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MODE OF ACTION OF α-CHYMOTRYPSIN IN ITS REVERSE REACTION AT pH 5.3

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SUMMARY

1. A peptic hydrolysate of soybean globulin was used as a substrate; its upper limit of mol. wt, estimated from its mobility in polyacrylamide gel electrophoresis, was approx. 2600. This substrate was incubated with α-chymotrypsin at a substrate concentration of 50% (w/v) in ethanol–water (1:9, by vol.).

2. The reverse reaction proceeded most favorably at pH 5.3 and the upper limit of mol. wt of the incubation mixture increased to 21 600 in 24 h.

3. At an initial stage a temporary decrease in the ninhydrin response was observed in the reaction system. An ^{18}O-exchange study also showed that at this stage the terminal carboxyl group of the substrate reacted actively with α-chymotrypsin to liberate water.

4. Comparative experiments using a methyl ester and an amide of the soybean globulin hydrolysate indicated that the reactivity of the carboxyl group was higher than the reactivity of the amide but lower than that of the ester.

5. Other experiments with N- and C-terminal inactivated substrates suggested that the transpeptidation reactions were not predominant.

6. From these results it is tentatively concluded that the condensation reaction mainly contributes to the initiation of the peptide chain elongation by this enzyme under the present experimental conditions.

INTRODUCTION

Peptide bond hydrolysis by proteinases has been generally studied at low substrate concentration systems. However, quite different conditions are observable in the examples of protein digestion in the intestine, protein autolysis in muscle, etc. In these biological systems it may happen that their pH values are not always the same as the optimal pH values for the proteinases to hydrolyze the peptide bonds. Under such unusual conditions an enzymatic reverse reaction may proceed which has been known as the plastein reaction.

In earlier days a series of research was carried out with the expectation that the plastein reaction might be relevant to protein biosynthesis [1–4], and plastein research, as well as the transpeptidation studies by the Fruton group [5], was continued until

the appearance of the modern concept of DNA–RNA-dependent peptide-chain elongation in vivo [1].

We have reinvestigated the plastein reaction to provide information worthy of its practical applications [6–9]. However, in the course of this study it has been found that various fundamental problems of research remain unsolved, especially concerning the mode of the reverse reaction. Although Determann and Köhler [10] have reported that several polycondensation products are formed as a result of a reverse reaction by pepsin in a model system containing a synthetic oligopeptide, there seems to be a general understanding that plastein synthesis by α-chymotrypsin from complex substrates such as protein hydrolysates proceeds mainly through the transpeptidation reaction [11].

This paper shows that the condensation is predominant over the transpeptidation in a reverse reaction by α-chymotrypsin at pH 5.3.

MATERIALS AND METHODS

Enzyme

A recrystallized preparation (Sigma Chemical Co.) of pepsin (EC 3.4.4.1) and a similar preparation (Miles Sevavac) of α-chymotrypsin (EC 3.4.4.5) were used.

Ordinary substrate

A soybean globulin fraction was prepared by the method of Wolf and Briggs [12]. This fraction was hydrolyzed with pepsin under the following conditions: protein concentration in water, 1% (w/v); pepsin–protein ratio, 1:100 (w/w); system pH value, 1.6 (adjusted with HCl); incubation temperature, 37 °C; and incubation time, 24 h. The mixture after the incubation was adjusted to pH 5.3 with NaOH and dialyzed through a cellophane membrane against a sufficient volume of water at 5 °C for 72 h. The dialyzed fraction was freeze-dried to obtain a hydrolysate (designated as NH_2–hydrolysate–COOH) which was to be subjected to the plastein reaction as its ordinary substrate. The average mol. wt of this substrate was obtained as 815 from the N-terminal amino group analysis by the Van Slyke's method [13] and the mol. wt upper limit estimated as approx. 2600 from the polyacrylamide gel electrophoresis mentioned below.

