THE STRUCTURAL AND FUNCTIONAL ROLES OF METAL IONS IN THERMOLYSIN

Authors: **Rodney S. Roche**
Department of Chemistry
The University of Calgary
Calgary, Alberta, Canada

Gerrit Voordouw
Department of Biochemistry
Agricultural University
Wageningen, The Netherlands

Referee: Brian W. Matthews
Institute of Molecular Biology
University of Oregon
Eugene, Oregon

1. INTRODUCTION

Thermolysin (EC 3.4.24.4) is a thermostable, calcium-binding zinc metalloendopeptidase excreted by the thermophile *Bacillus thermoproteolyticus.*[1] An understanding of the thermostability of this molecule is relevant to the general question of the molecular basis of thermophily as well as to current attempts to understand the mechanistic details of protein folding-unfolding processes. A number of thermostable proteins and enzymes from thermophilic microorganisms have been identified and have become the focus of intense interest.[54] Of these, thermolysin is the enzyme characterized in most detail. Its physicochemical properties,[2] amino acid sequence,[3] and three-dimensional structure to a nominal resolution of 2.3 Å[4,7] have all been reported. Recently, the mode of binding of a number of dipeptide inhibitors[55] and the naturally occurring inhibitor phosphoramidon[56] to thermolysin has been determined by X-ray crystallography. These results indicate the probable mode of binding extended substrates to thermolysin and also suggest a mechanism that is similar to one of the alternative mechanisms for peptide hydrolysis by carboxypeptidase A as suggested by Lipscomb and coworkers.[57] In this review we will focus on the structural and functional roles played by metal ions bound to thermolysin.

2. GENERAL DESCRIPTION OF THE MOLECULE

2.1. The Polypeptide Backbone

The overall conformation of the molecule (molecular weight 34,600) shows a folding in two distinct lobes (Figure 1) with most of the helical secondary structure in the carboxy-terminal half and most of the β-structure in the amino-terminal half of the molecule. Novel features of the structure include a γ-turn[47,48] and a short sequence, Asp 226; Asn 227; Gly 228; Gly 229, of left-handed α-helix which is the first to be observed in a globular protein.

2.2. The Metal Binding Sites

The two lobes of the molecule are separated by a deep cleft across the middle of the molecule. A zinc ion is bound at the bottom of this cleft; it is coordinated by the side chains of Glu 166, His 142, and His 146 (Figure 2A). The presence of one

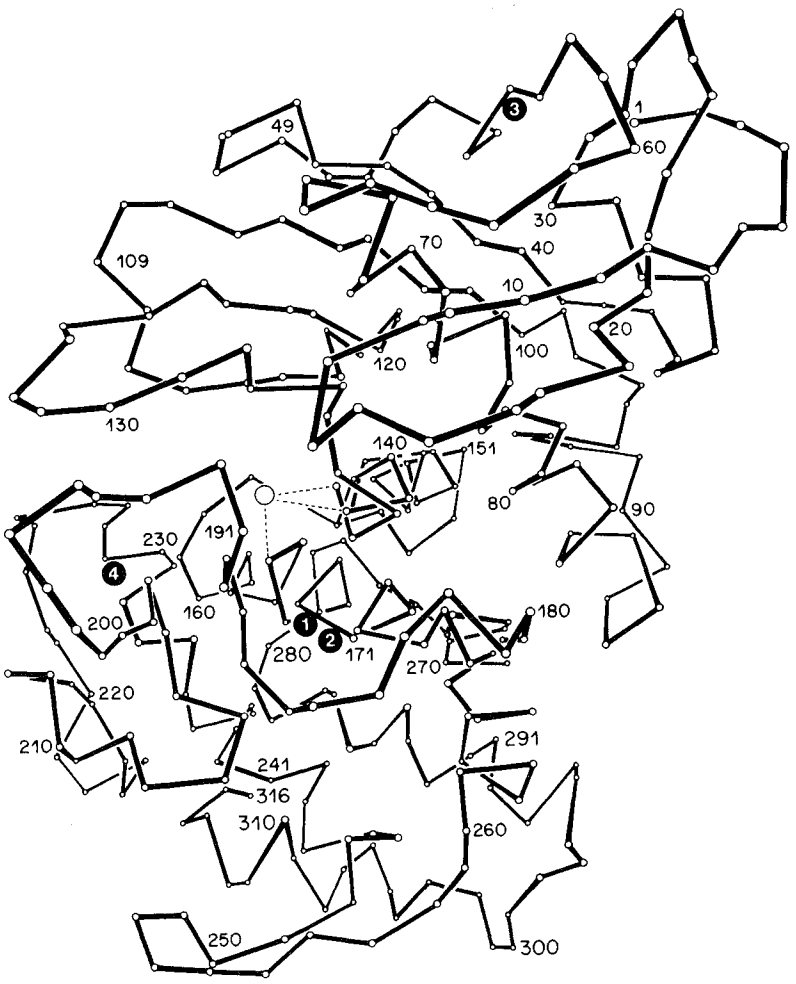
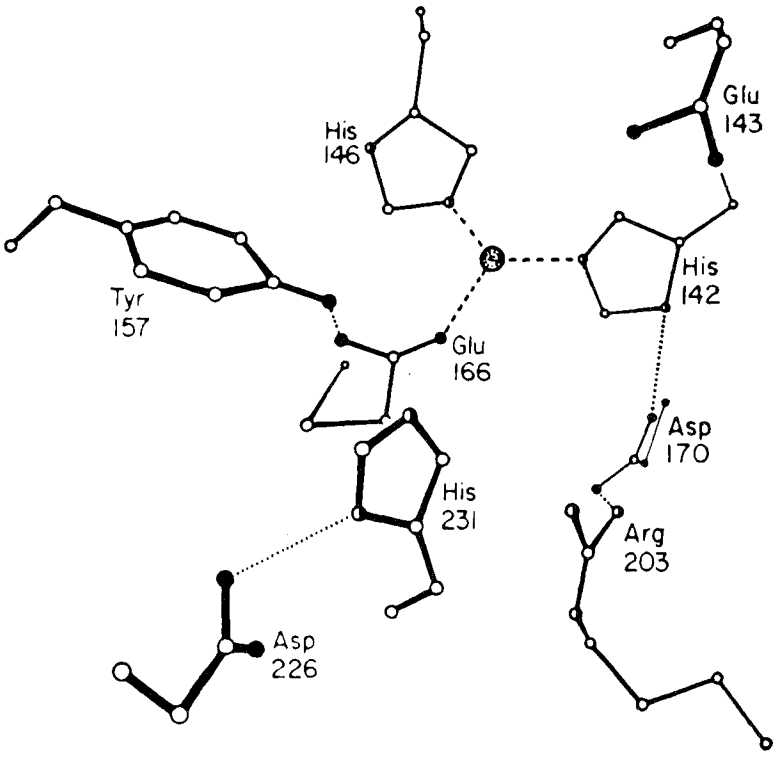


