On the Heterogeneity of Three-Times-crystallized α-Chymotrypsin*

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Evidence is presented for the existence of at least two active components in 3 ×-crystallized α-chymotrypsin. These components are shown by means of an "all-or-none" assay and by kinetic studies to have different specific activities and to be affected differently by incubation at various temperatures in the absence of Ca^{2+}. Both components are stabilized and activated to a similar degree by Ca^{2+} and can be separated by agar-gel electrophoresis in the absence of Ca^{2+}. They migrate as a single component when Ca^{2+} is added to the buffer solution. It is suggested that Ca^{2+} be incorporated in all buffer media used for studies on α-chymotrypsin.

It has been recognized for a number of years that Ca^{2+} enhances the proteolytic and esterolytic activities of α-chymotrypsin. Thus, for example, Green *et al.* (1952) reported a 50% increase in the rate of hydrolysis of ATEE[1] in the presence of 0.01 M Ca^{2+}. A similar effect was noted with casein and phenylalanine ethyl ester by Wu and Laskowski (1956), who reported, moreover, that the presence of Ca^{2+} increased the stability of α-chymotrypsin at 37°. Later studies by Jennings *et al.* (1958) showed that the extent of Ca^{2+} activation was dependent upon both the buffer and the substrate used in the assay.

During the development of an "all-or-none" assay for chymotrypsin which required incubation at 37° for 45 minutes (Erlanger and Edel, 1964), it was noted that constant but consistently low results were obtained unless a minimum of 0.02 M Ca^{2+} were present in the assay solution. It appeared as if there were at least two substances present in commercially available 3×-crystallized α-chymotrypsin and that one of them was unstable in the absence of Ca^{2+}. Since 3×-crystallized α-chymotrypsin is used extensively in studies on the mechanism of α-chymotrypsin, it seemed advisable to investigate the apparent heterogeneity further.

That preparations of α-chymotrypsin appear to be heterogeneous has been noted before. As a result of electrophoresis studies, Egan *et al.* (1957) reported the presence of two components in 4-7×-recrystallized α-chymotrypsin obtained from 8×-recrystallized chymotrypsinogen. Studies of Hofstee (1963), of Chervenka (1962), and of Martin and Frazier (1963) are also consistent with the conclusion that α-chymotrypsin is heterogeneous. The investigations reported in this paper make use of a kinetic and an all-or-none assay to confirm the presence of at least two components of different specific activities, heat stabilities, and electrophoretic mobilities. The effect of Ca^{2+} on each of the components is described and some suggestions are made relevant to studies on the mode of action of α-chymotrypsin.

EXPERIMENTAL[2]

Effect of Temperature upon Stability of α-Chymotrypsin.—(*a*) "ALL-OR-NONE" ASSAY.—A volume of 0.2 ml of a solution of 36 mg α-chymotrypsin in 1 ml of 0.001 N HCl was added to a test tube containing 1.5 ml of 0.05 M Tris-chloride buffer, *p*H 7.6, and incubated at the selected temperature for the specified time period. The tube was then placed in a 37° bath and 1.2 ml of 0.05 M Tris-chloride buffer, *p*H 7.6, containing 0.0375 M $CaCl_2$ was added. This was followed by the addition of 0.1 ml of a stock 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate solution prepared as described previously (Erlanger and Edel, 1964). The reaction mixture was transferred periodically to a cuvet and its absorbance at 410 mμ was determined in a Beckman DU spectrophotometer until a constant value was obtained. The maximal time required was 1.5 hours. Appropriate controls lacking enzyme were also set up.

Studies on the denaturation of α-chymotrypsin in the presence of Ca^{2+} were carried out in 1.5 ml of 0.05 M Tris-chloride buffer, *p*H 7.6, containing 0.02 M $CaCl_2$. After incubation for the selected time period, 1.2 ml of the same buffer was added and the usual assay procedure was carried out.

