pyridoxal 5′-phosphate represents such a form of metabolic regulation.

Acknowledgments

We are indebted to Mrs. Treasure Gallo and Mrs. Maria Tamasi for their excellent technical assistance. We also thank Dr. David Sigman for his many criticisms and suggestions.

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Studies on the Role of Calcium in Thermolysin*

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ABSTRACT: It has been found that the thermostable neutral protease from *Bacillus thermoproteolyticus*, thermolysin, contains four rather tightly bound calcium atoms per molecule. Removal of three to four of these calcium atoms has been achieved without effect on the catalytic properties of the enzyme but with marked reversible loss of thermostability. Addition of calcium to the low calcium enzyme results in restabilization of the molecule. The use of gel filtration over Sephadex G-25 and G-75 equilibrated with EDTA and 1,10-phenanthroline for simultaneous removal of calcium and zinc to yield low calcium inactive apoenzyme has permitted these manipulations which under other circumstances result in rapid autolysis of the enzyme. The stabilizing effect of additional calcium on the thermolysin was also studied.

The role of calcium in maintaining stability has been reported for a number of different enzymes. The stabilization of trypsin solutions by calcium has been reported (Bier and Nord, 1951; Gorini, 1951; Green *et al.*, 1952). Various α-amylases have been shown to be stabilized by calcium (Stein and Fischer, 1958; Stein *et al.*, 1964; Hsiu *et al.*, 1964) and Vallee *et al.* (1959) demonstrated the presence of at least one atom of calcium per molecule for certain of these enzymes. The maintenance of a taut enzyme molecule by calcium also has been reported for the *Escherichia coli* glutamine synthetase (Shapiro and Ginsburg, 1968). The bacterial metallo neutral proteases are particularly sensitive to the presence of calcium in solution for stability. The *Bacillus subtilis* neutral protease (McConn *et al.*, 1964; Tsuru *et al.*, 1966a,b) as well as the alkaline protease (Tsuru *et al.*, 1966a,b; Matsubara *et al.*, 1958) is stabilized by calcium. Similarly, calcium and other divalent cations stabilize the neutral proteases from *Bacillus megaterium* (Millet, 1969), *Streptomyces griseus* (Nomoto *et al.*, 1960), *Pseudomonas aeruginosa* (Morihara, 1963), and *Bacillus cereus* (J. Feder, 1971, unpublished data). The thermostable thermolysin also requires calcium ions for stability (Endo, 1962; Ohta *et al.*, 1965, 1966; Matsubara, 1967; Ohta, 1967). Removal of the calcium from the thermolysin molecule results in loss of the thermal stability of the enzyme. Since thermolysin and the other neutral proteases from the bacilli such as the *Bacillus subtilis* enzyme are very similar, with respect to specificity, zinc content and general catalytic properties as well as a dependence on the presence of calcium for

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stability and differ markedly in their thermal stability properties; they present an interesting model for the study of the role of calcium in stabilization. It has been found that thermolysin contains four rather tightly bound calcium atoms per molecule. Techniques have been developed for the reversible removal of three to four of these calcium atoms without loss of enzyme activity to yield a very unstable molecule. Characterization of these species with respect to thermal stability and the implication of these studies on the chemical role of calcium in protein structure is reported here.

Experimental Section

Materials

Thermolysin was purchased from Calbiochem and recrystallized three times before use by the method described by Matsubara (1970) with slight modification. Hepes[1] buffer was purchased from Sigma Chemical Co. The Hepes buffer was passed over a Dowex chelating resin (Sigma Chemical Co.) to remove contaminating metal ions. Particular care to maintain metal-free glassware was exercised including repeated soak and rinse with dithizone–carbon tetrachloride before use. Reagent 1,10-phenanthroline was purchased from Fisher Scientific Co.

Sephadex G-25-300 and G-75-120 were purchased from Sigma Chemical Co. The beads were equilibrated with pH 7.2 Hepes buffer (0.1–0.01 M) both with and without 1,10-phenanthroline and EDTA.

