TEMPERATURE-DEPENDENT ACTIVATION OF TRYPSIN BY CALCIUM

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We recently reported that calcium ions activate and stabilize a proteinase that is produced by a marine bacterium (Sipos and Merkel, 1967). More recently we observed a temperature-dependent activation of this proteinase with 0.02 M calcium ions, (Sipos and Merkel, 1968a). Because the phenomenon of calcium stabilization of trypsin against autolysis has been known for many years, (Nord and Bier, 1953; Green and Neurath, 1953) and the effects of calcium on the conformation of trypsinogen and trypsin have been studied more recently (Lazdunski and Delaage, 1965, 1967), we set out to see if trypsin also underwent a temperature-dependent activation by calcium, as indicated above.

In the present communication we wish to report that trypsin shows a temperature-dependent activation by calcium similar to that observed with a bacterial proteinase. The phenomenon was observed in trypsin catalyzed hydrolysis of urea-denatured hemoglobin, p-tosyl-L-arginine methyl ester (TAME), and benzoyl-L-arginine ethyl ester (BAEE). At pH 8.0 "calcium-free" trypsin (see Experimental) had its optimum activity for the hydrolysis of hemoglobin at 35°, but in the presence of calcium the optimum activity was shifted to 40°. With either TAME or BAEE as the substrate at pH 8.0, the temperature optimum was shifted from 45° to 60°. In the presence of calcium the enzyme activity at the temperature optimum was always greater than controls without calcium, and the activating effect of calcium always occurred at or

after the transition temperature (temperature optimum) for "calcium-free" trypsin. This indicated that some change occurred to trypsin at the transition temperature and the altered form of the molecule in the presence of calcium was stable to higher temperatures and possessed greater enzyme activity than "calcium-free" trypsin. We also noted that esterolytic activity of trypsin could be separated from the proteolytic activity. Proteolytic activity was inhibited above 50°, but esterolytic activity reached its optimum at 60-65°.

EXPERIMENTAL

Trypsin used in the present experiments was a 3x recrystallized, salt-free preparation (TRL 100 S) purchased from Worthington Biochemical Corp., Freehold, N.J.; "calcium-free" trypsin was obtained by dialyzing the commercial preparation against 5 l of 0.001 M HCl at 4°C over a 20 hr. period. The concentration of trypsin in 0.001 M HCl solution was determined by its optical density at 280 mμ, using 0.694 as the optical factor for converting the absorbance to mg/ml concentration (Davie and Neurath, 1955). TAME was obtained from the Nutritional Biochemical Corp., Cleveland, Ohio; BAEE was purchased from the Mann Research Laboratories, New York; and the hemoglobin substrate was purchased from Worthington Biochemical Corp. The preparation of the marine bacterial proteinase will be described in a future publication (Merkel and Sipos, 1968).

The spectrophotometric method of Schwert and Takenaka (1955) was used to follow BAEE hydrolysis, and the method of Hummel (1959) was used for TAME hydrolysis. A typical enzyme velocity determination involved the addition of 1.5 ml of 0.2 M calcium chloride and 11.5 ml of 0.02 M Tris buffer at pH 8.0, to 1.5 ml of 0.01 M TAME or BAEE immersed in a constant temperature bath. After 3 minutes preincubation, 0.5 ml of ice cold enzyme solution (12.5 μg/ml in 0.001 M HCl) was added and immediately mixed. At fixed time intervals 2.5 ml portions were removed and transferred to a silica cuvette

(1 cm light path) and the optical density was immediately recorded (at 254 mμ for BAEE and 247 mμ for TAME) on a Bausch & Lomb Spectronic 600 using an enzyme-free blank to correct for any spontaneous hydrolysis of the substrates. Determinations of the enzyme activity were made with each of the three substrates at various temperatures, pH's, and in the presence and absence of calcium chloride.