Modified substrates

From the ordinary substrate were prepared its methyl ester (NH_2–hydrolysate–COOMe), amide (NH_2–hydrolysate–$CONH_2$), acetyl derivative (Ac–NH–hydrolysate –COOH), and carboxyl-reduced derivative (NH_2–hydrolysate–CH_2OH) by the methods of Chibnall et al. [14], Smith and Slonim [15], Späth and Lederer [16] and Chibnall and Rees [17], respectively. Also, another substrate in which its carboxyl O atoms were labeled with ^{18}O was prepared as follows.

^{18}O labeling

A given amount of NH_2–hydrolysate–COOMe was suspended in a 100-fold amount of 0.05 M $Ba(OH)_2$ in $H_2{}^{18}O$ (isotopic enrichment, 3%; Miles Laboratories, Inc.) for 10 h at room temperature. The mixture was then adjusted to pH 5.3 with H_2SO_4 and centrifuged. The supernatant was freeze-dried, the resulting powder

suspended in a large amount of natural water and the suspension freeze-dried again. This procedure was duplicated until the $H_2{}^{18}O$ content in the suspension reached a natural level (0.204%); the method of its determination is described below. A carboxyl-O labeled substrate, designated as NH_2-hydrolysate-$C^{\ddagger}{}^{*}O^{\ddagger}{}^{*}OH$, was thus obtained —it is known that in such a case ^{18}O is located proportionally in these two oxygen parts [18].

Substrate checks

Infrared spectroscopic checks [19] for the substrates in film warranted that there was no significant difference between NH_2-hydrolysate-COOH and NH_2-hydrolysate-$C^{\ddagger}{}^{*}O^{\ddagger}{}^{*}OH$, and also that NH_2-hydrolysate-COOMe, NH_2-hydrolysate-$CONH_2$, and NH_2-hydrolysate-CH_2OH did not bear any carboxyl groups. On the other hand, a spot test showed that Ac-NH-hydrolysate-COOH gave no coloring response when treated with a ninhydrin reagent [20]. These substrates, though not completely soluble in water, were solubilized by ethanol-water (1:9, by vol.).

Reverse reaction

Each substrate was dissolved in ethanol-water (1:9, by vol.). By adding an appropriate concentration of NaOH in ethanol-water (1:9, by vol.), the solution was adjusted to pH 5.3 and at the same time to 50% (w/v) substrate concentration. To the adjusted solution was added α-chymotrypsin which amounted to 1% (w/w) of the substrate, and the resulting mixture was incubated at 37 °C.

Probes for time-course changes. Samplings were made at appropriate intervals during the reverse reaction and each sample was tested for solubility in aqueous trichloroacetic acid [6], ninhydrin response [20] and polyacrylamide gel electrophoretical mobility [21]. Details are mentioned in the legends to the figures.

Determination of leaving groups

To determine the leaving group, $H_2{}^{18}O$, from NH_2-hydrolysate-$C^{\ddagger}{}^{*}O^{\ddagger}{}^{*}OH$, 0.2 ml of the sample water was equilibrated with CO_2 at 25 °C for 2 days using an apparatus proposed by Cohn and Urey [22], and the gaseous phase was injected into a mass spectrometer (Hitachi model RMS-4). The $H_2{}^{18}O$ quantity was obtained from the observed abundance ratio, *m/e* 46 vs *m/e* 44. Other leaving groups, methanol from NH_2-hydrolysate-COOMe and ammonia from NH_2-hydrolysate-$CONH_2$, were determined by injecting the sample solutions directly into a gas chromatograph (Shimadzu GC-4A) and into an amino acid analyzer (Hitachi KLA-5), respectively.

RESULTS AND DISCUSSION

It has been known that an extremely high substrate concentration is required for the effective formation of plastein [2]. Besides this, the reaction system should be slightly acidic (pH 4–6), irrespective of the pH optima of enzymes for protein hydrolysis [23].

