terminal analysis and by rates of denaturation in 8 *M* urea (*60*). Only one autolytic species was produced from each of the isomers. After incubation at 4°C in 0.1 *M* sodium phosphate buffer, pH 7.1, α_1-CT autolyzed to γ-CT. Autolytic conversion was complete in about one week. The autolysis of κ-CT was more rapid and formed α-CT in about 2 days. It was also noted that the presence of 1 *M* ammonium sulfate in the incubation media accelerated the rate of autolytic conversion twofold.

At neutral pH and 4°C, the rate of autolytic conversion of κ-CT to α-CT was about four times slower than the rate of isomerization of PMS-κ-chymotrypsin to PMS-α_1-chymotrypsin (*60*). If rates of isomerization of the PMS derivatives were the same as that for the nonderivatized enzyme, then one might expect a significant proportion of κ-chymotrypsin incubated at pH 7.1 to be isomerized to α_1-chymotrypsin. Nevertheless, all of the κ-chymotrypsin was converted to α-chymotrypsin (*60*). Our interpretation is that the kinetics of isomerization of the nonderivatized enzymes, compared with PMS derivatives, are much slower. This view is entirely consistent with the study of Corey *et al.* (*56*), who found that acyl derivatives of α- and γ-chymotrypsin underwent pH-dependent isomerizations in measurable periods of time, while the isomerizations of the nonderivatized enzymes were orders of magnitude more slow. Thus, there emerges an important limitation which must be considered when discussing routes by which the α_1-κ-chymotrypsin protein conformers are converted to the α-γ-chymotrypsin conformers by autolytic cleavage of the –Asn_{148}–Ala_{149}– peptide bond (*60*). Any practical route in the genesis of α-chymotrypsin at neutral pH cannot include γ-chymotrypsin as an intermediate species.

In 1978, a revised scheme for the activation of bovine chymotrypsinogen A (*60*) was published which accomodated the relationship of α_1-chymotrypsin with κ-chymotrypsin and their respective autolysis products, γ- and α-chymotrypsin. A symmetrical, partially hypothetical activation grid was proposed (*60*). Based on the condition that the autolytic cleavage of the –Tyr_{146}–Thr_{147}– bond must always precede cleavage of the –Asn_{148}–Ala_{149}– bond, the scheme contained 12 chemically unique protein species, instead of 16 protein species, as suggested earlier (*37*). It predicted that rapid activation of Ala-neoCTG should yield γ-CT, just as Thr-neoCTG produced α_1-CT (*68*). But a few months later, when the rapid activation of Ala-neoCTG was studied, the activation product turned out not to be γ-CT but rather α-chymotrypsin (*69*).

ISOLATION AND CHARACTERIZATION OF μ- AND ω-CHYMOTRYPSIN

As mentioned above, alanine-neochymotrypsinogen was activated to α-chymotrypsin (*69*) and threonine-neochymotrypsinogen was converted to α_1-CT by elimination of the $-\mathrm{Ser}_{14}-\mathrm{Arg}_{15}-$ dipeptide in a two-step reaction that is analogous to that observed in the rapid activating CTG to δ-CT (*24, 37, 68*). It was speculated (*37, 60*) that π-chymotrypsin-like transient intermediates should be produced after the initial cleavage of the $-\mathrm{Arg}_{15}-\mathrm{Ile}_{16}-$bond. More recently (*61*) the two transient species, μ- and ω-chymotrypsin, produced during the rapid activation of Thr-neoCTG and Ala-neoCTG, respectively, have indeed been found. Two different procedures were employed to trap the transient enzyme species. 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*. Under acid conditions and 4°C, autolysis of μ-CT to α_1-CT or autolysis of ω-CT to α-CT was supressed. Both trapping procedures were equally successful and both yielded comparable results (*61*).

Both μ- and ω-chymotrypsin were purified and partially characterized (*61*). The immediate protein species obtained from activated Thr-neoCTG, μ-CT, had Thr, Ile, and half-cystine as amino-terminal amino acids. Similarly, the immediate species of Ala-neoCTG, μ-CT, had Ala, Ile, and half-cystine as amino-terminal amino acids. The first-order rate constants of denaturation in 8 *M* urea under standard conditions (see below for discussion) for these two new enzyme species were 0.8 min^{-1} for μ-chymotrypsin and 1.4 min^{-1} for ω-chymotrypsin. These rates constants differ from all previously known species of chymotrypsin (*59, 60, 69*).

After the isolation and characterization of μ- and ω-chymotrypsin, the autolytic products were studied (*61*). 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 hr. As expected, the stable autolytic product of μ-CT was confirmed to be α_1-CT. In a parallel study, the autolysis product of ω-CT was shown to be α-CT (*61*).