(*b*) ASSAY WITH GPANA.[3]—α-Chymotrypsin (16.5 mg) was dissolved in 4.0 ml 0.05 M Tris-chloride buffer, *p*H 7.6, and placed in a 37° bath. At the specified time intervals 0.5-ml aliquots were withdrawn and added to 3.8 ml of ice-cold 0.05 M Tris-chloride buffer, *p*H 7.6 containing 0.02 M $CaCl_2$. The diluted aliquots were kept in an ice bath until all were collected. They were then assayed for activity in the following way:

A substrate solution was prepared by dissolving 77 mg of GPANA in 4 ml of methanol and bringing the volume to 200 ml with 0.05 M Tris-chloride buffer, *p*H 7.6, containing 0.02 M $CaCl_2$. Five-ml quantities of this substrate solution were pipetted into test tubes and were equilibrated at 25°. One-ml aliquots of the enzyme solutions were added, followed, after 20 minutes of incubation at 25°, by 1 ml of 30% acetic acid (to stop the reaction). The absorbance at 410 mμ was then determined. The extinction coefficient of *p*-nitroaniline at this wavelength is 8800 (Erlanger *et al.*, 1961).

Effect of Ca^{2+} upon Component Remaining after 1-Hour Incubation at 37° in Absence of Ca^{2+}.—Enzyme stock solution was 40 mg α-chymotrypsin in 2 ml 0.001 N HCl. Substrate stock solutions (GPANA) were prepared as described in the previous paragraph, except that one additional stock solution was prepared using 0.05 M Tris-chloride buffer, *p*H 7.6, lacking Ca^{2+}.

A volume of 0.25 ml of the chymotrypsin stock solution was brought to 7.5 ml by the addition of 0.05 M Tris-chloride buffer, *p*H 7.6. Two such solutions were prepared. One was incubated at 37° for 1 hour;

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[1] Abbreviations used in this work: ATEE, acetyl L-tyrosine ethyl ester; GPANA, glutaryl-L-phenylalanine *p*-nitroanilide.

[2] All α-chymotrypsin preparations were Worthington, 3 × crystallized.

[3] The synthesis and properties of this specific substrate of chymotrypsin will be described in a paper now in preparation.

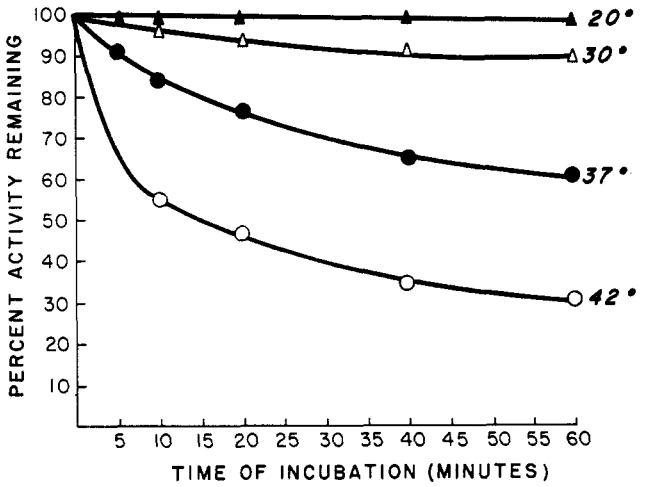

FIG. 1.—Effect of incubation at various temperatures upon activity of 3×-crystallized α-chymotrypsin as determined by all-or-none assay. Details under Experimental.

the other was kept in an ice bath for the same period of time. Both solutions were assayed by the addition of 0.2 ml of the enzyme solution to 2.5 ml of the substrate solution which had been allowed to equilibrate at 25° in temperature-controlled Beckman DU spectrophotometer. The release of *p*-nitroaniline was measured at 410 mμ during the course of the reaction. The points were plotted and the rate was determined by measurement of the slope (the plot is linear). Controls lacking enzyme were always run.

Agar-Gel Electrophoresis.—A Shandon electrophoresis tank (Shandon Scientific Co., Ltd.) was used. Agar slides were prepared according to the methods described by Grabar and Burtin (1960). One and 2% enzyme solutions were applied to the slides. Electrophoresis was carried out for 3 hours with a current of 9.5–12 ma per slide. Buffer was 0.076 N Veronal acetate, *p*H 6.12.