In the present study it was also confirmed that, although the α-chymotryptic hydrolysis of the soybean globulin in ethanol-water (1:9, by vol.) proceeded most effectively at pH 7.8, the effective condition for the reverse reaction was acidic, especially at pH 5.3 (Fig. 1). At this pH, a plastein (trichloroacetic acid-insoluble

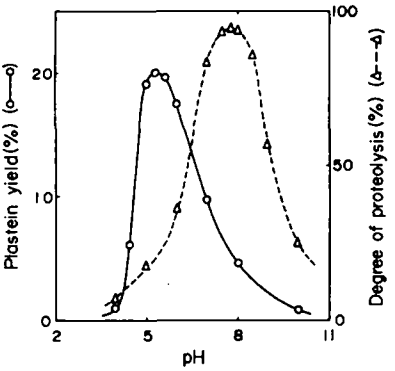

Fig. 1. Difference in pH dependence between protein hydrolysis by α-chymotrypsin and its reverse reaction. Soybean globulin was hydrolyzed with α-chymotrypsin under the following conditions: substrate concentration, 1% (w/v); enzyme–substrate ratio, 1:100 (w/w); incubation temperature, 37 °C; and incubation time, 30 min. The hydrolysate was treated with an equal volume of 20% trichloroacetic acid. The degree of hydrolysis was given as a ratio (%) of the trichloroacetic acid-soluble N vs total N. The reverse reaction was carried out as described in the text. After the reverse reaction the incubation mixture was treated with a 100-fold vol. of 10% trichloroacetic acid. The plastein yield was given as a ratio (%) of the trichloroacetic acid-insoluble N vs total N.

fraction) was formed with the time-course curve shown in Fig. 2. Investigation by polyacrylamide gel electrophoresis indicated that during the course of such a reverse reaction the upper limit of mol. wt of the entire incubation mixture changed from 2600 to 21 600 with a slower rate of increase (Fig. 2).

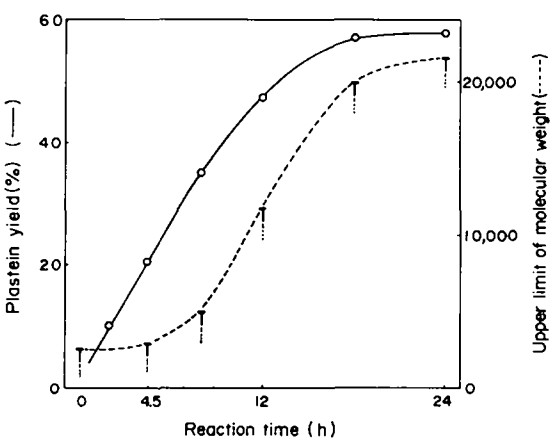

Fig. 2. Time-course increases in the plastein yield and in the upper limit of mol. wt. The reverse reaction was carried out with the ordinary substrate at pH 5.3. The plastein yield was determined as described in Fig. 1. The upper limit of mol. wt was estimated as follows: Samplings were made at appropriate intervals during the reaction and each sample was electrophoresed on 7.5% polyacrylamide gel at 3 mA/cm for 7 h at 20 °C by use of phenol–acetic acid–water (1:1:1, by vol.). After the electrophoresis the gel was stained with Amido black 10B in the usual manner. The shortest migration distance of the stained zone was compared with the migration distances of several markers. Oxidized insulin B-chain (mol. wt, 2575), cytochrome *c* (mol. wt, 11 700), trypsin (mol. wt, 23 500) and ovalbumin (mol. wt, 45 000) were used as the markers, their mobilities being 9.8 cm, 6.8 cm, 4.9 cm and 2.1 cm, respectively, under the same conditions of electrophoresis.

When another time-course change was investigated by the ninhydrin response test, it was found that there was a clear decrease in the coloring response at an earlier stage (i.e. within 30 min) of the incubation (Fig. 3). This result suggests that a condensation reaction between substrate peptides occurred most actively at this stage. It is also supposed that in the subsequent stage the condensation reaction still proceeds along the dotted curve in Fig.3, although the ninhydrin response of the reaction system begins to increase gradually, probably because of the partial hydrolysis of the condensation products.

