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ON THE ACTIVATION OF BOVINE CHYMOTRYPSINOGEN A

PREPARATION OF ALANINE-NEOCHYMOTRYPSINOGEN AND ITS ACTIVATION TO α-CHYMOTRYPSIN *

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Summary

Alanine-neochymotrypsinogen was prepared by incubating 20 parts bovine pancreas chymotrypsinogen A with one part α-chymotrypsin in a solution containing 1 M $(NH_4)_2SO_4$, 0.1 M sodium acetate, 0.05 M Tris buffer (pH 8.0) and 0.5 mg/ml soybean trypsin inhibitor. Optimal yields of NH_2-terminal alanine were obtained after 60 h incubation at 4°C. Ala-neochymotrypsinogen was isolated from the reaction mixture by affinity chromatography and ion-exchange chromatography on carboxymethyl-cellulose. As expected, the purified preparation was enzymatically inactive and, compared to chymotrypsinogen, had one additional NH_2-terminal group identified as alanine.

Ala-neochymotrypsinogen was activated by incubating with trypsin at a zymogen : trypsin ratio of 30 : 1 in 0.1 M phosphate buffer, pH 7.6 at 4°C for 1 h. The fully active, stable species was identified as α-chymotrypsin.

Introduction

The activation of bovine chymotrypsinogen A involves the selective cleavage of the Arg_{15}-Ile_{16} bond by trypsin, thus freeing an NH_2-terminal isoleucine. It is this residue which is involved in formation of a functional binding site in chymotrypsin [1]. During the activation process three other bonds in chymotrypsinogen are also susceptible to cleavage: they are Leu_{13}-Ser_{14}; Tyr_{146}-

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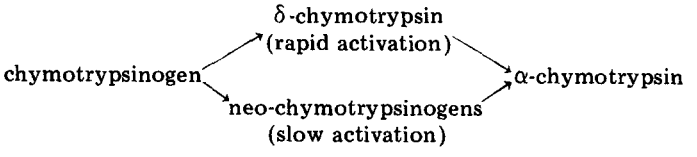
Thr_{147} and Asn_{148}-Ala_{149} [2—4]. Depending upon which of these last three bonds are cleaved, π-, δ, κ-, and α-chymotrypsin are generated [2—9]. If any of these last three bonds in chymotrypsinogen are cleaved prior to the tryptic cleavage of the Arg_{15}-Ile_{16} bond, a series of inactive neochrymotrypsinogens are generated [3]. These neochymotrypsinogens can then be activated with trypsin to yield various active chymotrypsins including α- and α_1-chymotrypsin [3,4,10—12].

In 1960, Desnuelle [4] proposed a unified scheme for the in vitro activation of bovine chymotrypsinogen A with trypsin:



It was noted that one obtains different active forms of chymotrypsin depending upon the amount of trypsin added to the chymotrypsinogen. At high ratios of trypsin to chymotrypsinogen (rapid activation) [2,5], the first bond hydrolysed is Arg_{15}-Ile_{16} and all subsequent derivatives produced by autolysis reactions are also active enzymes. At lower trypsin to chymotrypsinogen ratios (slow activation) [13,14], the activation process is about fifty times slower and, after going through several uncharacterized neochymotrypsinogen intermediates, eventually forms a single active species, α-chymotrypsin. Although aspects of the Desnuelle scheme have been challenged from time to time [15,16], others have reported that the Desnuelle activation sequence is valid [8,9]. Recently, more detailed activation schemes have appeared [9,17].

In this report aspects of the slow activation process are examined. Alanine-neochymotrypsinogen, a previously suspected intermediate of slow activation [3] is purified and characterized. A symmetrical, partially hypothetical activation grid proposed by Sharma and Hopkins [9] predicted that rapid activation of alanine-neochymotrypsinogen should yield γ-chymotrypsin, just as the activation of threonine-neochymotrypsinogen produces α_1-chymotrypsin [3,9,12]. The findings in the present paper do not support this prediction. Dispite the fact that γ-chymotrypsin is reported to be the stable form of the α-(γ-) conformer at neutral pH values [27] it was α-chymotrypsin that was produced when alanine-neochymotrypsinogen was activated with trypsin at neutral pH.

Materials and Methods

Crystalline preparations of bovine pancreas chymotrypsinogen A, α-chymotrypsin, and soyabean trypsin inhibitor were purchased from Mann Biochemicals and Sigma Chemical Co. Threonine-neochymotrypsinogen was prepared according to the method of Valenzuela and Bender [12]. Enzyme activity was measured spectrophotometrically using *N*-acetyl-L-tyrosine ethyl ester as the substrate [18]. Protein concentration was determined at 280 nm using a molar extinction coefficient of $5 \cdot 10^9\ M^{-1} \cdot cm^{-1}$ [19,20]. Free amino

groups of protein samples were determined quantitatively using the trinitrobenzene-sulfonic acid assay [21]. A modified dansylation procedure described by Zanetta et al. [22], was used to determine the free NH_2-terminal amino groups. In this procedure the NH_2-terminal half-cystine, (Cys), present in chymotrypsinogen is not determined. This residue is assumed to be present in all members of the chymotrypsinogen family.