Staining for protein was carried out by immersing slides overnight in a Ponceau S solution prepared by diluting 1 ml Ponceau S (Consolidated Labs., Inc., Chicago) in 125 ml of 3% trichloroacetic acid. Staining was followed by washing for several hours in an aqueous solution containing 2% acetic acid and 15% glycerol.

Staining for esterase activity was accomplished by immersing the slides in a β-naphthylacetate solution prepared by dissolving 5 mg β-naphthylacetate (Mann Research Labs.) in 0.25 ml acetone and diluting to 25 ml with 0.067 M potassium phosphate buffer, *p*H 7.4. Present in this solution, in addition, was 10 mg diazo blue (Mann Research Labs.). The solution was filtered before use. It was usually necessary to soak the slides for 60–90 minutes. This is essentially the procedure of Uriel (1961).

Staining for amidase activity was carried out by immersion of the slide for 2 hours in a solution of succinyl-L-phenylalanine β-naphthylamide (Erlanger, in preparation) which was prepared by dissolving 15 mg in 0.25 ml acetone and diluting to 25 ml with 0.067 M phosphate buffer, *p*H 7.4. This was followed by immersion for 1 hour in 0.04% diazo blue.

RESULTS

Shown in Figure 1 is the effect of temperature upon the stability of α-chymotrypsin in the absence of Ca^{2+} at *p*H 7.6, as measured by the all-or-none assay. In this assay, the quantity of active enzyme present after various periods of incubation is being measured. Only negligible loss of activity occurred at 25°. At higher temperatures, however, inactivation was noted and was 70% complete after 1 hour at 42°.

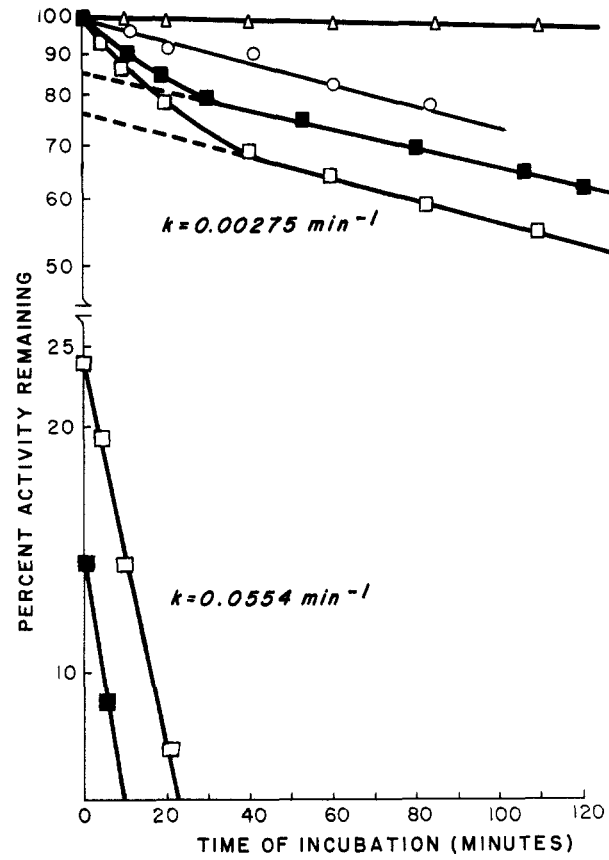

FIG. 2.—Stability of α-chymotrypsin at 37° in the presence of 0.02 M Ca^{2+} and in the absence of Ca^{2+}. Buffer = 0.05 M Tris-chloride, *p*H 7.6. Δ—Δ, in presence of 0.02 M Ca^{2+} (all-or-none assay); □—□, no Ca^{2+} present (all-or-none assay); ■—■, no Ca^{2+} present (GPANA assay); O—O, "stable fraction," in absence of Ca^{2+} (all-or-none assay).