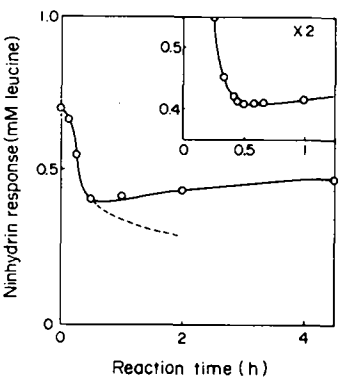

Fig. 3. A decrease in the ninhydrin response during the reverse reaction. Samplings were made at appropriate intervals and each sample was, after dilution with a 1000-fold vol. of 0.1 M HCl, treated with the ninhydrin reagent. The color intensity was measured at 570 nm with a Hitachi recording spectrophotometer and the result expressed in terms of the leucine equivalent.

The intermediate peptidyl chymotrypsin formation may be inevitable in advance of the completion of the condensation reaction. It is known that the peptidolysis, amidolysis, and ester hydrolysis reactions by α-chymotrypsin involve the acyl–enzyme formation which takes place at the Ser-195 residue [24, 25]. Tanimoto et al. [26] have reported that in the plastein reaction the Ser-195 residue of α-chymotrypsin acts as a catalytic center, probably as a target of the acylation (peptidylation).

When the condensation reaction between two peptides is completed with the aid of α-chymotrypsin, it is necessary that the carboxyl group of one peptide reacts with this enzyme, with the liberation of water, and the resulting peptidyl–enzyme is subsequently attacked by the amino group of the other peptide. As to the water liberation an expected result was obtained that $H_2{}^{18}O$ left a substrate, NH_2–hydrolysate–$C^{\ddagger}{}^{*}O^{\ddagger}{}^{*}OH$, with a rapid rate as is shown in Fig. 4. The liberation curve reaches a plateau after 2 h, showing that approximately 5/6 parts of the carboxyl groups of this substrate have reacted at least once during this time. Thus, the results in Figs 3 and 4 indicate that the condensation reaction actively occurs at an earlier stage of the plastein reaction, contributing to the initiation of the peptide chain elongation.

When NH_2–hydrolysate–COOMe or NH_2–hydrolysate–$CONH_2$ was used instead of the ordinary substrate, a leaving group, methanol or NH_3, was formed with a higher or lower liberation rate at the earlier stage of the plastein reaction (Table I). From the differences in the liberation rates among the leaving groups (water, methanol and NH_3) it is estimated that the reactivity of the terminal carboxyl for forming the

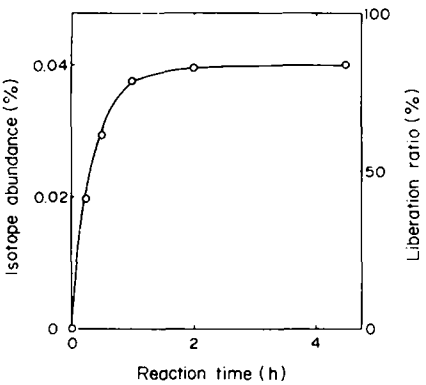

Fig. 4. Liberation of $H_2{}^{18}O$ during the course of the reverse reaction.

TABLE I

LIBERATION RATES OF LEAVING GROUPS AND RATES OF PLASTEIN FORMATION FROM MODIFIED SUBSTRATES AT THE INITIAL STAGE OF THE REVERSE REACTION BY α-CHYMOTRYPSIN

Type of substrate		Liberation rate of leaving group†	Rate of plastein formation††
N-Terminal	C-Terminal		
NH_2-CH(R)-CO-	-NH-CH(R)-$C^{(1/2)*}O^{(1/2)*}$OH	1.873†††	5.422
NH_2 CH(R)-CO-	-NH-CH(R) $COOCH_3$	2.899	9.024
NH_2-CH(R)-CO-	-NH-CH(R)-$CONH_2$	0.800	2.275
NH_2-CH(R)-CO-	-NH-CH(R) CH_2OH		1.515
CH_3CO-NH-CH(R)-CO-	-NH-CH(R)-COOH		0.000

† μmole/mg enzyme per min at the reaction stage of 30 min.
†† mg plastein/mg enzyme per h at the reaction stage of 4.5 h.
††† Calculated from Fig. 4.

peptidyl–chymotrypsin is higher than the reactivity of the amide though lower than that of the methyl ester. α-Chymotrypsin possesses both esterase and amidase activities, and the former is generally higher than the latter [27, 28]. Accordingly, it is speculated that the activity of α-chymotrypsin in its peptidylation by the carboxyl group lies in a position between the esterase and amidase activities of this enzyme, so far as the present experimental conditions are concerned.