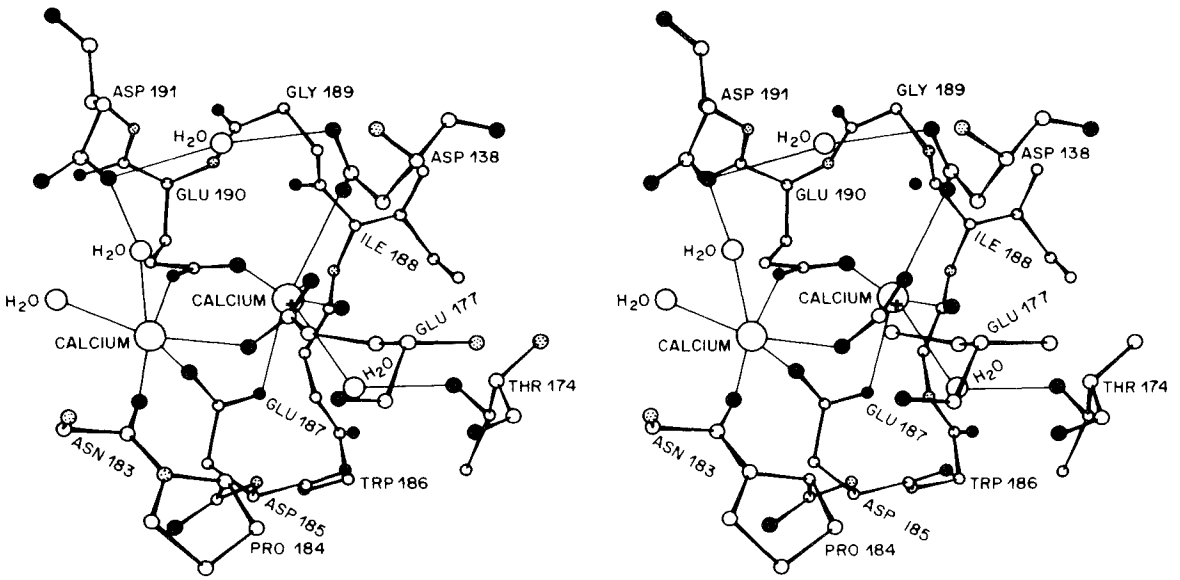
FIGURE 1. Perspective drawing illustrating the backbone conformation of the thermolysin molecule. The zinc (○) and calcium ions (●) are also indicated. (Adapted from Colman, P. M., Jansonius, J. N., and Matthews, B. W., *J. Mol. Biol.*, 70, 701, 1972. With permission.)

tightly bound zinc per mole of enzyme, essential for catalytic activity and previously demonstrated by solution studies,[8] is thus confirmed and characterizes thermolysin as a zinc metalloenzyme, showing neutral endopeptidase activity.[1,3] Interestingly, the approximately tetrahedral coordination of the zinc ion, with a water molecule acting as a fourth ligand, is similar to that of the active site zinc of carboxypeptidase A, which employs the same ligands (Glu 72, His 69, His 196, H_2O).[6,7,35]

X-Ray crystallographic studies have also demonstrated the presence of four calcium binding sites in the thermolysin structure[5-7,12] in agreement with solution studies.[10,11] These calcium ions, binding in positions relatively distant from the active site zinc ion (Table 1), are not important for the enzymatic activity, but play a significant role in stabilizing the structure of the molecule. Their contribution to the kinetic thermal stability of thermolysin has recently been elucidated in some detail[33] (see Section 4.5). The four sites are denoted as follows:[6] S(1), the inner of the double site; S(2), the outer of the double site; S(3), the single site at Asp 57; and S(4), the single site at Asp 200. The two calcium ions present in the double site bind very close together (Figure 2B and Table 1). They share the coordina-



A



B

FIGURE 2. (A) The zinc-binding site; (B) the calcium-binding double site S(1)-S(2); (C) Site S(3); (D) Site S(4). (Figure 2a from Colman, P. M., Jansonius, J. N., and Matthews, B. W., *J. Mol. Biol.*, 70, 701, 1972. With permission. Figures 2b, 2c, and 2d adapted from Matthews, B. W. and Weaver, L. H., *Biochemistry*, 13, 1719, 1974. With permission.)