The thermolysin substrate 3-(2-furylacryloyl)glycyl-L-leucinamide (FA-Gly-Leu-NH_2) was synthesized as described (Feder and Schuck, 1970).

Methods

The enzyme-catalyzed hydrolysis of FA-Gly-Leu-NH_2 was monitored spectrophotometrically at 345 mμ using a Cary 14 PM recording spectrophotometer with a thermostated cell compartment at 25.0 ± 0.1°, as described (Feder, 1968; Feder and Schuck, 1970). All reactions were run at a substrate concentration ($S_0 = 9.76 \times 10^{-4}$ M) much below the K_m exhibiting pseudo-first-order kinetics which yielded rate constants, $k = k_{cat}(E_0)/K_m$. The molar enzyme concentration was calculated from the first-order rate constants using a k_{cat}/K_m value of 17.2×10^3 M^{-1} sec^{-1} for the reaction in pH 7.2 Hepes buffer (0.1 M). The enzyme concentration was also determined from the absorbance at 280 mμ using a value of $E^{280}_{1\%} = 17.6$ (Ohta *et al.*, 1966). Good agreement was obtained by both methods.

Zinc and calcium were determined by atomic absorption using a Beckman atomic absorption accessory with an acetylene–air laminar flow burner attached to a Beckman DU spectrophotometer and a 10-in. recorder. Zinc and calcium measurements were carried out at 213.9 and 422.2 mμ, respectively, with 4.8 psi of acetylene and 20 psi of supporting air and 8 and 15 mA through the zinc and calcium hollow cathode lamps, respectively. Standard curves were over the range of 25–100 ppb for zinc and 50–300 ppb for calcium. The removal of calcium from crystalline thermolysin preparations without concomitant denaturation and autolysis was achieved by gel filtration over Sephadex G-25-300 or G-75-120, equilibrated with pH 7.2 Hepes buffer containing 2×10^{-3} M 1,10-phenanthroline and 10^{-4} M EDTA. This yielded an inactive, zinc-free, low-calcium enzyme. Assays for the enzyme activity were carried out using FA-Gly-Leu-NH_2 containing 3×10^{-5} M $ZnCl_2$ and 10^{-2} M $CaCl_2$.

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; FA, furylacryloyl.

TABLE I: Calcium Content of Crystalline Thermolysin.

	$E \times 10^5$ M		
Ca^{2+} (ppb)	From Absorbance	From Activity	Ca^{2+}/Mole
2450	1.55	1.50	3.95

Results

Two approaches were employed to determine the calcium content of thermolysin. Since the enzyme was purified and crystallized from calcium acetate solutions, it was necessary to separate the calcium which was not an intrinsic part of the molecule. Crystalline thermolysin was recrystallized three times from calcium-free buffers. The crystals were dried and the calcium content was determined as well as the enzyme concentration. The molarity was determined both from the protein content using the absorbance at 280 mμ assuming a molecular weight of 37,500 (Ohta *et al.*, 1966) and from the pseudo-first-order rate constant for the enzyme-catalyzed hydrolysis of FA-Gly-Leu-NH_2. As shown in Table I, 4 atoms of calcium were found per molecule of thermolysin. A second approach that was used employed gel filtration over Sephadex G-75 to remove extraneous calcium. The gel was washed and equilibrated with pH 8.0 Hepes buffer (0.1 M) which had been passed over a Dowex chelating resin to remove contaminating metal ions. Table II shows the calcium and zinc content of the peak fractions, 7 and 8, together with the molar enzyme concentration obtained from the activity toward FA-Gly-Leu-NH_2. This method also yielded about four calcium atoms per molecule of enzyme.

