THERMOLYSIN: A ZINC METALLOENZYME

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Metal analyses and inhibitor studies have shown that thermolysin, a neutral protease from B. thermoproteolyticus, is a zinc metalloenzyme. The relevance of this finding to the active site characteristics of other bacterial neutral proteases and to those of alkaline proteases is considered.

INTRODUCTION: A catalytically essential zinc atom has been found at the active sites of a number of proteolytic enzymes (1). Thermolysin, an endopeptidase from B. thermoproteolyticus, has been purified recently (2), and some of its chemical and kinetic properties (3,4), as well as its specificity (5,6,7,8) have been described. The enzyme is stabilized by Ca^{2+} (2) and inhibited by EDTA and 1,10-phenanthroline (6), though no data as to its metal content are on record as yet. The present studies demonstrate that thermolysin is a zinc metalloenzyme.

MATERIALS AND METHODS: Thermolysin was obtained from Diawa Kasei (Osaka, Japan) and Calbiochem (Los Angeles, California). This material was recrystallized three or more times before use, either by dissolving at high pH, followed by recrystallization at neutrality (2) or by dissolving the enzyme in 5 M NaBr at neutral pH and dialyzing to low ionic strength to promote crystallization. Both the metal content and the specific act-

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ivities of material obtained by either procedure are virtually the same.

Metal analyses were performed both by spark emission spectrography (9) and by atomic absorption spectrometry (10,11). Data are reported on the basis of dry weight for samples which were ashed at 450° in an electric muffle furnace before analysis. The results of zinc analyses were similar when performed by atomic absorption spectrometry on native material and based on the extinction coefficient of the enzyme (3).

Activity was assayed spectrophotometrically based on a procedure for neutral protease of B. subtilis (12; Feder, personal communication), utilizing the decrease in absorbance at 345 mμ on hydrolysis of furylacryloylglycyl-L-leucine amide (Cyclo Chemical Company, Los Angeles, California). At initial substrate concentrations of 1 mM, well below the substrate K_m, all rates of hydrolysis were first order for at least two half lives. Values of k_{obsd}, the observed first order rate constant, were proportional to enzyme concentration. Reactions were performed in 0.05 M Tris, 0.01 M Ca^{2+}, pH 7.5, μ = 0.1 M (NaCl) at 25° and were followed to completion (10 half lives) in several experiments. Rate constants are expressed as $k = k_{obsd}/[E]$ in $M^{-1}min^{-1}$ with enzyme concentration based on $OD_{280}^{1cm} = 17.65$ for a 1% solution of an enzyme of molecular weight 37,500(3).

Inhibition studies with metal binding agents were performed by the addition of stock enzyme to a reaction cuvette containing an equilibrated solution of substrate and inhibitor. The first readings of absorbance were taken one minute after enzyme addition. Products of the hydrolysis, furylacryloylglycine and leucine amide, were identified by thin layer chromatography (methanol-$CHCl_3$, 1:9) on fluorescent silica gel (Eastman).

All inhibitors and chemicals were of reagent grade or recrystallized several times before use. Buffers were rendered metal-free by dithizone extraction. Acid cleaned glassware or nalgene tubes were used throughout and appropriate precautions to prevent metal contamination were observed(13).

RESULTS: The metal analyses of five different preparations of recrystallized thermolysin are shown in Table I. Preparations contained about 2000 µg/g zinc, 3800 µg/g calcium on the average,and the concentration of other metals found was insignificant.

The addition to reaction mixtures of agents known to bind zinc ions in solution inhibited hydrolysis instantaneously. Prolonged preincubation of the enzyme with these agents gave identical results. Figure 1 shows the effect of increasing concentrations of 1,10-phenanthroline, thioglycolic acid and imidazole on enzyme activity. Values of pK_I for 2,2'-bipyridine, mercaptoethylamine, mercaptoacetic acid, and cyanide were 3.4, 3.1, 2.4 and 2.2, respectively. Azide did not inhibit at concentrations up to 0.08 M. The addition of zinc ions to enzyme solu-

Table I.

Emission Spectrographic Analyses of Three Times Recrysallized Thermolysin

Metal Content (µg/g)

Sample	Zn‡	Ca	Fe	Al	Ba
1	2100 ± 200	3400	*	*	1
2	2000 ± 300	3900	310	50	*
3	2000 ± 300	3500	350	100	20
4	1850 ± 200	4000	80	25	2
5	1900 ± 300	4400	100	20	*

‡ Includes atomic absorption measurements. All zinc measurements were performed at least in quadruplicate; all other metals were measured at least in duplicate. The values given are the averages.

* = Not detected; also Cd, Co, Cr, Mg, Mn, Mo, Ni, Pb, Sr. Copper was not determined due to the method employed (9).

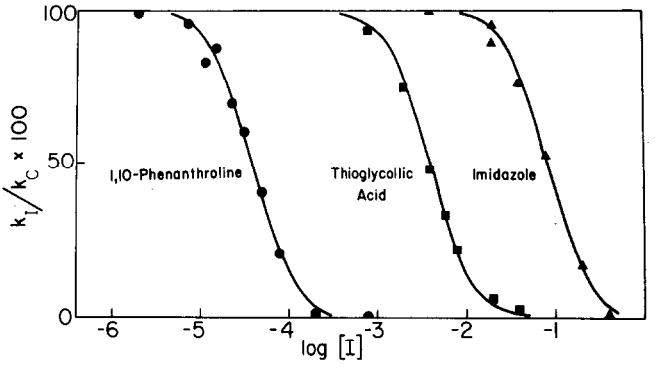

Figure 1: Effect of Metal Binding Agents on the Activity of Thermolysin. Initial substrate concentration was 1 mM in .05 M Tris-Cl, .01 M $CaCl_2$, and NaCl to ionic strength of 0.1 at 25°. Activity is expressed as 100 times the ratio of the inhibited rate constant, k_I to the control k_c, determined as described under Materials and Methods. k_c under the above conditions is $9 \pm 1 \times 10^5 M^{-1} min^{-1}$.