Affinity chromatography. The affinity matrix, *N*-ε-amino caproyl-D-tryptophan methyl ester coupled to Sepharose was a commercial preparation from Miles-Yeda and was identical to that described by Cuatrecasas et al. [23], for the isolation of active chymotrypsin. The stated capacity for the resin was 7 mg chymotrypsin per ml of resin. Affinity chromatography was carried out in a column (10 × 1.2 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 8, at 4°C.

Ion-exchange chromatography. Carboxymethyl-cellulose ion-exchange resin (Watman CM 32, microgranular) was used to separate one-, two- and three-chained neochymotrypsinogens [11]. The column (60 × 1 cm) was thoroughly equilibrated with 5 mM Tris and 100 mM acetate buffer, pH 8.5, at 4°C and 10—15-mg protein samples were eluted using the same buffer system. 2-ml fraction volumes were collected at a flow rate of 5.2 ml/h.

Preparation of alanine-neochymotrypsinogen. Two separate solutions of chymotrypsinogen (40 mg/ml) and α-chymotrypsin (20 mg/ml), both in 1 M $(NH_4)_2SO_4$, 0.1 M sodium acetate and 0.05 M Tris, pH 8.0, were incubated at 4°C in the presence of 0.5 mg/ml of soyabean trypsin inhibitor. After 30 min 0.1 ml of the α-chymotrypsin was added to 1 ml of the chymotrypsinogen solution and the mixture was incubated at 4°C for 60 h. The reaction was stopped by removing α-chymotrypsin from the reaction mixture by affinity chromatography using *N*-ε-amino caproyl-D-tryptophan methyl ester coupled to Sepharose. The enzymatically inactive material eluting in four column volumes of 0.05 M Tris buffer, pH 8, was dialyzed against three changes of 1 mM HCl and lyophilized. As expected, the α-chymotrypsin was tightly bound to the column and was removed only after washing the column with 0.1 M acetic acid, pH 3 [23].

Denaturation kinetics. The time-dependent increase in protein fluorescence during denaturation in 8 M urea, 0.25 M sodium phosphate buffer, pH 7.3 [8,24] was measured on a Perkin-Elmer MPF-3 spectrophotofluorimeter (excitation at 290 nm and emission at 390 nm) fitted with a strip chart recorder and a water-jacketed cell holder thermostated at 30 ± 0.1°C. Solutions of urea were prepared fresh within 1 h of the experiment. Denaturation was initiated by rapid mixing of 100 μl of protein sample (5 mg/ml) into 1.5 ml buffered 8.54 M urea. The final area concentration in the cuvette was 8 M. Graphic analysis of the kinetic data [25] was used to obtain first-order rate constants of denaturation in 8 M urea (k_u).

Results and Discussion

Preparation and characterization of alanine-neochymotrypsinogen

Alanine-neochymotrypsinogen was prepared by limited proteolysis of chymotrypsinogen A with chymotrypsin in a manner similar to that described by Rovery et al. [3].

A variety of experimental conditions were examined in the preparation of alanine-neochymotrypsinogen by limited proteolysis of bovine chymotrypsinogen A with α-chymotrypsin. Only incubations done in the presence of relatively high concentrations of ammonium sulphate produced useful amounts of alanine-neochymotrypsinogen. Optimal conditions with respect to the yield of NH_2-terminal alanine and absence of non-specific autolysis during the proteolytic digestion process were a chymotrypsinogen to α-chymotrypsin ratio of 30 : 1 (w/w), incubated at 4°C for 60 h in buffered 1 M $(NH_4)_2SO_4$.

Alanine-neochymotrypsinogen was separated from any unreacted chymotrypsinogen remaining in the reaction mixture by ion-exchange column chromatography on carboxymethyl-cellulose [11,26]. In this chromatography procedure, which proved to be quite effective for separation of several members of the chymotrypsinogen A family, those proteins which have undergone limited proteolysis eluted earlier from the column than the parent zymogen. Fig. 1 shows the elution pattern of a model mixture of α-chymotrypsin, threonine-neochymotrypsinogen and chymotrypsinogen. In this particular run, β-phenylpropionate, a competitive inhibitor of α-chymotrypsin was included in the elution buffer to prevent proteolysis during chromatographic development. The order of elution was α-chymotrypsin (three chains), threonine-neochymotrypsinogen (two chains) followed by chymotrypsinogen (one chain). These elution positions served to calibrate the ion-exchange column.