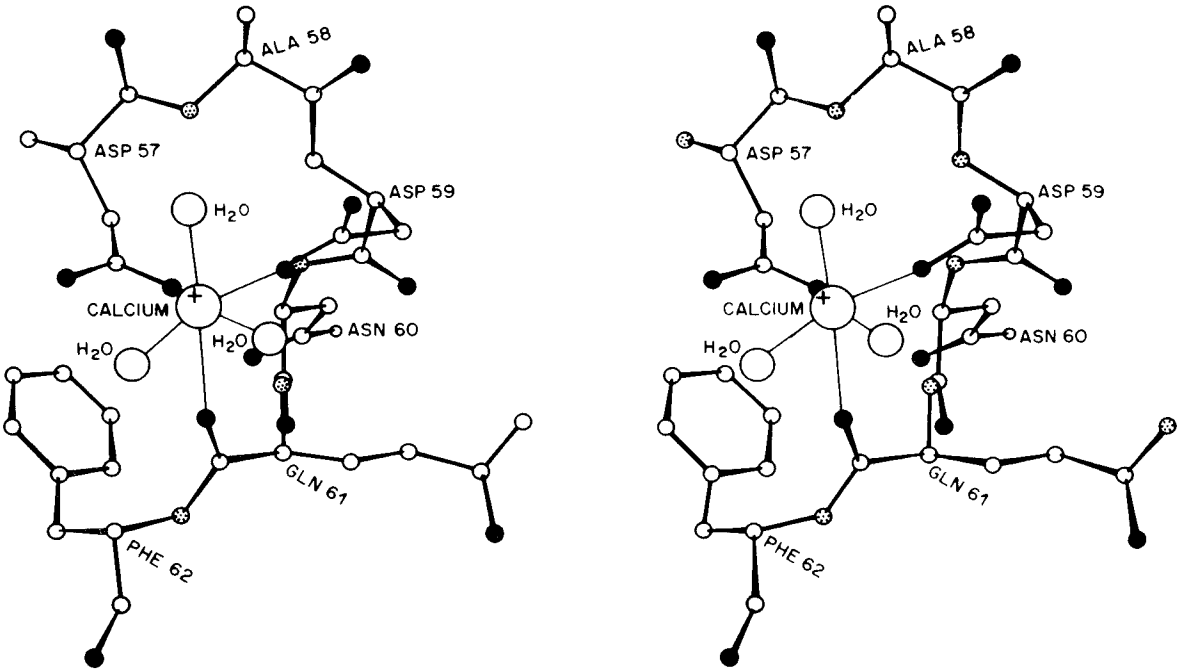


FIGURE 2C

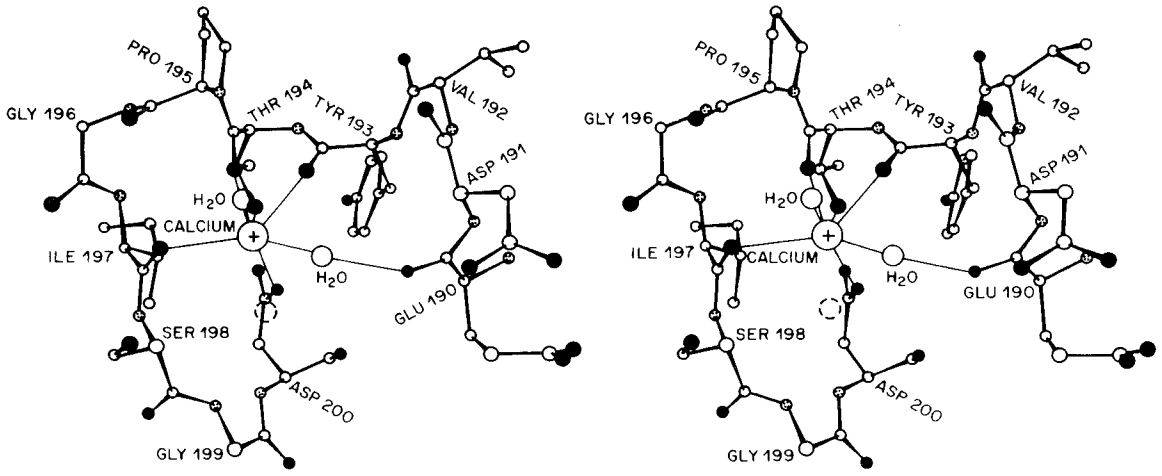


FIGURE 2D

TABLE 1
Ion-Ion Distances (Å) in Thermolysin[a]

	Ca(1)	Ca(2)	Ca(3)	Ca(4)
Ca(2)	3.7			
Ca(3)	29.6	28.7		
Ca(4)	10.9	11.7	29.6	
Zn	13.7	17.2	29.9	19.1

[a]Calculated from the coordinates given by Matthews et al.[7]

tion of three carboxyl groups (Glu 177, Asp 185, and Glu 190) which act as bridging ligands between the two ions, thus creating a *single* site accommodating *two* ions. The inner calcium ion, Ca(1), is shielded from the solvent and in addition to the bridging ligands is coordinated by the carboxyl group of Asp 138, the backbone carbonyl oxygen of Glu 187, and an internal water molecule. The outer calcium ion, Ca(2), is additionally coordinated by the backbone carbonyl oxygen of Asn 183 and two water molecules.[7] The coordination of both calcium ions is distorted octahedral. From a structural point of view, the double site seems to be positioned at a strategic position in the thermolysin molecule. It is located near the cleft that runs between the two lobes of the molecule and provides a connection between them.[7] The other two calcium ions, Ca(3) and Ca(4), are each bound in a single, extended loop of polypeptide chain. The roughly octahedral coordination of Ca(3) is provided by the carboxyl groups of Asp 57 and Asp 59, the backbone carbonyl oxygen of Glu 61, and three water molecules (Figure 2C). Similarly, Ca(4) is coordinated by the backbone carbonyl oxygens of Tyr 193, Thr 194, and Ile 197; the hydroxyl group of Thr 194, the carboxyl of Asp 200, and two water molecules (Figure 2D).

It is clear that the structural features of the five metal binding sites in thermolysin have been elucidated in considerable detail; the atomic coordinates having been reported for the structure refined to a nominal resolution of 2.3 Å.[7] These data, together with the possibility of focusing on specific sites by suitable metal ion substitutions as described below (Section 4.3), place thermolysin in a unique position for the study of the roles of metal ions in protein structure.

3. THE ACTIVE SITE ZINC ION

3.1. Inhibition by Zinc Chelating Agents

It can be readily demonstrated that bound zinc is essential for enzymatic activity since chelating agents which bind zinc but not calcium ions inhibit thermolysin. For all of the chelating inhibitors examined thus far,[8,14] the inhibition appears to operate by a single predominant mechanism in which the inhibitor lowers the free zinc concentration by chelation to levels below the dissociation constant of the zinc-emzyme complex. The other possibility is inhibition by formation of a ternary inhibitor-zinc-enzyme complex. The following suggests that the first of these two possibilities is operative:

1. The free inhibitor concentration at half maximal inhibition (K_I) decreases proportionately with the dissociation constants of the complexes that are known to form between the zinc ion and a range of chelating inhibitors.[15,16]

2. Upon dialysis of holothermolysin* against inhibitor solutions of sufficiently high concentration, inactive apothermolysin** is indeed obtained.

3. In the case of 1,10-phenanthroline, attempts to spectrophotometrically detect a ternary complex even as an intermediate in the inhibition process were unsuccessful[14] in contrast to other zinc metalloenzymes.[37,38]

The insignificant contribution of mixed complexes to the inhibition has also been established by kinetic methods for the inhibitor 1,10-phenanthroline.[17,18] The rates of hydrolysis of the synthetic substrate furylacryloylglycyl-L-leucinamide (FAGLA) in the presence (v) and absence (v_o) of 1,10-phenanthroline can be expressed by Equation 1:[17]

$$\left(\frac{v}{v_o - v}\right)\left\{1 + K_{ZnL_1}[L]_T + K_{ZnL_1}K_{ZnL_2}[L]_T^2 + K_{ZnL_1}K_{ZnL_2}K_{ZnL_3}[L]_T^3\right\} = K_{ZnE}[Zn]_T - K_{ZnE}[E]_T\frac{v}{v_o}\Bigg) \quad (1)$$

where $[L]_T$, $[Zn]_T$, and $[E]_T$ are the total 1,10-phenanthroline, zinc ion, and thermolysin concentrations, respectively; K_{ZnL_1}, K_{ZnL_2}, and K_{ZnL_3} are the known stability constants of the zinc-1,10-phenanthroline complexes formed;[15,16] and K_{ZnE} is the stability constant of the zinc-enzyme complex under the given experimental conditions. K_{ZnE} can be determined by measuring v_o and v as a function of $[L]_T$ at constant, known $[E]_T$

*The enzyme form binding 1 mol of zinc and 4 mol of calcium ions per mole.
**The zinc-free enzyme form binding 4 mol of calcium per mole.

and $[Zn]_T$ and plotting the data according to Equation 1. This leads to $K_{ZnE} = 2.1 \times 10^{11}$ M^{-1} at pH 7.50, 25°C, and ionic strength 0.1.[17] The high value of K_{ZnE} ensures complete saturation of the active site and full enzymatic activity of thermolysin at very low free zinc levels (10^{-8} to 10^{-9} M).