At 37° the inactivation process appeared to be biphasic. Extension of the incubation period at 37° to 110 minutes gave the data shown in Figure 2, in which the log of the remaining activity is plotted against time. The curve can be explained as being the result of the simultaneous inactivation of two components of unequal stability. The first-order rate constants for the inactivation of the components, as derived from the linear plots in Figure 2, are 0.0554 min^{-1} and 0.00275 min^{-1}, respectively. Also shown in Figure 2 is the rate of inactivation at 37° in the presence of 0.02 M Ca^{2+}. The presence of Ca^{2+} has markedly increased the stability of both components of the α-chymotrypsin preparation.

Shown in Figure 2, as well, is the effect of incubation at 37° upon the activity of chymotrypsin as measured against the specific substrate GPANA. The curve representing the loss of activity parallels the one derived from the all-or-none assay and also behaves as if there were two components denaturing at different rates. The calculated rates (from the slopes) are the same as those calculated from the all-or-none assay. It should be noted, however, that, although one component represents about 23% of the enzyme mixture (by all-or-none assay), its denaturation decreases the overall activity against GPANA by only about 14%. The implication of this is that the more unstable component is about 60% as active against GPANA as the stable one.

Since inactivation at 37° for 1 hour should completely destroy the more labile fraction, it was of interest to determine the heat stability of the fraction remaining. α-Chymotrypsin (864 mg) was incubated for 1 hour at

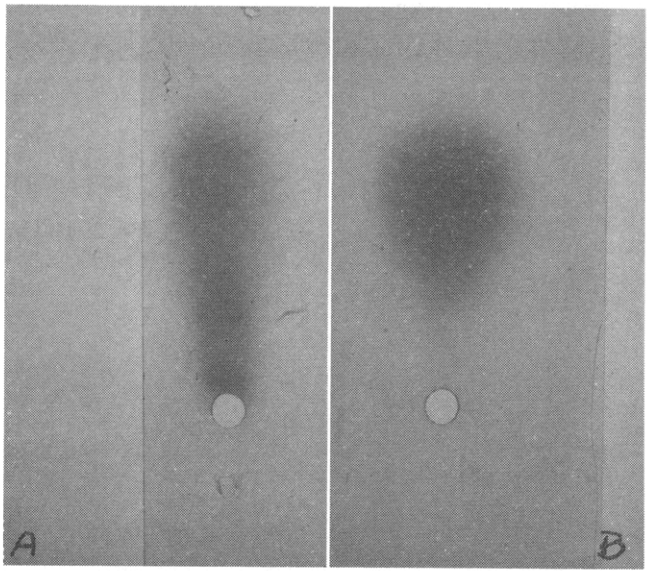

FIG. 3.—Agar-gel electrophoresis of 2% solutions of 3×-crystallized α-chymotrypsin (A) in absence and (B) in presence of 0.03 M Ca^{2+}. Stained for esterase activity using β-naphthylacetate. Migration is toward cathode. See text for details.

37° in 0.05 M Tris buffer, *p*H 7.6, in the absence of Ca^{2+}. After 24 hours of dialysis against 0.001 M HCl, the remaining material was recovered by lyophilization. The yield was 589 mg. Assay of this preparation using 2 - nitro - 4 - carboxyphenyl - *N*,*N* - diphenylcarbamate showed it to possess, on a weight basis, 82.6% of the activity of the original α-chymotrypsin. The results of a study of its inactivation at 37° in the absence of Ca^{2+} are shown in Figure 2 (open circles). The stock solution contained 43 mg of enzyme per ml instead of 36 mg (see Experimental), so that the concentration of active enzyme was the same as in the experiments with native enzyme. Unlike undenatured α-chymotrypsin, its loss of activity followed first-order kinetics throughout the 80-minute incubation period.