As seen in Fig. 1, the inactive product of limited proteolysis, subjected to chromatography under identical conditions but in the absence of β-phenylpropionate, eluted as a single peak in a position expected from a two-chained product. Neither unreacted chymotrypsinogen nor three-chained neochymotrypsinogens were detected in the preparation.

Quantitative NH_2-terminal analysis of the pooled material ('A' in Fig. 1) revealed a new NH_2-terminal amino group, alanine (0.82 mol/mol protein). Also the trinitrobenzene-sulfonic acid assay for free amino groups indicated that sixteen free amino groups were present, one more free amino group than found in the parent compound, chymotrypsinogen. This confirmed that the

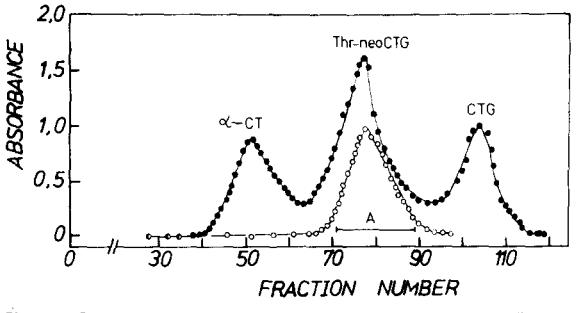


Fig. 1. Chromatography on carboxymethyl-cellulose CM-32 of proteins derived from chymotrypsinogen (CTG). The column was equilibrated and developed at 4°C with 5 mM Tris and 100 mM acetate, pH 8.5, with or without 12 mM β-phenylpropionate. 2-ml fraction sizes were collected at a flow rate of 5.2 ml/h. Protein was determined by absorbance at 280 nm. •, elution profile of an artificial mixture of α-chymotrypsin (12 mg), threonine-neochymotrypsinogen (22 mg) and chymotrypsinogen (10 mg) applied to the column (60 × 1 cm) in the presence of 12 mM β-phenylpropionate; ○, elution profile of an 12 mg aliquot of the neochymotrypsinogen preparation after removal of α-chymotrypsin by affinity chromatography.

TABLE I

CHARACTERISTICS OF CHYMOTRYPSINOGEN AND TWO NEOCHYMOTRYPSINOGENS

Zymogen	Number of chains	NH_2-terminals (residues/mol protein)			Free amino groups [b]	k_u (min^{-1}) [c]
		Cys [a]	Thr	Ala		
Chymotrypsinogen	1	1.00	0.00	0.00	15.1	0.37 [d]
Thr-neochymotrypsinogen	2	1.00	0.88	<0.06	16.0	0.60
Ala-neochymotrypsinogen	2	1.00	0.06	0.82	16.1	1.00

[a] The NH_2-terminal half-cystine (Cys) pre-existing in native chymotrypsinogen is assumed to be present in all preparations. This residue is refractory to the method of NH_2-terminal analysis used in the present work.

[b] Determined by trinitrobenzene-sulfonic acid assay [21].

[c] Denaturation conditions: 8 M urea, 0.25 M sodium phosphate, pH 7.3, at 30°C.

[d] Value reported by Avery and Hopkins [8].

inactive protein is two-chained and that the material meets the structural requirements for alanine-neochymotrypsinogen. These results are tabulated in Table I along with data for threonine-neochymotrypsinogen and chymotrypsinogen for comparison.

Rates of denaturation in 8 M urea of the two-chain neochymotrypsinogens, threonine-neochymotrypsinogen and alanine-neochymotrypsinogen, were measured. A comparison of the rates of denaturation in 8 M urea of the two-chain neochymotrypsinogens (Fig. 2) indicate that the cleavage of the Tyr_{146}-Thr_{147}