One can also derive from Equation 1 how the total inhibitor concentration at half maximal inhibition ($[L]_T = K_I$, $v/v_o = 0.5$) depends on $[Zn]_T$ and $[E]_T$.

$$\{K_{ZnL_1} K_I + K_{ZnL_1} K_{ZnL_2} K_I^2 + K_{ZnL_1} K_{ZnL_2} K_{ZnL_3} K_I^3\} = K_{ZnE}[Zn]_T - 0.5 K_{ZnE}[E]_T - 1 \quad (2)$$

Equation 2 provides a quantitative expression for the finding[14,17] that K_I depends on $[Zn]_T$ and $[E]_T$. Equations 1 and 2 do not apply when there is a significant contribution of mixed complexes to the inhibition.[17]

3.2. The Conformation of Apothermolysin

The conformational changes that accompany the dissociation of the zinc ion from the active site are very small. A 2.3-Å resolution electron density difference map between native thermolysin and thermolysin crystals soaked in 1,10-phenanthroline solution shows very little perturbation of the protein structure upon removal of the zinc and suggests only small localized adjustments in the three zinc ligands (His 142, His 146, and Glu 166) and in the side chains of Glu 143 and His 231.[7] In solution, the similarity of the structures of holo- and apothermolysin is indicated by the absence of detectable differences in the peptide and aromatic circular dichroism spectra.[14] A small conformational adjustment can be shown, however, by UV difference spectroscopy in the aromatic region of the spectrum; the absorbance of apothermolysin at 288 nm decreases slightly upon addition of zinc.[14]

($\Delta\epsilon = -230\ M^{-1}\ cm^{-1}$)

3.3. Replacement of Zinc by Other Metal Ions

Apart from the zinc ion, a number of other metal ions can be incorporated in the active site of thermolysin by stoichiometric addition to the apoenzyme. The Co^{2+} and Mn^{2+} derivatives are particularly interesting because of their characteristic spectral properties (sections 3.5 and 5). Interestingly, whereas the reconstituted holoenzyme displays 100% enzymatic activity, the Co^{2+} and Mn^{2+} derivatives show 200 and 10% of the holoenzyme activities, respectively, when assayed with FAGLA.[14] From enzyme assays with varying concentrations of Zn^{2+} and Co^{2+} present in the solution, the stability constant of the Co^{2+}-enzyme complex has been estimated to be 600-fold smaller than that of holothermolysin.[14] A similar ratio between the stability constants of the Zn^{2+} and Co^{2+} derivatives was found for carboxypeptidase A,[19] further illustrating the similar chelating properties of the active sites of these two enzymes. Addition of Fe^{2+} to apothermolysin restores 60% of the holoenzyme activity.[14] In contrast to the Co^{2+}- and Mn^{2+}-induced activities, the Fe^{2+} activity is observed only after addition of a 30-fold molar excess of Fe^{2+}. Although a much larger number of other ions has been tested (Mg^{2+}, Cr^{2+}, Ni^{2+}, Cu^{2+}, Mo^{2+}, Pb^{2+}, Hg^{2+}, Cd^{2+}, Fe^{3+}, Nd^{3+}, Pr^{3+}, and Dy^{3+}), none restores activity.[14] Although this may simply reflect a failure of the given ion to bind to the active site of the apoenzyme, X-ray crystallographic studies have shown full occupancy of the active site in the Hg^{2+} derivative.[7] The Hg^{2+} site does not coincide exactly with the Zn^{2+} site in holothermolysin, but is displaced from it by approximately 0.7 Å.

3.4. Inhibition of Thermolysin by Metal Ions and the Mechanism of Enzyme Action

Holothermolysin is inhibitied by concentrations of Zn^{2+} in excess of 10^{-5} M[9,14]. This inhibition is the result of binding to a second zinc binding site. The latter site has a stability constant of approximately 10^5 M^{-1} under the conditions examined (25°C, pH 7.50).[9,14] The dual role played by zinc leads to the bell-shaped pZn-activity profile shown in Figure 3, which has been calculated for the range $2.0 < pZn < 14.0$ using the binding constants $K_1 = 2.1 \times 10^{11}$ M^{-1} and $K_2 = 10^5$ M^{-1} for the active site[17] and second zinc binding site,[9] respectively. The location of the second zinc site is suggested by the X-ray crystallographic identification of a site at His 231 which binds Hg^{2+} as well as Ag^+.[6,7] Both Ag^+ and Hg^{2+} are known to reversibly inhibit holothermolysin activity, which suggests that Zn^{2+} inhibits by binding at the same

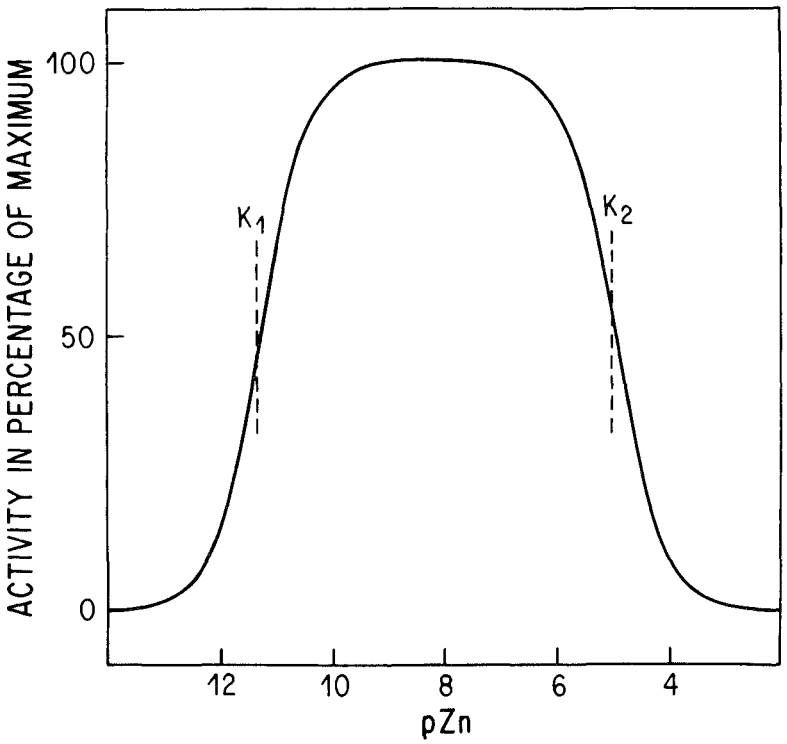


FIGURE 3. The pZn-activity profile calculated from $K_1 = 2.1 \times 10^{11}\ M^{-1}$ and $K_2 = 10^5\ M^{-1}$.

site and implicates His 231 in the catalytic mechanism.* The observation that mercury compounds protect the protein against covalent inhibition by ethoxyformic anhydride further implicates His 231 in the proposed mechanism of thermolysin.[9] Chemical modification with ethoxyformic anhydride indicates that a single peptide residue with a pKa of 7.6 is essential for enzyme activity. Although a modified peptide could not be identified by peptide-mapping techniques, it is thought to be an active site histidine.[39,40] Of the three histidine residues in the active site, only His 231 is free to participate in the catalytic mechanism; His 142 and His 146 are coordinated to the essential zinc atom. It is proposed[9] (Figure 4) that the protonated form of His 231 could serve as a proton donor to the amino nitrogen of the peptide undergoing cleavage. Sundberg and Martin[58] have pointed out, however, that the peptide nitrogen in the ground state cannot act as a proton acceptor. The proposed proton transfer from His 231 to the nitrogen of the scissile peptide bond must, therefore, occur in the transition state in which both the nitrogen and the carbonyl carbon of the hydrolysed peptide are tetrahedral. The function of His 231 is thought to be similar to that of Tyr 248 in carboxypeptidase A; otherwise, the active site residues of carboxypeptidase A and thermolysin are remarkably similar.[56] Both enzymes have one glutamyl residue, Glu 270 and Glu 143, respectively, located in precisely the same spatial relation to the active site zinc atom, which is similarly coordinated in both enzymes. The proposed function of this glutamyl residue[9,35] is that of a generalized base attacking nucleophilically, via a water molecule, the carbonyl carbon of the susceptible peptide bond, which in turn is polarized by coordination of its oxygen to the zinc.[9] The transfer of a proton from Tyr 248 in carboxypeptidase A or from His 231 in thermolysin is thought to complete the scission of the substrate peptide bond. The active sites of both enzymes have a hydrophobic pocket (identified crystallographically by the binding of com-

*Holothermolysin is reversibly inhibited by Hg^{2+} which replaces the active site zinc ion or binds to His 231 or both.