It was of interest, now, to determine the effect of Ca^{2+} upon the activity of the more stable component and to compare it with the intact enzyme preparation. As shown in Table I, Ca^{2+} has the same effect upon the

TABLE I
EFFECT OF CALCIUM ION ON THE RATE OF HYDROLYSIS OF GPANA BY COMPONENTS OF 3×-CRYSTALLIZED α-CHYMOTRYPSIN[a]

Enzyme Preparation	v_o (no Ca^{2+})	v_o (0.03 M Ca^{2+})	v_o(0.03 M Ca^{2+}) / v_o (no Ca^{2+})
Native	3.82	4.94	1.29
Heated at 37°, 1 hour	3.18	3.97	1.23

[a] $[E_o]$ = 1.97 × 10^{-6} M (mw = 25,000); $[S_o]$ = 8.81 × 10^{-4} M; buffer = 0.05 M Tris-chloride, *p*H 7.6; Ca^{2+} = 0.03 M when present; t = 25°; v_o is in units of 10^{-6} M per 100 seconds.

stable component as it does upon the mixture. We can deduce from this, therefore, that both components of 3×-crystallized α-chymotrypsin are activated by Ca^{2+} to a similar extent.

Finally, attempts were made to separate the two components or at least to demonstrate their existence by electrophoretic techniques. Although 3×-crystallized α-chymotrypsin migrated as a single substance with Sepraphore III (Gelman Instrument Co.) as the supporting medium, two components could be separated by agar-gel electrophoresis using 0.076 M Veronal-acetate buffer, *p*H 6.12 (Fig. 3). Both components were active against β-naphthylacetate and succinyl-L-phenylalanine β-naphthylamide. Upon the addition of 0.03 M Ca^{2+} to the buffer, only one component was observed. This component migrated with the mobility of the fast-moving fraction (see Fig. 3).

DISCUSSION

Our findings support the conclusion that 3×-crystallized α-chymotrypsin is not homogeneous but, in fact, consists of at least two active components of unequal stability. The major component is the more stable one; the minor component(s) can be stabilized by the addition of Ca^{2+}. Both components can be activated by Ca^{2+} presumably because Ca^{2+} decreases the K_m of the enzyme-substrate interaction (Inagami and Sturtevant, 1960; B. F. Erlanger and A. G. Cooper, paper in preparation). Agar-gel electrophoresis patterns show two active components migrating toward the cathode at *p*H 6.12 in the absence of Ca^{2+}, the minor labile component apparently having a lower mobility. Addition of Ca^{2+} to the buffer results in an electrophoretically homogeneous system, the minor component now migrating with the major component. This indicates that the minor component is more negatively charged, perhaps possessing an aspartic or glutamic acid residue which is present in the major component as an asparagine or a glutamine.

According to the findings of Winzor and Scheraga (1963) and Gilbert (1955, 1959), the two spots observed after agar-gel electrophoresis need not be an indication of two separate components but can result from a system in which two forms are in rapid equilibrium. In the case of α-chymotrypsin they would be monomer and dimer molecules (Schwert, 1949; Smith and Brown, 1952; Steiner, 1954; Massey *et al.*, 1955; Dreyer *et al.*, 1955; Rao and Kegeles, 1958; Tinoco, 1957). If this were true, we must conclude that the monomer-dimer equilibrium is greatly affected by the presence of Ca^{2+}. However, in preliminary ultracentrifuge studies on α-chymotrypsin in the presence and absence of Ca^{2+} (Erlanger and Castleman, unpublished), no marked difference in the sedimentation patterns could be observed. We tentatively conclude, therefore, that the electrophoretic pattern shown in Figure 3 is an indication of heterogeneity.

The results of the large scale incubation at 37° in the absence of Ca^{2+} suggest that, during incubation, the minor component was digested by the major one. This conclusion is based upon the finding that only 68% of the original α-chymotrypsin could be recovered after dialysis. Whether the digestion occurred prior to or after denaturation of the minor component was not determined.

None of the results in this paper can explain the manner in which Ca^{2+} activates 3×-crystallized α-chymotrypsin. Some preliminary experiments have shown, however, that activation can be reversed by 3 × 10^{-4} M *o*-phenanthroline, although it is unaffected by the presence of the same concentration of Versene.

Egan *et al.* (1957) have expressed the view that the impurity in α-chymotrypsin is β- or γ-chymotrypsin. Studies on the stability of 2×-crystallized Worthington γ-chymotrypsin carried out in this laboratory appear to eliminate it as a possibility since its activity decreases only 40% after 60 minutes at 37°.