Previous attempts to remove the calcium from thermolysin and from some of the other neutral proteases resulted in very rapid inactivation of the enzyme. For example, treatment of the *B. subtilis* neutral protease with 0.1 M EDTA at basic pH resulted in very rapid autolysis of the enzyme. This was evidenced by a change in the elution profile of the enzyme over Sephadex G-75 to yield a low molecular weight peak. Analysis for N-terminal amino acids yielded leucine and phenylalanine typical of what was expected by autolysis of a neutral protease from its specificity. In order to study the effect of the

TABLE II: Calcium Content of Thermolysin Obtained on Gel Filtration over Sephadex G-75.[a]

Fraction	Ca^{2+} (ppb)	Zn^{2+} (ppb)	$E \times 10^5$ M[b]	Ca^{2+}/Mole of E	Ca^{2+}/Zn^{2+}
7	2200	830	1.27	4.3	4.5
8	2710	930	1.42	4.7	4.1

[a] Sephadex G-75 equilibrated with pH 8.0 Hepes buffer (0.1 M) and eluted with same; column 24 × 1 cm. [b] Enzyme molarity determined from first-order rate constant, $k = k_{cat}(E_0)/K_m$, for enzyme-catalyzed hydrolysis of FA-Gly-Leu-NH_2, assuming $k_{cat}/K_m = 17.2 \times 10^3$ M^{-1} sec^{-1}.

TABLE III: Removal of Calcium from Thermolysin by Gel Filtration over Sephadex G-25 Equilibrated with 1,10-Phenanthroline and EDTA.[a]

Fraction	Ca^{2+} (ppb)	$E \times 10^5$ M[b]	Ca^{2+}/Mole
6	1000	2.03	1.23
7	1040	3.34	0.78
8	825	1.07	1.93

[a] Sephadex G-25-300 equilibrated with pH 7.2 Hepes buffer (0.1 M) containing 2×10^{-3} M 1,10-phenanthroline and 10^{-4} M EDTA and eluted with same; column 24 × 1 cm. [b] Fractions assayed with FA-Gly-Leu-NH_2 (9.76×10^{-4} M) in pH 7.2 Hepes buffer (0.1 M) containing 3×10^{-5} M $ZnCl_2$ and 10^{-2} M $CaCl_2$. Enzyme molarity calculated from first-order rate constant, $k = k_{cat}(E_0)/K_m$, assuming $k_{cat}/K_m = 17.2 \times 10^3$ M^{-1} sec^{-1}.

removal of calcium from thermolysin, it was necessary to devise an approach which would prevent autolysis. Gel filtration over Sephadex G-25-300 or G-75-120 equilibrated with pH 7.2 Hepes buffer containing 2×10^{-3} M 1,10-phenanthroline and 10^{-4} M EDTA proved successful for this purpose. The 1,10-phenanthroline provided a very rapid method for removal of the zinc atom yielding an inactive apoenzyme (Feder and Garrett, 1971; Feder *et al.*, 1971) which did not autolyze. Table III shows the calcium content of the enzyme obtained in the fractions across the enzyme peak. The fractions were assayed with FA-Gly-Leu-NH_2 substrate containing 3×10^{-5} M zinc and 10^{-2} M calcium to determine the molar enzyme concentration. The calcium content dropped to 1–2 atoms/molecule. In other experiments values as low as 0.5 atom of calcium/molecule of enzyme have been obtained. Attempts to prepare completely calcium-free enzyme were limited by the solubility of the enzyme and the contaminating level of calcium on the column. This enzyme exhibited the same activity toward FA-Gly-Leu-NH_2 as the original enzyme containing 4 atoms of calcium. Likewise, the presence or absence of high

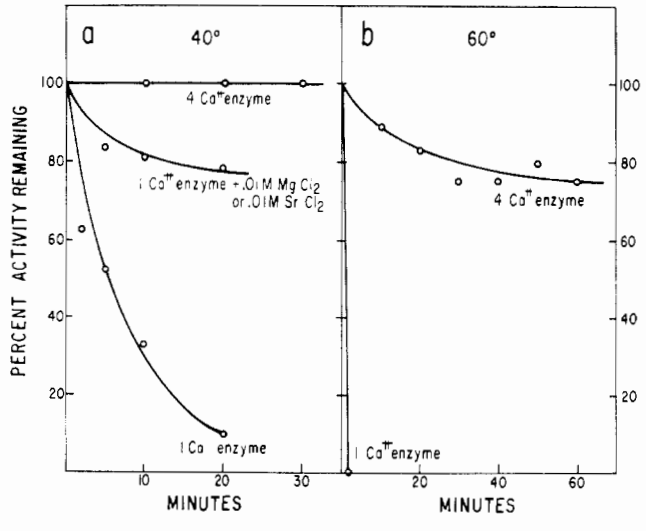

FIGURE 1: Comparison of the thermal stability of the four-calcium and one-calcium thermolysins. (a) (E_0) = $5.8–7.0 \times 10^{-7}$ M in pH 7.2 Hepes buffer (0.1 M); (b) (E_0) = $3.8–6.7 \times 10^{-7}$ M in pH 7.2 Hepes buffer (0.1 M); details of assay in text.