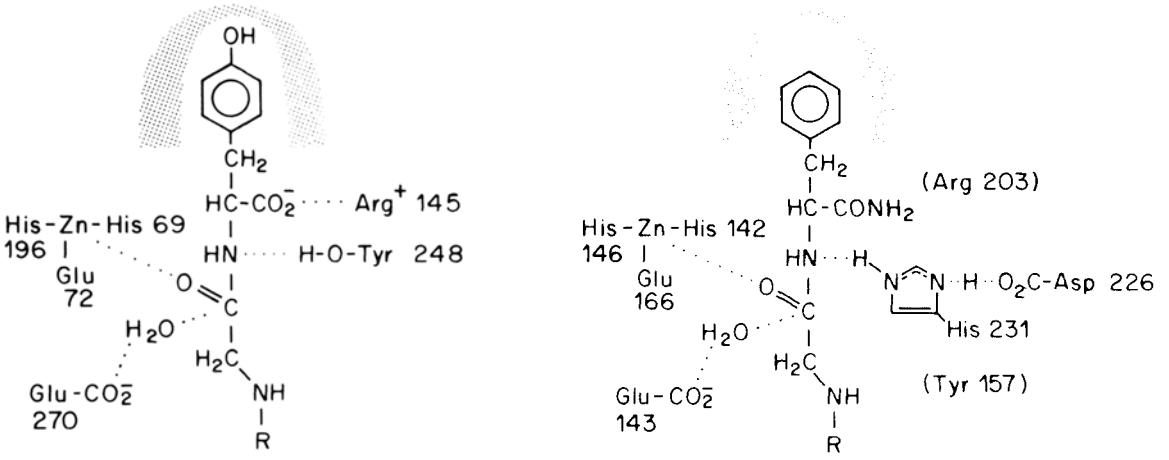


FIGURE 4. Comparison of the proposed mechanisms of action of carboxypeptidase A. (From Pangburn, M. K. and Walsh, J. A., *Biochemistry*, 14, 4050, 1975. With permission.)

petitive inhibitors) and an arginine (Arg) residue. In carboxypeptidase A, Arg 145 forms a salt bridge with the C-terminal carboxyl group of the substrate and thus presumably assists in its binding and orientation; the function of Arg 203 in thermolysin, an endopeptidase, is unclear. Recently, considerable support for these mechanistic proposals has been adduced by Matthews and co-workers from their studies of the binding of a number of dipeptide inhibitors[55] and the naturally occurring inhibitor phosphoramidon.[56] The observed binding of the latter resembles that of the presumed transition state and provides further evidence supporting the mechanism in which Glu 143, acting as a general base, promotes the attack of water on the carbonyl carbon, rather than the alternative mechanism in which Glu 143 attacks the carbonyl carbon directly, forming an anhydride intermediate.[57] Recently, Werber[49] has suggested that the water molecule bound to the active site zinc in thermolysin may play a role analogous to that proposed for the zinc-bound water molecule in carbonic anhydrase.

3.5. Spectroscopic Studies of Manganese (II) and Cobalt (II) Thermolysin

It is well known that for a variety of systems, replacement of the optically transparent Zn^{2+} by either Co^{2+} or Mn^{2+} yields derivatives with considerably more spectral information. Co^{2+}-thermolysin displays a characteristic absorption spectrum in the visible region. The spectrum (Figure 5) is characterized by a maximum at 555 nm (ϵ = 90 *M*/cm) and a broad shoulder near 500 nm.[14] The transitions are optically active, the molar ellipticity being positive with maxima: $[\theta]_{500}$ = 1080° and $[\theta]_{550}$ = 879° cm²/dmol. The magnetic CD spectrum of Co^{2+}-thermolysin exhibits major negative bands at 580 and 510 nm with $[\theta]_M$ = −3500 and $[\theta]_M$ = −1300° cm²/dmol at 40 kG, respectively.[14] The spectra shown in Figure 5 all disappear when Co^{2+} is replaced by Zn^{2+}. Based on the conclusions of other studies,[41] they indicate that the coordination of the cobalt ion at the active site has a distorted tetrahedral geometry[14] in agreement with X-ray diffraction results.[6,7] The profound changes in the spectra observed upon binding the competitive inhibitor β-phenylpropionyl-L-phenylalanine (Figure 5, dotted lines) indicate that the latter binds at the active site and interacts with the cobalt. The overall coordination geometry of the cobalt is not affected by the inhibitor binding, however.[14]

The incorporation of 1 mol of the paramagnetic Mn^{2+} ion per mole of apothermolysin can readily be shown by electron spin resonance studies.[20] Nuclear magnetic resonance (NMR) studies have also shown that a single exchangeable water molecule binds in the first coordination sphere of Mn^{2+}. This result is consistent with the X-ray crystallographic data[7] and is similar to that obtained from NMR studies of Mn^{2+}-carboxypeptidase. The magnetic resonance correlation time for the interaction between H_2O and Mn^{2+} in both