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tions, fully inhibited by 1,10-phenanthroline or 2,2'-bipyridine, resulted in complete reactivation. Concentrations of zinc ions, in excess of those which reactivated the enzyme completely, caused inhibition, in agreement with the effect of excess zinc ions on the native enzyme.

Dialysis versus three changes of 0.001 M 1,10-phenanthroline, 0.05 M HEPES, pH 7, 0.4 M NaCl, 0.01 M $CaCl_2$ for eight hours each,

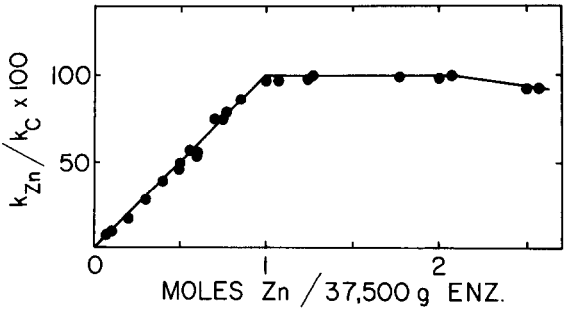

Figure 2: Return of Enzymatic Activity to Apothermolysin upon Readdition of Zinc. Apothermolysin, prepared as described in the text, contained five per cent of the initial zinc content, and possessed six per cent of the activity of the native enzyme. Activity data are expressed at the ratio of k_{Zn}, the rate observed with zinc added to the apoenzyme, to k_c, the rate of the native enzyme.

followed by dialysis against three changes of buffer for eight hours each, reduced enzymatic activity to less than 6% and zinc content to 5% of the control. As shown in Figure 2, activity was completely reestablished by the addition of approximately 1 gram atom of zinc per 37,500 grams of apoenzyme. Moreover, as judged by regeneration of activity upon zinc addition, the apoenzyme was stable on storage in such buffers at 5° C for at least two weeks.

DISCUSSION: The criteria for the identification of metalloenzyme have been delineated (9,14), and the present data establish that thermolysin is a zinc metalloenzyme. Recrystallized thermolysin consistently contains from 1700 to 2300 μg per gram of zinc, averaging about 2000 μg per gram (Table I). The metal is essential to the function of the enzyme. A number of chelating agents, known to bind transition and group IIB ions in solution, effectively inhibit thermolysin (Figure 1). Further, zinc ions effectively compete with enzyme zinc for the inhibitors, thereby reversing the inhibition. The product resulting from dialysis of the enzyme against 1,10-phenanthroline did not contain zinc and was inactive. Thus, this agent inhibits the enzyme through interaction and removal of zinc. Further, the reincorporation of the metal atom restores activity (Figure 2). When stored and assayed in buffers containing calcium, the apoenzyme was inactive within the limits of experimental error. Thus, calcium ions do not seem involved directly in catalytic activity.

While for single chain proteins the stoichiometry of the number of metal atoms per protein molecule has been established with relative ease, such efforts have been problematic when investigating multichain metalloenzymes (15). For thermolysin a preliminary molecular weight of 37,500 has been reported (3), but it is not known whether or not the enzyme has subunit structure. Hence, at present it would seem advisable to express the zinc content either in μg/gm (Table I) or moles of zinc per 37,500

grams of thermolysin (Figure 2). A detailed investigation of the protein structure of thermolysin is required before a stoichiometry of zinc/enzyme can be assigned definitively. This would appear imperative also in view of the large amounts of calcium which have been found consistently in all of the preparations of thermolysin here described. Though there is no evidence for the direct participation of Ca^{2+} in function of the enzyme, metal atoms can serve functional, structural, or both purposes (15) and, hence, such possibilities will have to be investigated rigorously. Conjectures as to the role of calcium must await more precise definition of the molecular architecture of the enzyme.

In recent years, an increasing number of mammalian proteolytic enzymes have been found to be zinc enzymes as exemplified by the carboxypeptidases and their precursors and by certain aminopeptidases (16). Similarly, the neutral protease from B. subtilis (17) and that from B. thermoproteolyticus (16) now constitute examples of bacterial zinc proteases. B. aeruginosa, S. griseus, A. oryzae (18) and B. megatherium (19,20) also contain neutral proteases. That from B. megatherium, megatheriopeptidase, is inhibited by EDTA, 2,2'-bipyridine and 1,10-phenanthroline, which suggested that this neutral protease may also be a metalloenzyme (20). Thus, a systematic study of neutral proteases from bacteria might well reveal such common chemical features, contrasting with alkaline bacterial proteases which generally have been found to have DFP reactive seryl residues (21).

Thus, certain bacterial strains contain at least two classes of peptidases which differ both in pH optimum and the detailed chemistries of their active sites, in some ways reminiscent of the proteolytic enzymes of mammalian pancreas. Here, a DFP reactive seryl residue is characteristic of certain endopeptidases while some exopeptidases avail themselves of a zinc atom in their mechanism of action. Could the preservation of distinctive chemical properties at the active sites of

two types of proteolytic enzymes in lower and higher forms of life contain information concerning the evolution of enzymatic specificities and mechanisms of action? It could be that changes in primary, secondary and tertiary structure including those dependent upon ions such as, e.g. Ca^{2+}, have been employed to modulate functions to which the zinc atom in one class and the active seryl residue in the other are invariant. Comparative enzymological studies along such lines might have relevance both to the evolution of these classes of enzymes and the elucidation of the details of their mechanism of action.

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