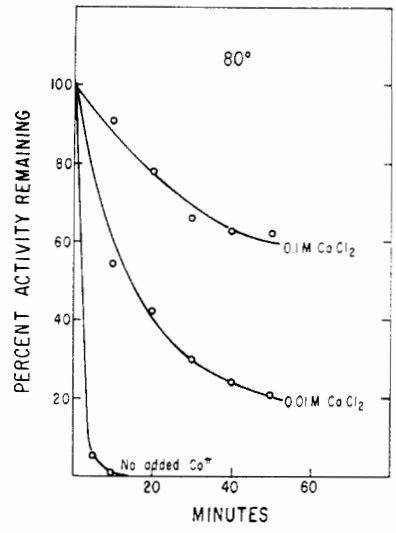

FIGURE 2: Effect of calcium on the thermal stability of the four-calcium thermolysin. (E_0) = $3.6–7.4 \times 10^{-7}$ M in pH 7.2 Hepes buffer (0.1 M); details of assay in text.

calcium (0.1 M) in the reaction did not affect the rate constant. Since no apparent catalytic effect was observed with the enzyme containing high or low calcium, the thermostability of these preparations was studied. Although the stabilizing effect of calcium ions on thermolysin has been reported (Endo, 1962; Ohta *et al.*, 1965, 1966; Matsubara, 1967; Ohta, 1967) no relationship between stability and calcium content has been shown. The thermostability of the crystalline enzyme containing 4 atoms/molecule was determined and compared to the low calcium enzyme. The enzyme was incubated at various temperatures and at intervals aliquots were withdrawn and assayed at 25° with FA-Gly-Leu-NH_2 containing 3×10^{-5} M $ZnCl_2$. Figure 1 shows the thermostability of a 4-calcium and 1.2-calcium thermolysin at 40 and 60° in the absence of added calcium. The loss of 3 atoms of calcium decreased the thermostability of the enzyme considerably. The 4-calcium enzyme retained 100 and 76% of its activity after 1 hr at 40° and 60%, respectively, in contrast to the low calcium enzyme

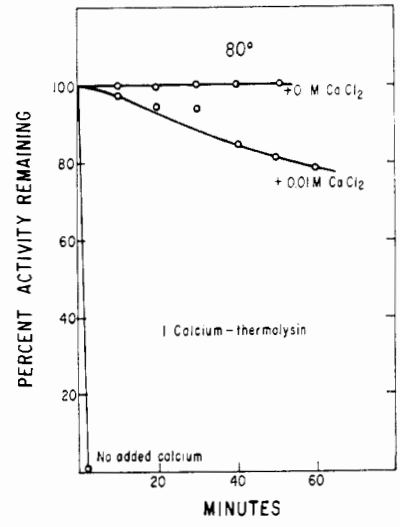

FIGURE 3: Effect of calcium on the thermal stability of the one-calcium thermolysin. (E_0) = 1.03×10^{-6} M in pH 7.2 Hepes buffer (0.1 M).

(1.2 calcium/molecule) which lost 90% of its activity after 20 min at 40° and was totally inactivated at 60° within 2 min. The effect of the calcium removal was reversible, since the addition of calcium to the incubation buffer reestablished the thermal stability. The addition of magnesium and strontium ions also stabilized the enzyme but to a lesser extent than calcium (Figure 1a).