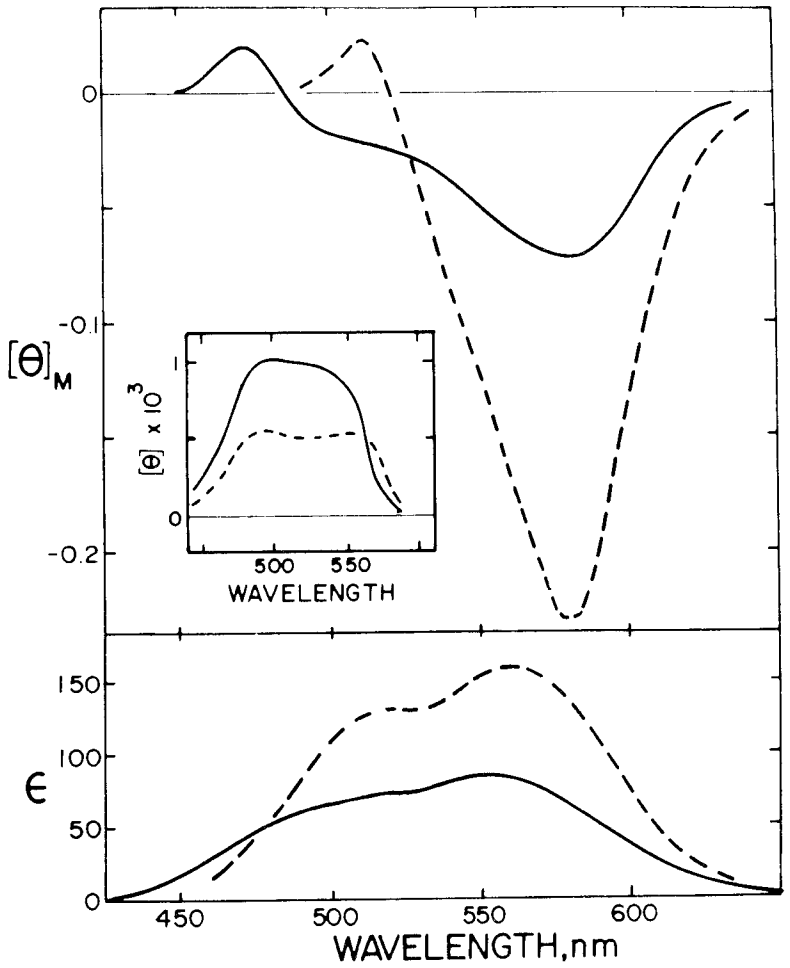


FIGURE 5. Magnetic circular dichroism, $[\theta]$, absorption, ϵ, and circular dichroism, $[\theta]$ (inset), spectra of cobalt thermolysin, 2.23×10^{-4} in 3 *M* NaBr, pH 6.8 in the absence (—) and presence (- - -) of the competitive inhibitor β-phenylpropionyl-L-phenylalanine, 0.025 *M*. MCD measured at 40 kG and expressed in degrees cm^2 $decimole^{-1}$ G^{-1}. (From Holmquist, B. and Vallee, B. L., *J. Biol. Chem.*, 249, 4601, 1974. With permission.)

systems is determined mainly by the electron spin relaxation time, τ_c. The field-dependent τ_c has nearly identical values for the two enzymes.[20,21] These results serve as a further illustration of the conformational homology of the active sites of the two enzymes. Studies of the effect of inhibitor binding on the water relaxation rate enhancement by the bound Mn^{2+} ion show that the water molecule is displaced completely by phenylalanyl-L-phenylalanylamide, but not by the inhibitor carbobenzoxy-L-phenylalanine. This indicates the possibility of different binding modes for the latter reversible inhibitor.[20] The latter possibility has been confirmed by direct X-ray crystallographic determination of the mode of binding of these inhibitors.[55]

4. THE BOUND CALCIUM IONS

4.1. Preliminary Considerations

Thermolysin offers a unique opportunity to study the role of bound calcium ions in protein structure, since the structures of its four calcium binding sites have been elucidated in detail by X-ray crystallographic methods.[6,7,12] The sites, which are located in different parts of the mole-

cule, are clearly nonidentical in size* and the number and disposition of binding ligands involved (Figures 2B, 2C, and 2D), implying that a given bound calcium ion may not necessarily play the same role as the others. In principle, therfore, one may expect to be able to distinguish the different sites and resolve the roles of each by appropriate methods. How can such an objective be achieved?

Of the various strategies one can adopt, the following appears to be the most informative. First, one determines the equilibrium calcium-binding isotherm. If the calcium affinities of the structurally distinct sites are significantly different, they can be resolved in this way. Second, one probes the changes in the equilibrium conformation of the molecule that are coupled to the binding of calcium ions. A variety of techniques including absorption, fluorescence, and CD spectroscopy,[5 9] can be used for this purpose. Combining the data in this way with the structural data may lead to an understanding of specific binding sites in terms of their affinity for calcium. The replacement of calcium by ions that substitute isomorphously, e.g., Sr^{2+}, Ba^{2+}, and especially the lanthanides,[3 6,5 0-5 3] may also provide a further resolution of the roles of the various sites, particularly when a given ion can substitute specifically at certain sites and not others, e.g., Eu^{3+} at S(1).[6,7,1 2]

Having obtained a measure of the affinities of the various sites, one can then interpret the modulating effects of calcium in a variety of reactions, e.g., hydrogen exchange, autolytic degradation, thermal or chemical denaturation. The results obtained so far with the approach outlined above are presented in the next sections and discussed in the context of the available structural information.

4.2. Studies on Calcium Binding and Coupled Conformational Changes

One of the problems in quantitative studies of the binding of calcium ions to thermolysin is that their removal increases the susceptibility of the enzyme to autolytic degradation.[1 1,4 3] It is essential, therefore, that inactive thermolysin preparations be used for the binding analysis. This has been achieved by including the zinc-chelating agent tetraethylene pentamine (TEP) in the equilibrating buffer, thus converting holothermolysin to the inactive apoenzyme.[1 1] TEP does not chelate calcium significantly. The binding data presented in Figure 6 have been obtained by equilibrium gel filtation at 25 and 6°C[1 1,4 2] under otherwise identical conditions (see legend to Figure 6). Since only small conformational changes are associated with the removal of the active site zinc ion (Section 3.2), it seems reasonable to infer that these data may also reflect the calcium-binding behavior of the holoenzyme. The data at 25°C show that all four sites are saturated at free calcium ion concentrations above 10^{-4} M indicating that each has a high affinity for calcium. Below 10^{-4} M dissociation occurs; two calcium ions dissociate in the rather narrow span of only one pCa^{2+} unit ($4 < pCa^{2+} < 5$). At $pCa^{2+} < 5.0$, the isotherm appears to flatten, indicating that the other two calcium ions are of still higher affinity. Unfortunately, the calcium-binding isotherm cannot be evaluated at $pCa^{2+} < 5$ since thermolysin appears to precipitate under these conditions. The binding data have been analyzed by the present authors on the assumption that only two of the four calcium ions dissociate in the range $4.0 < pCa^{2+} < 5.0$.[1 1] The latter analysis leads to a parabolic Scatchard plot (Figure 7) and a linear Hill plot (Figure 8) with a slope of 2.0, indicating that these two ions bind with complete positive cooperativity with an overall binding constant of 2.8×10^{9} M^{-2}.[1 1] The pCa^{2+} dependence of the spectral perturbations, observed upon dissociation of calcium in the range $4.0 < pCa^{2+} < 5.0$, can be described by the same binding constant as that obtained in the analysis of the binding isotherm. This suggests that the conformational change, which causes the observed spectral perturbation, is coupled to the dissociation of these ions. The magnitude of the spectral perturbations suggests that the conformational change is small, which is indicated by the absence of calcium-dependent changes in the sedimentation coefficient and the peptide CD of thermolysin.[2 4] Also, the spectral titrations (Figure 9), which reflect the calcium-coupled conformational changes, are complete at $pCa^{2+} \geq 5.0$. The inference that only two calcium ions dissociate in the range $4.0 \leq pCa^{2+} \leq 5.0$ therefore appears to be reasonable.

A more extensive treatment of the binding data

*"Size" is here defined as the average distance (Å) from the bound calcium ion to the liganding atoms: S(1), 2.87; S(2), 3.13; S(3), 2.48; S(4), 2.53.