Hemoglobin-digesting activity of the enzymes was studied by a slight modification of Anson's method (Anson, 1938). The reaction mixtures contained 2.5 ml of urea-denatured hemoglobin (20 mg/ml in 0.2 M Tris buffer, pH 7.0 or 8.0) and 0.5 ml of enzyme (43 μg/ml in the case of trypsin). The substrate was equilibrated at the desired temperature prior to the addition of enzyme. Enzyme reactions were stopped after 5 min. by the addition of 4 ml of 5% trichloroacetic acid, and the absorbances at 280 mμ of the soluble peptides were read against controls that received the TCA before the addition of enzyme.

RESULTS AND DISCUSSION

Figure 1 shows typical temperature-velocity curves obtained with given amounts of the bacterial proteinase and hemoglobin substrate. The most significant feature of these temperature profiles is the appearance of a secondary peak in the presence of calcium. The secondary peak occurs at a higher temperature and with a greatly increased enzyme activity. The experiment has been repeated numerous times and the activation effect of calcium always occurred at or after the temperature optimum (transition temperature) for "calcium-free" enzyme, which indicated that calcium activation required significant structural changes of the enzyme molecule. The shoulder in the temperature optimum curve of calcium-treated enzyme indicates that calcium is doing more than simply protecting the enzyme against heat denaturation. It appears that in the range of 40°, the enzyme molecule undergoes changes in its structure, and in the presence of high concentrations of calcium a new conform-

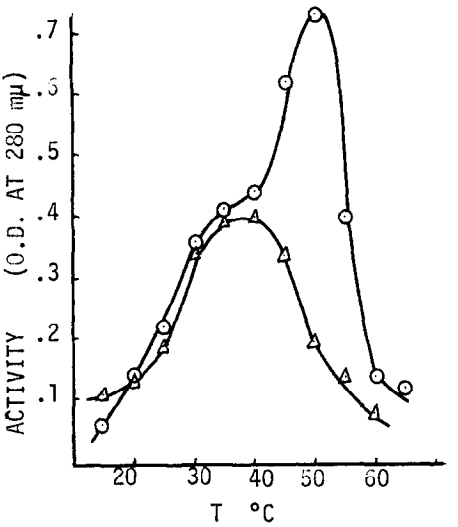

Fig.1.Temp.dependent activation of a bacterial proteinase by calcium.
Substrate: hemoglobin, pH 7.0
(-o-o-) with calcium, 0.02 M
(-Δ-Δ-) without calcium

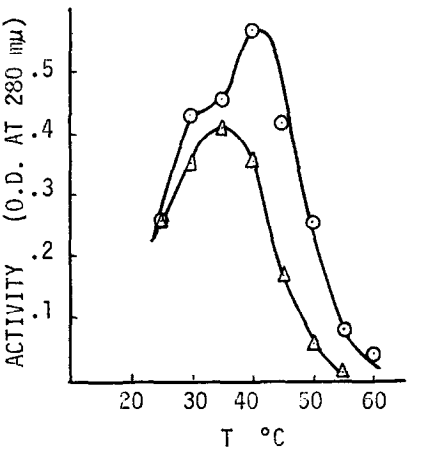

Fig.2.Temp. dependent activation of trypsin by calcium.
Substrate: hemoglobin, pH 8.0
(-o-o-) with calcium, 0.02 M
(-Δ-Δ-) without calcium

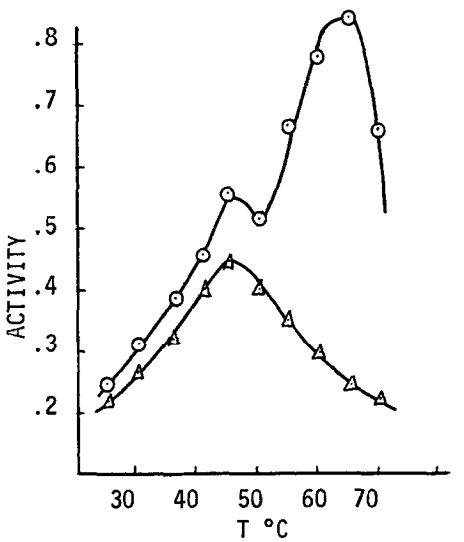

Fig.3.Temp.dependent activation of trypsin by calcium.
Substrate: TAME, pH 8.0
(-o-o-) with calcium, 0.02 M
(-Δ-Δ-) without calcium
Activity: mM TAME hydrol./min/mg trypsin