As Table I shows, the rates of the liberation of water, methanol and NH_3 are comparable to the rates of the plastein formation from NH_2–hydrolysate–COOH,

NH_2–hydrolysate–COOMe and NH_2–hydrolysate–$CONH_2$, respectively, at a reaction time of 4.5 h. Since the deacylation steps are similar in that the aminolysis reaction takes place in each case, the difference in the mode of the acylation (peptidylation) is considered to be relevant to the rate of the plastein formation.

Another substrate, NH_2–hydrolysate–CH_2OH, is inactive at the C-terminal and the cleavage of an interior peptide bond is necessary for the peptidylation of α-chymotrypsin. Thus, a C-terminal carboxyl group of the cleaved peptide is transferred to the enzyme (C-transpeptidation). However, such a C-transpeptidation reaction seems to have a minor effect on plastein synthesis (at pH 5.3), since the plastein is formed more slowly from this substrate than from the ordinary substrate (Table I).

On the other hand, an N-terminal inactive substrate, Ac–NH–hydrolysate–COOH, has a property similar to the ordinary substrate regarding the mode of acylation and quite different from any other cases in respect to the mode of deacylation. The deacylation step of this acetyl substrate consists of a transfer of an interior amido group (N-transpeptidation). However, the occurrence of such an N-transpeptidation reaction was ruled out since no significant amount of plastein was obtainable from Ac–NH–hydrolysate–COOH (Table I).

Earlier workers have conducted the α-chymotryptic plastein synthesis in the neighborhood of pH 7 [3, 11], and it is considered that at this pH region the C-transpeptidation reaction is probably predominant over the condensation reaction since the terminal carboxyl groups are mostly ionized. The lower the system pH, the more favorable the reaction of the carboxyl groups. A reverse relation holds when the terminal amino groups react at the aminolysis step. Accordingly, the most favorable pH condition should be intermediate, such as pH 5.3 (Fig. 1) which may correspond to an isoionic point of a neutral oligopeptide.

As is well known, the rate of protein hydrolysis by α-chymotrypsin shows a maximum near pH 7.8, and the pH–rate profile in this case takes such a shape as is surrounded by the titration curves of the Ile-16 and His-57 residues of α-chymotrypsin. Since both these residues are also reported to be responsible for plastein synthesis [26], a similar pH rate profile should hold the plastein reaction. However, for plastein synthesis, two other factors, i.e. the amino and carboxyl groups of substrate, are additionally responsible. Thus, the pH rate profile (Fig. 1) may take such a shape as is surrounded by the titration curves of Ile-16 and His-57, and at the same time by the titration curves of the terminal amino and carboxyl groups.

A significant part of the carboxyl groups is thought to be in an unionized state at pH 5.3, especially at an extremely high substrate concentration (50%). It has been postulated that the carboxyl group, unless ionized, is as reactive as its ester in the acyl–enzyme formation [29].

From the results obtained from the present study, it is concluded that the C-terminal carboxyl group of a substrate peptide is sufficiently active in the reverse reaction by α-chymotrypsin and the resulting peptidyl–chymotrypsin is attacked by the N-terminal amino group of another substrate peptide to form a condensation product. Such a condensation reaction is much more active at the initial stage of the incubation and, thereafter, a similar mode of the reverse reaction may be gradually repeated until the product reaches a higher molecular weight as shown in Fig. 2.

It is possible that this type of proteinase reverse reaction generally proceeds in the examples of protein digestion in the intestine, protein autolysis in muscle, etc.

The reverse reaction study may provide basic information fundamental to the possibility of these processes occurring in such biological systems with high protein concentrations.

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