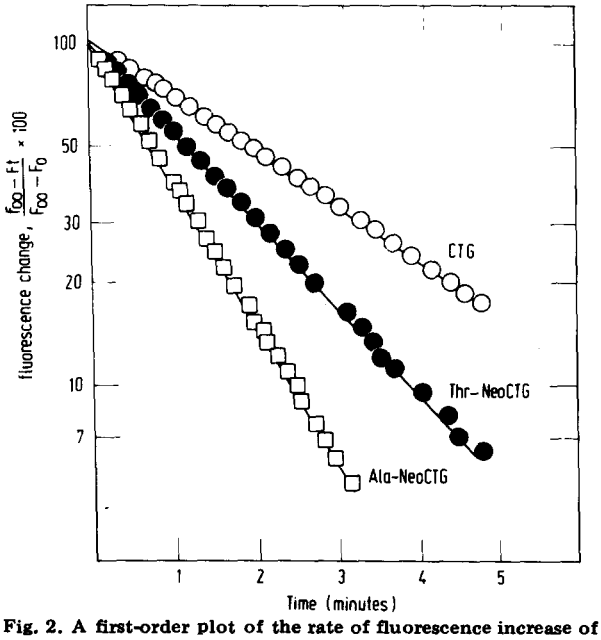


Fig. 2. A first-order plot of the rate of fluorescence increase of chymotrypsinogen and two inactive derivatives, threonine-neochymotrypsinogen and alanine-neochymotrypsinogen, upon exposure to 8 M urea, 0.25 M sodium phosphate, pH 7.3, at 30°C. F, F_t and F_∞ are the respective fluorescence intensities in 8 M urea at zero time, time t and at a time where complete unfolding has occurred.

and Asn_{148}-Ala_{149} bonds in chymotrypsinogen lead to an increased sensitivity to denaturation in 8 M urea. Similar conclusions were made by Avery and Hopkins [8] who measured the rates of denaturation in 8 M urea of several active species of the chymotrypsinogen A family. The semilogarithmic plot in Fig. 2 also demonstrates that the denaturation of each neochymotrypsinogen species followed a single first-order transition. A biphasic denaturation curve would have indicated that more than one protein species was present in the preparation [25]. The apparent first-order rate constants of denaturation (k_u) of alanine-neochymotrypsinogen and threonine-neochymotrypsinogen are included in Table I along with that of chymotrypsinogen.

Taken together the data indicate that alanine-neochymotrypsinogen, an inactive product of limited chymotryptic proeolytis of chymotrypsinogen, can be formed in good yield in the presence of 1 M $(NH_4)_2SO_4$. That ammonium sulfate enhances the susceptibility of the Asn_{148}-Ala_{149} bond to chymotryptic cleavage has been noted before [3,11]. The long period of incubation was essential under our experimental conditions. Reaction mixtures incubated for shorter periods contained mixtures of threonine-neochymotrypsinogen and alanine-neochymotrypsinogen. Presumably, then cleavage at the Tyr_{146}-Thr_{147} bond preceded cleavage of the Asn_{148}-Ala_{149} bond.

In a recent review Bender and Killheffer [17] proposed a pathway for the conversion of chymotrypsinogen to α-chymotrypsin under conditions of classical slow activation. Their scheme included three neochymotrypsinogens: chymotrypsinogen → threonine-neochymotrypsinogen → alanine-neochymotrypsinogen → alanine, serine-neochymotrypsinogen → α-chymotrypsin. The results reported here can be taken as evidence for the existence of the first two steps. However, we found no evidence that alanine, serine-neochymotrypsinogen was an intermediate in the genesis of α-chymotrypsin from chymotrypsinogen. In the present study neochymotrypsinogens having NH_2-terminal serine were not detected. Nor were any three-chain neochymotrypsinogens detected on ion-exchange column chromatography on CM-cellulose. These data indicate that the Leu_{13}-Ser_{14} bond is resistant to proteolytic attack at 4°C, a conclusion in agreement with Rovery and Bianchetta [11].

Activation of alanine-neochymotrypsinogen

Considering the structure of alanine-neochymotrypsinogen, it is apparent that the final product of tryptic activation of this zymogen could be either α- or γ-chymotrypsin. In order to find out which of these two enzyme conformers is produced, alanine-neochymotrypsinogen was activated with trypsin under classical rapid activation conditions and the stable product was characterized by quantitative NH_2-terminal analysis and denaturation kinetics. Quantitative NH_2-terminal analysis of the activation product showed isoleucine (0.88 mol/mol protein), alanine (0.86 mol/mol protein) and cysteine (assumed). The rate of denaturation in 8 M urea was kinetically indistinguishable from crystalline α-chymotrypsin (k_u of 1.8 min^{-1}) but clearly different from crystalline γ-chymotrypsin (k_u of 3.5 min^{-1}). These results establish that the fully active, stable product of rapid activation of alanine-neochymotrypsinogen is α-chymotrypsin.

Alanine-neochymotrypsinogen is activated to α-chymotrypsin by elimination

of the Ser_{14}-Arg_{15} dipeptide in a reaction that is analogous to that observed in the rapid activation of chymotrypsinogen to δ-chymotrypsin or threonine-neochymotrypsinogen to α_1-chymotrypsin [2,4,5,7,9,12]. Transient, active enzyme species should be produced after the initial cleavage of the Arg_{15}-Ile_{16} bond and, indeed, the presence of the transient species, π-chymotrypsin, in the rapid activation of chymotrypsinogen to δ-chymotrypsin has been recognized for some time [2]. The transient chymotrypsins produced during the rapid activation of threonine-neochymotrypsinogen and alanine-neochymotrypsinogen have now been isolated and will be described in a companion paper in this series.

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