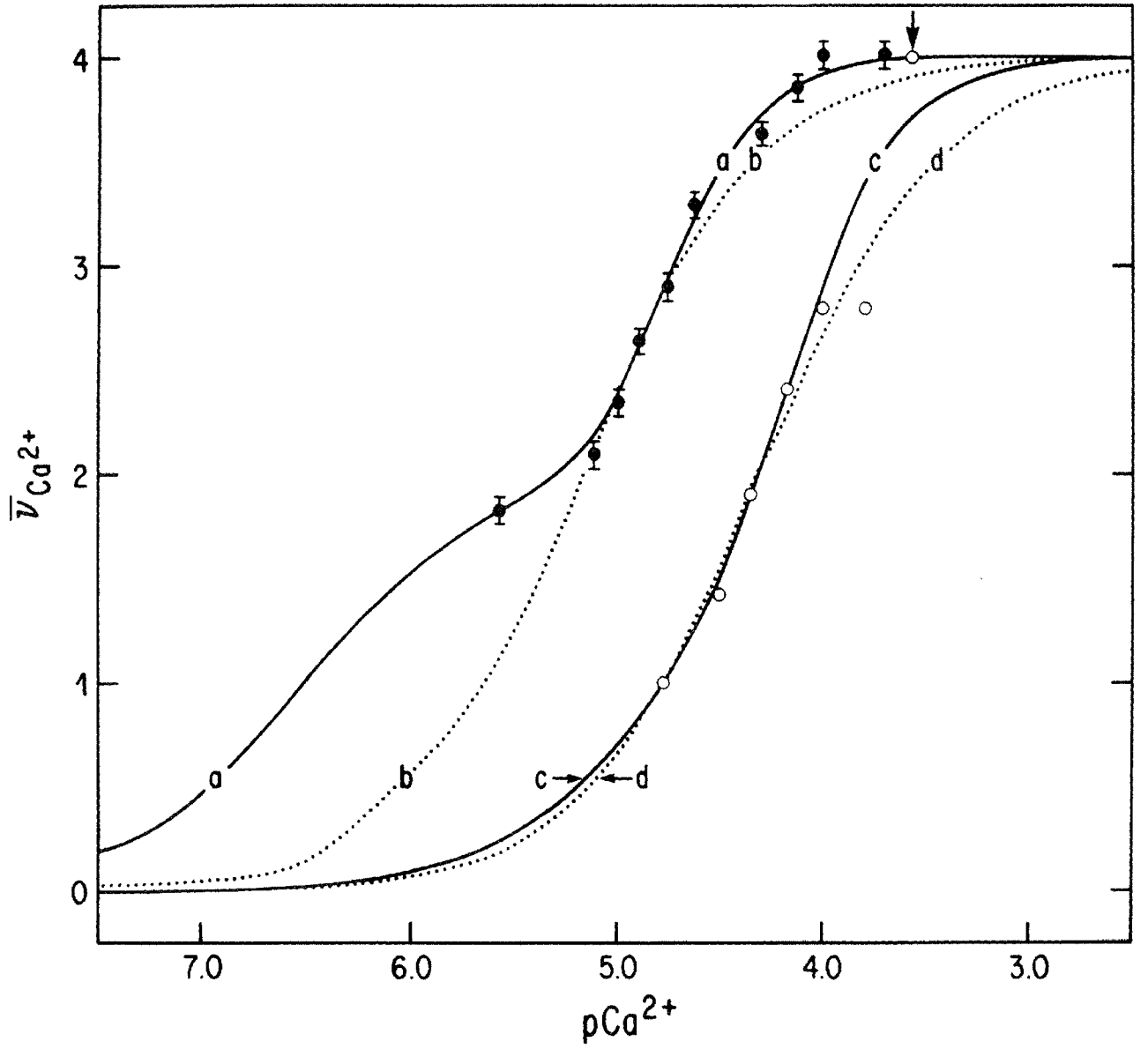


FIGURE 6. Calcium-binding isotherms of apothermolysin in 0.1 *M* NaCl, 0.01 *M* tetraethylenepentamine, pH 9.0 at 25°C[11] (●) and 6°C[42] (○).

(a) $$\bar{v}_{Ca^{2+}} = \frac{2K_{1,2}\,C^2}{1 + K_{1,2}\,C^2} + \frac{K_3 C}{1 + K_3 C} + \frac{K_4 C}{1 + K_4 C}$$

with $K_{1,2} = 2.8 \times 10^9\ M^{-2}$ and $K_3 = K_4 = 3.2 \times 10^6\ M^{-1}$

(b) $$\bar{v}_{Ca^{2+}} = \frac{4K_o C}{1 + K_o C}$$

with $K_o = 1.5 \times 10^5\ M^{-1}$

(c) $$\bar{v}_{Ca^{2+}} = \frac{2K_{1,2}\,C^2}{1 + K_{1,2}\,C^2} + \frac{K_3 C}{1 + K_3 C} + \frac{K_4 C}{1 + K_4 C}$$

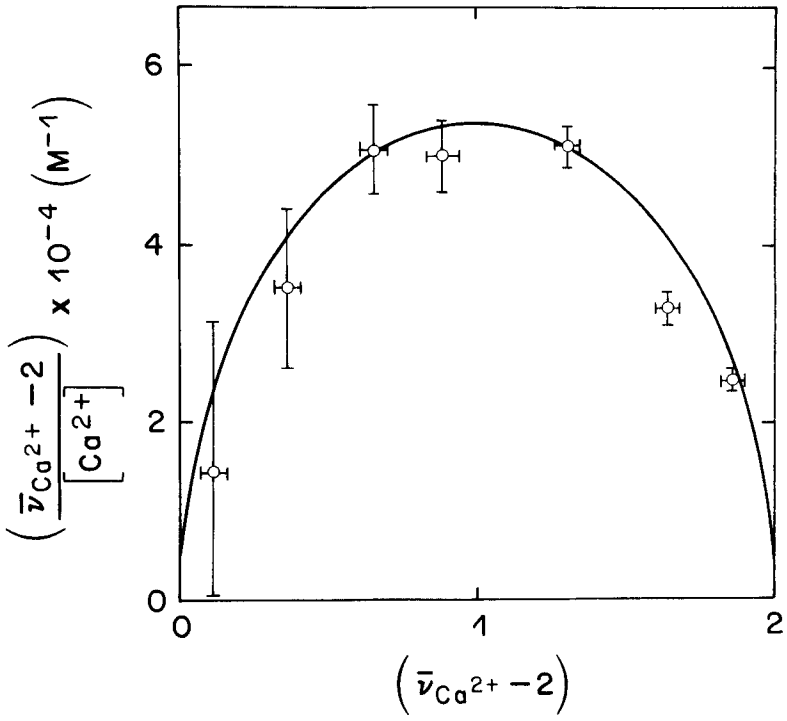


FIGURE 7. Scatchard plot using the data of Figure 6(a), for the range $0.1 < (\bar{\nu}_{Ca^{2+}} - 2) < 1.9$. The error bars have been calculated from the measured standard deviation in $\bar{\nu}_{Ca^{2+}}$ (±0.08). The curve is the theoretical Scatchard plot for the case of complete positive cooperative binding of *two* calcium ions: $y = [Kx(2 - x)]^{1/2}$ where $y = x/c$; $x = \bar{\nu}_{Ca^{2+}} - 2$, and $K = 2.8 \times 10^9\ M^{-2}$. (From Voordouw, G. and Roche, R. S., *Biochemistry*, 13, 5017, 1974. With permission.)