In conclusion, the studies (*61*) support the pathways

$$\text{Thr-neoCTG} \rightarrow \mu\text{ - CT} \rightarrow \alpha_1\text{-Ct} \qquad \text{and} \qquad \text{Ala-neoCTG} \rightarrow \omega\text{ - CT} \rightarrow \alpha\text{ - CT.}$$

CHARACTERIZATION OF MEMBERS OF THE CHYMOTRYPSINOGEN FAMILY BY DENATURATION RATE CONSTANTS

Some time ago a sensitive kinetic technique of characterizing chymotrypsin derivatives in solution, based on the difference in the fluorescence spectra of proteins in the native and denatured state, was described by Hopkins and Spikes (*72, 73*). Later, Avery and Hopkins (*59*) characterized all members of the chymotrypsin family known at the time using first-order rate of denaturation in 8 *M* urea (k_u) as kinetic markers. They noticed that these proteins could be grouped into two classes. Two-chain enzymes such as π- and δ-chymotrypsin unfolded slowly, while three-chain enzymes such as α-, β-, and γ-chymotrypsin unfolded at rates that were approximately 10 times more rapid. Examining newer species of chymotrypsin we find (*60*) that α_1-CT will join the previously studied three-chain proteins (*59*) as a rapidly unfolding species but κ-CT, which is a three-chain protein, denatures in 8 *M* urea at rates typical of two-chain enzymes. Therefore, the correlation between the rate of denaturation in urea and the number of chains in chymotrypsin is no longer considered to be valid (*60*). A summary of the first-order rates of denaturation in 8 *M* urea (k_u) for all members of the chymotrypsinogen family is given in Table 3.

It is interesting that each protein examined has a unique rate of denaturation in 8 *M* urea (k_u). Moreover, in the zymogens or in the active enzymes, cleavage of the $-\mathrm{Tyr}_{146}-\mathrm{Thr}_{147}-$ bond as well as the $-\mathrm{Asn}_{148}-\mathrm{Ala}_{149}-$ bond always results in an increase in sensitivity to urea denaturation. Apparently, after the second dipeptide, $-\mathrm{Thr}_{147}-\mathrm{Asn}_{148}-$, is eliminated, the protein undergoes a structural change reflected as an increase in its sensitivity to denaturation in 8 *M* urea. It is likely that this conformational change also affects the enzymatic properties. For example, it is known that the integrity of the substrate binding site in three-chained α-CT is far more labile in the alkaline pH region than it is in two-chained δ-CT or in three-chain chymotrypsins which retain the $-\mathrm{Thr}_{147}-\mathrm{Asn}_{148}-$ dipeptide in their structure, such as α_1- and κ-CT (*68*). The conformational changes accompanying the activation of bovine chymotrypsinogen have been reviewed elsewhere (*23*).

The underlying molecular events in the family of structurally similar enzymes

TABLE 3

A SUMMARY OF FIRST-ORDER DENATURATION RATE CONSTANTS IN 8 *M* UREA[a] (k_u) OF VARIOUS KNOWN MEMBERS OF THE CHYMOTRYPSINOGEN A FAMILY

Protein	k_u (min^{-1})	Reference(s)
CTG	0.37	*59, 69*
Thr-neoCTG	0.60	*69*
Ala-neoCTG	1.00	*69*
π-CT	0.20	*59*
μ-CT	0.80	*61*
ω-CT	1.40	*61*
δ-CT	0.11	*59*
κ-CT	0.24	*60*
α_1-CT	1.60	*60*
α-CT	1.80	*59, 60*
γ-CT	3.50	*59, 60*

[a] Standard conditions for denaturation are 8 *M* urea in 0.25 *M* sodium phosphate (pH 7.3) at 30.0 ± 0.1°C.

(α- or γ-CT and κ- or α_1-CT) which are manifested in different rates of denaturation are not known. It is clear, however, that the α_1-κ- conformer (*60*) and the α-γ-conformer (*56*) can assume two fully active forms which can only be distinguished in solution by their different rates of denaturation. While dimerization properties and crystal morphology also reveal differences in the protein–protein interactions of α-CT compared to other active forms of chymotrypsin (*57, 58*), only rate measurements of denaturation in 8 *M* urea appeared to be sensitive enough to permit differentiation of each member of the bovine chymotrypsinogen A family (*59–61, 69*). Likewise, the kinetics of urea denaturation technique have proven useful in monitoring subtle changes in conformation of enzymes reacting with enzyme inhibitors and other chemical ligands (*74–76*).

CURRENT ACTIVATION SCHEME OF BOVINE CHYMOTRYPSINOGEN A

Our three-dimensional scheme (*60*) proposed in 1978 retained the important features of the Desnuelle scheme and others (*29, 37, 51, 57, 58*) and, at the time, successfully accomodated all known active and zymogen species of the bovine chymotrypsinogen A family. Subsequent studies (*61*), however, required a revision, and the final activation scheme (*61*) is shown in Fig. 3. In this scheme only *proven* pathways and well-characterized members of the chymotrypsinogen family are incorporated.