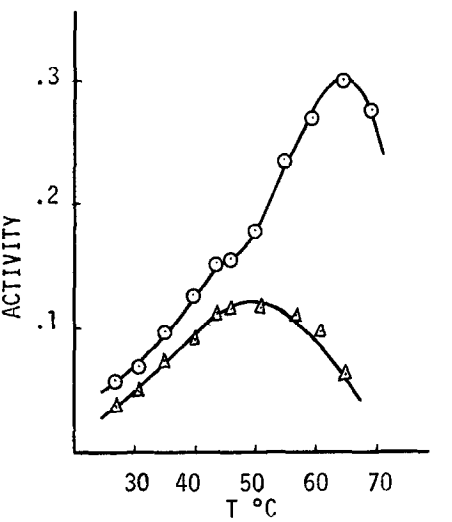

Fig.4.Temp.dependent activation of trypsin by calcium.
Substrate: BAEE, pH 8.0
(-o-o-) with calcium, 0.02 M
(-Δ-Δ-) without calcium
Activity: mM BAEE hydrol./min/mg trypsin

ation is stabilized. The new conformation seems to endow new modes of activity as evidenced by the increased activity, stability, and, as we shall see below in the case of trypsin, a separation of esterolytic and proteolytic activity.

Attempts to replace calcium with other metal ions (Mg^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Sr^{2+}, Mn^{2+}, Cu^{2+}, Fe^{3+}) of comparable ionic strength failed to show similar activation effects, when the bacterial enzyme activity was tested with hemoglobin substrate.

When the enzyme activity of dialyzed trypsin was tested with hemoglobin substrate in the presence or in the absence of calcium ions, the patterns shown in Fig. 2 were obtained. The activating effect of calcium was similar to that observed with the bacterial proteinase, but under the conditions employed, the effect was not as pronounced. This latter fact may account for the failure of other investigators to note the activating effect. Another factor that must be taken into consideration if one wants to observe a difference in activity of calcium and "calcium-free" preparations, is the need to remove all metals from most commercial enzyme preparations by dialysis. Using dialyzed trypsin, the activating effect of calcium is reproducible.

Activation of trypsin by calcium at 40° was found to be concentration and pH dependent. These effects will be discussed in a succeeding publication.

Figures 3 and 4 illustrate the influence of 0.02 M calcium ions on the hydrolysis of TAME and BAEE by trypsin at various temperatures. In the presence of calcium (upper curves) the appearance of the secondary peak with a greater enzyme activity and a higher temperature optimum clearly indicates that the temperature-dependent calcium activation phenomenon is similar to that obtained with the bacterial proteinase. The result of the apparent molecular transition that occurs around 45° is intensified in the presence of these synthetic substrates. We attribute the small differences between the BAEE and TAME curves to the variation in sensitivity of each reaction to the presence of metal ions. Of the three substrates used in the current set of

experiments, BAEE was the most sensitive to metal ion activation. Thorough dialysis of trypsin, and metal-free Tris buffer were required for the results shown in Fig. 4.

A comparison of the curves in Fig. 2 with those in Figs. 3 and 4 reveals another striking feature of the influence of calcium on trypsin. A separation of proteolytic and esterolytic activity was achieved at 65°. At this high temperature trypsin showed no proteolytic activity against hemoglobin substrate, even in the presence of calcium, while esterolytic activity reached its maximum with TAME and BAEE.

The above observations indicate that calcium probably functions to maintain a specific conformation of the enzyme molecules which is necessary for their catalytic activity. The transitions in molecular conformation that normally occur at the optimum temperature for each of the enzymes that we studied, appear in some way to be stabilized by high concentrations of calcium and the new structures impart increased, and modified, enzyme activity to the molecules. We are presently studying the conformational changes that occur in the enzyme molecules under the influence of temperature and calcium ions, and the preliminary results will be published soon (Sipos and Merkel, 1968b).

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