All of these studies were concerned with the stability of the 4-calcium or low-calcium (1–2 atoms/molecule) enzyme in a buffer free of additional calcium ions. These calciums were rather firmly bound to the molecule since they were not removed by gel filtration over Sephadex G-25 or G-75. The presence of additional calcium ions in solution; however, markedly stabilized the enzyme. Figures 2 and 3 show the effect of various calcium concentrations on the stability of the 4-calcium and 1–2-calcium enzymes, respectively. Greater thermal stability was obtained as the calcium concentration was increased. It appeared, however, that the low-calcium enzyme was significantly more stabilized than the 4-calcium enzyme at any given concentration of calcium. If some of the inactivation at elevated temperature was due to increased autolysis in addition to thermal denaturation, the presence of an inhibitor would stabilize the enzyme. The presence of inhibitory 1,10-phenanthroline in the low-calcium preparation might thus exhibit a stabilizing effect. Thus, it was possible to remove three calciums from the enzyme, retain all of its catalytic activity toward FA-Gly-Leu-NH_2 but markedly alter its thermal stability and then return to its original state by the addition of calcium.

Discussion

The presence of 4 atoms of calcium/molecule of thermolysin of a molecular weight of 37,500 which has been found in these studies is in good agreement with the calcium content reported by Latt *et al.* (1969) for their crystalline preparations of this enzyme. This calcium appeared to be firmly bound to the enzyme and was not removed by gel filtration. Likewise, this species exhibited a characteristic thermal stability. Gel filtration over EDTA- and 1,10-phenanthroline-equilibrated Sephadex G-25 or Sephadex G-75 resulted in the removal of 2–3 of these calcium atoms to yield a thermolysin species which upon addition of zinc retained all of its catalytic activity but was considerably more thermolabile than the native enzyme. This was, however, a reversible process and addition of calcium to this low-calcium enzyme resulted in a restabilization of the enzyme. Whether the remaining single calcium atom is more tightly bound than the rest or whether this represents the limiting level of calcium contamination is speculative at this point.

In addition to these two enzyme species the addition of increasing concentrations of calcium ions up to 0.1 M to the enzyme solution resulted in progressively greater thermal stability. This suggested additional binding sites for the divalent cation with poorer binding constants, which result in a more stable conformation of the enzyme. Preliminary optical rotatory dispersion studies have indicated that the conformation of the molecule becomes markedly more random in the presence of EDTA. Ohta (1967) reported that the removal of calcium by dialysis against EDTA caused loss of activity and a decrease in the helical conformation. Likewise, Drucker and Yang (1969) reported a drop in the helical content of thermolysin in the absence of calcium in the solution. These preliminary studies strongly suggest that the calcium plays an important role in these enzymes in maintaining the integrity of its tertiary structure. Considering that the thermolysin molecule is a single polypeptide chain containing about 315 amino acids without any disulfide bridges, these results lead one to propose that some of the divalent cations might perform this bridging function within the molecule. Hsiu *et al.* (1964) have proposed intramolecular cross-linking involving alkaline earth metals, similar in function to disulfide bonds for some α-amylases. A similar function for calcium in glutamine synthetase from *E. coli* has been proposed by Shapiro and Ginsburg (1968).

The neutral proteases from *Bacillus*, including those from *B. subtilis*, *B. megaterium*, *B. cereus*, and *B. thermoproteolyticus*, have no disulfide bonds and are markedly stabilized by calcium. Since they share common enzymatic properties such as specificity, zinc requirement, and pH dependence, but differ considerably with respect to thermal stability, they offer a unique opportunity for the study of the role of calcium in maintaining the conformation of proteins.

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Rare Earth Metal Ions as Substitutes for the Calcium Ion in *Bacillus subtilis* α-Amylase*

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ABSTRACT: Rare earth metal ions have been used to replace the calcium ion in *Bacillus subtilis* α-amylase. The lanthanide α-amylases were found to be enzymatically active. The effectiveness of various lanthanide ions to activate apoamylase was found to follow the order: $Ca^{2+} \sim Lu^{3+} > Yb^{3+} > Er^{3+} \sim Y^{3+} \sim Dy^{3+} \sim Tb^{3+} > Sm^{3+} > Nd^{3+} > Pr^{3+} > La^{3+}$. The smallest rare earth ions were most effective as calcium ion substitutes whereas the larger lanthanides were less effective. The circular dichroic spectra of the native calcium-containing α-amylase, apoamylase, and Nd^{3+}–amylase are identical in the spectral region from 200 to 250 nm. This suggests there are no large structural changes in the enzyme upon removal of the calcium ion from native amylase.