The arrangement of α_1- and κ-chymotrypsin with respect to δ-chymotrypsin in Fig. 3 requires further comment. Miller *et al.* (*58*) have shown that δ-CT autolyzes to κ-CT at acid pH. On the other hand, no evidence exists to support an autolysis

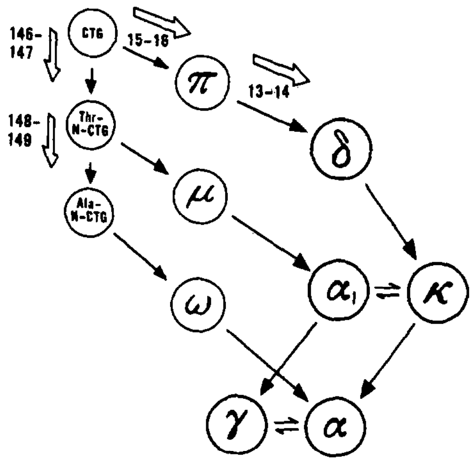

FIG. 3. Current scheme of activation of bovine chymotrypsinogen A (*61*). Peptide bonds cleaved are indicated by residue numbers drawn by the top and left side of the activation scheme. Also shown are two reversible reactions (⇄) not involving peptide bond cleavage. These later reactions are pH-dependent conformational isomerizations of chemically identical enzyme species (*56, 60*). Reproduced with permission from the publisher.

pathway at neutral pH, where δ-CT autolyzes to α_1-CT. Since there are two peptide bonds cleaved in the conversion of δ-CT to α-CT at neutral pH (*58–60*), a transient enzyme species must be produced during the autolysis process. While in theory this transient enzyme species can be either κ- or α_1-CT, only a route where δ-CT autolyzes to κ-CT and then to α-CT is compatible with recent experimental results (*59, 60*).

Some previously proposed pathways of activation must be rejected. Activation of Thr-neoCTG to α_1-CT cannot proceed via the route Thr-neoCTG → μ-CT → κ-CT → α_1-CT, because the rate of isomerization of κ-CT to α_1-CT, although favored at neutral pH, is far too slow to account for the complete conversion within 90 min of Thr-neoCTG to α_1-CT (*60, 68*). A similar argument can be applied in dismissing the activation sequence. Ala-neoCTG → ω-CT → γ-CT → α-CT. Again, the rate of isomerization of γ-CT to α-CT is orders of magnitude too slow and, furthermore, the isomerization is not favored at neutral pH (*56*).

The transient nature of π-, μ-, and ω-chymotrypsin in neutral solutions (*24, 61*) indicates that these chymotrypsins have a high affinity for cleavage of their own $-\mathrm{Leu}_{13}-\mathrm{Ser}_{14}-$ bond. Once the $\mathrm{Ser}_{14}-\mathrm{Arg}_{15}$ dipeptide is removed, the evolving enzymes (δ-, α_1-, and α-CT) display decreased specific activities (*24, 60, 61, 68*) and relatively good resistance to further autolysis (*61*). Apparently the autolytic cleavage of peptide bonds in the bovine chymotrypsin is not random. The preferred order of autolytic cleavage is the $-\mathrm{Leu}_{13}-\mathrm{Ser}_{14}-$ bond followed by the $-\mathrm{Tyr}_{146}-\mathrm{Thr}_{147}-$ bond and then the $-\mathrm{Asn}_{148}-\mathrm{Ala}_{149}-$ bond. This sequence is best illustrated in the known pathway: $\pi \rightarrow \delta \rightarrow \kappa \rightarrow \alpha$ (*24, 58, 60*). If special reaction 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. This is illustrated by the recently substantiated pathways: $\mu \rightarrow \alpha_1 \rightarrow \gamma$ and $\omega \rightarrow \alpha$ (*61*).

We now consider pathways for the classical slow activation of bovine chymotrypsinogen A (CTG A). The exact sequence of bond cleavage during slow activation of bovine CTG A to α-CT has been obscure for a long time. It has been generally believed that one or more uncharacterized neochymotrypsinogen species were intermediates in the activation process (*29, 37, 51, 57–61*). While Thr-neoCTG and Ala-neoCTG are confirmed intermediate species, no experimental evidence has been found for Ser-neoCTG being an intermediate species. This is consistent with the findings of Rovery and Bianchetta (*54*), who showed that the $-\mathrm{Leu}_{13}-\mathrm{Ser}_{14}-$ bond in chymotrypsinogen is quite resistant to proteolytic attack at 4°C.

Other refinements in the activation scheme for the conversion of CTG 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-CT is neither autolyzed nor isomerized to any significant extent in the 24- to 48-hr period of the slow activation process (*18, 62, 63*). Likewise, all hypothetical pathways which include γ-CT are unsuitable because of the extremely slow isomerization of γ-CT to α-CT (*56*). This leaves the following description of the slow activation of chymotrypsinogen (see also Fig. 2).

The initial activation step is the tryptic cleavage of some of the CTG to π-CT. The π-CT then rapidly autolyzes to δ-CT. As δ-CT accumulates in the reaction mixture, the main reaction shifts the formation of Thr-neoCTG from the remaining CTG. Further proteolysis of Thr-neoCTG by δ-CT leads to Ala-neoCTG. Finally, tryptic attack of the Ala-neoCTG forms ω-CT followed by rapid autolysis gives α-CT (Fig. 3). 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 autolysis to α-chymotrypsin with the intermediate in this two-step process being κ-chymotrypsin (*58–60*).

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