The findings reported in this paper are of significance for any study of 3×-crystallized α-chymotrypsin carried out in the absence of Ca^{2+}. Under these conditions it must be assumed that the experiments are being per-

formed on a heterogeneous system. There is always danger, therefore, that results derived from such studies may be a reflection of this heterogeneity. This is particularly relevant to experiments carried out at temperatures above 30°. For example, in their investigations on the relative effects of temperature on the conformations of α-chymotrypsin and diisopropylphosphoryl α-chymotrypsin, Havsteen and Hess (1962, 1963) found large changes in the optical rotation of the active enzyme at temperatures above 40°. Since Ca^{2+} was absent it is pertinent to ask whether these changes may have been caused solely by denaturation of the more labile component of α-chymotrypsin.

Since only in the presence of Ca^{2+} does α-chymotrypsin behave as a homogeneous system by the few parameters studied (electrophoretic mobility and stability), it is suggested that Ca^{2+} be present in any investigation of the mechanism of action of this enzyme.

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The Ribonuclease-catalyzed Hydrolysis of Uridine-2',3'-phosphate*

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The values for k_o, K_o, and K_p for the ribonuclease-catalyzed hydrolysis of uridine-2',3'-phosphate at 25° and at various *p*H values are as follows: *p*H 5.0, $k_o = 0.96 \pm 0.15$ sec^{-1}, $K_o = 0.51 \pm 0.35$ mM, $K_p = 0.047 \pm 0.033$ mM; *p*H 6.0, $K_o/k_o = 0.35 \pm 0.11$ mM sec, $K_p = 0.056 \pm 0.018$ mM; *p*H 7.0, $K_o/k_o = 1.45 \pm 0.11$ mM sec, $K_p = 0.47 \pm 0.08$ mM; *p*H 7.5, $k_o = 3.94 \pm 0.08$ sec^{-1}, $K_o = 19.9 \pm 1.2$ mM, $K_p = 2.16 \pm 0.21$ mM; *p*H 8.0, $K_o/k_o = 14.3 \pm 0.4$ mM sec. Inhibitor-free uridine-2',3'-phosphate is hydrolyzed more rapidly than previously reported and at rates comparable to those of cytidine-2',3'-phosphate. Product inhibition is marked at all *p*H values and the ratio K_o/K_p is nearly independent of *p*H and temperature, and equal to values of *ca.* 10. Uridine-2'-phosphate is a more potent inhibitor than uridine-3'-phosphate at low *p*H values. Adenosine-2'-phosphate is a weak inhibitor of ribonuclease. No inhibitory action toward ribonuclease is exhibited at *p*H 5.0 by any of the following compounds: D-ribose; DL-α-glycerophosphate; β-glycerophosphate; D-glucosamine; D-galactose; β-D-glucose; D-gluconic acid; D-fructose; D-fructose-1,6-diphosphate; adenosine; deoxyadenosine; deoxyadenosine-5'-monophosphate; adenosine-3'-phosphate. The temperature dependences of the kinetic parameters at *p*H values 5.0 and 7.0 were studied. The entropy of activation for the decomposition of enzyme-substrate complex to enzyme-product complex is −32 eu/mole at *p*H 5.0 and −29 eu/mole at *p*H 7.0. Uridine-2',3'-phosphate is subject to slow nonenzymic hydrolysis at *p*H 7.0 to yield exclusively uridine-3'-phosphate. Adenosine-2',3'-phosphate is not appreciably hydrolyzed under these conditions.

A detailed study (Herries *et al.*, 1962) of the ribonuclease (RNAase)-catalyzed hydrolysis of cytidine-2',3'-phosphate (C-2',3'-P) has recently been published. The kinetics of this hydrolysis were shown to be consistent with a mechanism involving product inhibition:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_o} EP \overset{K_p}{\rightleftharpoons} E + P$$

where ES and EP are the enzyme-substrate and enzyme-product complexes, respectively, and K_p is the enzyme-product-complex dissociation constant. The kinetics followed the rate law:

$$v = -d(S)/dt = k_o(E)_t(S)/\{(S)(1 - K_o/K_p) + K_o[1 + (S)_i/K_p]\}$$

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