Research on the nature of the interaction of the calcium ion with proteins has lagged far behind studies of the nature of transition metal ion interactions with proteins. This situation has come about simply because it is difficult to obtain any spectroscopic or magnetic information about the calcium ion which can be used to reveal the nature of its interaction with proteins. We have recently proposed that the rare earth metal ions should make good isomorphous replacement substitutes for the calcium ion (Darnall and Birnbaum, 1970; Birnbaum *et al.*, 1970). In contrast to the calcium ion, however, the lanthanide ions can be scrutinized by a variety of magnetic and spectral methods and hence should make good probes for the calcium ion binding sites in proteins. Williams (1970) has recently come to similar conclusions.

We have shown previously that the neodymium ion binds to trypsin and trypsinogen with concomitant changes in the visible absorption spectrum of the neodymium ion (Darnall and Birnbaum, 1970). Neodymium ion was shown to function in a manner similar to the calcium ion acceleration of the activation of trypsinogen to trypsin.

If information concerning the lanthanide ion binding sites in proteins is to be extrapolated to the calcium ion binding sites in proteins, it must be shown that the lanthanide ions are isomorphic replacements for the calcium ion. Strong evidence that this obtains in the α-amylase system is presented herein.

The α-amylases of various origins so far investigated have been found to contain at least one atom of calcium firmly and specifically bound to the enzyme (Stein *et al.*, 1964, and references therein). *Bacillus subtilis* α-amylase, which contains two apparently identical subunits, has been shown to bind 4–5 calcium ions/mole of enzyme of mol wt 48,000 (Stein *et al.*, 1964; Imanishi, 1966; Connellan and Shaw, 1970). It has been shown by Hsui *et al.* (1964) and Imanishi (1966) that when the calcium is removed from *B. subtilis* α-amylase, enzymatic activity is lost. Upon addition of calcium back to the apoamylase full enzymatic activity is recovered.

We have prepared calcium-free α-amylase and tested the lanthanide ions as possible activators of the apoenzyme. We have been able to observe reactivation of *B. subtilis* α-amylase with many of the rare earth metal ions, and indeed we have observed a correlation of enzyme activation with the crystal ionic radius of the rare earth metal ion tested.

Experimental Section

Enzyme. Crystalline *B. subtilis* α-amylase (lot 108B-0590, Sigma) was used without further purification. The enzyme sedimented as a single symmetrical boundary in the ultracentrifuge and gave a single component upon polyacrylamide electrophoresis. Before calcium was removed from the enzyme, it was always treated with 10^{-3} M phenylmethylsulfonyl fluoride for 12–20 hr at 4° to inactivate traces of proteolytic enzymes (Hsui *et al.*, 1964). Unless these contaminants were thoroughly inactivated, α-amylase was irreversibly inactivated by proteolysis upon removal of the calcium. After treatment with phenylmethylsulfonyl fluoride, the enzyme was dialyzed against the desired solution and centrifuged at 15,000 rpm, and the protein content determined by absorption at 280 nm using $E^{1\%}_{1\,cm} = 25.3$ (Hsui *et al.*, 1964). If the solution was to serve directly for enzymic studies it was diluted in 0.02 M maleic acid–Tris buffer (pH 6.0) to approximately 2 μg/ml. If the enzyme was to be electrodialyzed or dialyzed against EDTA for calcium removal, it was not diluted with buffer, but immediately put in the appropriate vessel for electrodialysis.

Calcium Analysis. Calcium concentrations were analyzed by means of a Perkin-Elmer 303 atomic absorption spectrometer. The procedure followed was that outlined by the manufacturer, except that the purity of our water eliminated the need for a 1% lanthanum chloride solution in the analysis. Calcium ion concentration was determined at 422 nm from

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