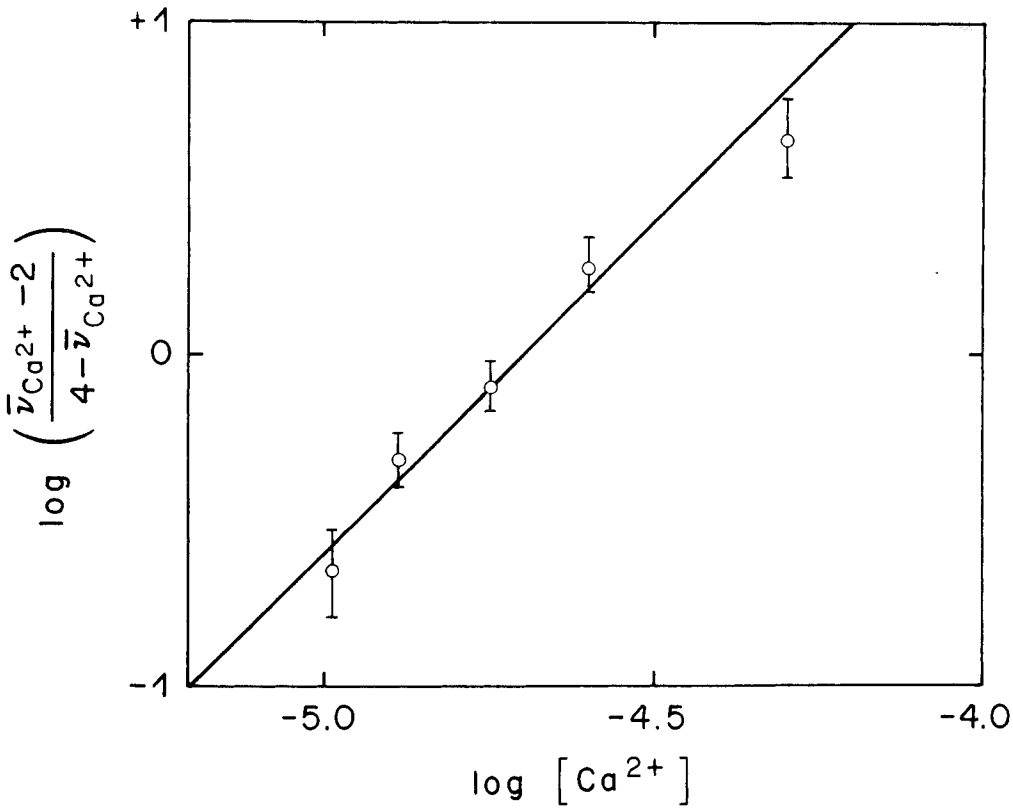


FIGURE 8. Hill plot using the data from Figure 6(a) for the range $0.2 < (\bar{\nu}_{Ca^{2+}} - 2) < 1.8$. The line drawn has a slope of 2.0. The error bars have been calculated as in Figure 7.

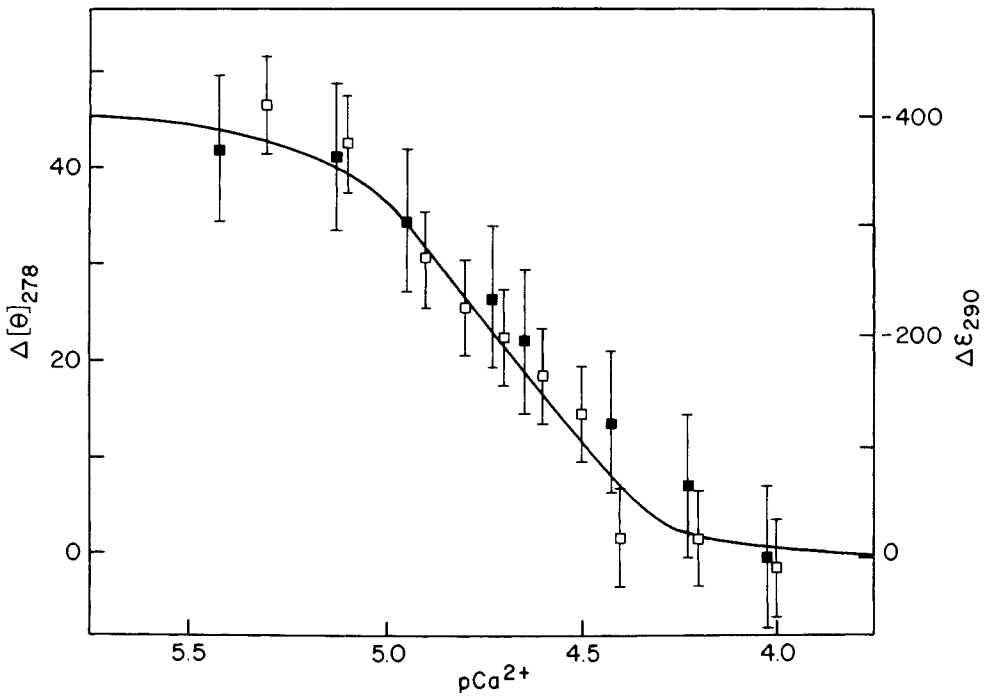


FIGURE 9. Dependence of the difference molar ellipticity at 278 nm, $\Delta[\theta]_{278}$ in degrees cm^2 per decimole (□) and the difference molar extinction coefficient at 290 nm. $\Delta\epsilon_{290}$ in $(M\ cm)^{-1}$ (■), on pCa^{2+}. Differences are for apothermolysin with $pCa^{2+} = 4.0$ as reference in 0.1 *M* NaCl – 0.01 *M* TEP (pH 9.00). (From Voordouw, G. and Roche, R. S., *Biochemistry*, 14, 4667, 1975.)

than that given previously,[11] which includes all the calcium ions, can be based on the following binding isotherm:

$$\bar{\nu}_{Ca^{2+}} = \frac{2\,K_{1,2}\,C^2}{1 + K_{1,2}\,C^2} + \frac{K_3\,C}{1 + K_3\,C} + \frac{K_4\,C}{1 + K_4\,C} \quad (3)$$

where C is the free calcium ion concentration, and it has been assumed that two calcium ions bind with complete positive cooperativity ($K_{1,2}$) and two bind independently (K_3 and K_4). A very satisfactory fit is obtained, using $K_{1,2} = 2.8 \times 10^9$ M^{-1}[11] and $K_3 = K_4 = 3.2 \times 10^6$ M^{-1}, which accounts for all of the available data (Figure 6, Curve a). The assumption that $K_3 = K_4$ has been made for simplicity since the lack of data in the range $0 < \nu_{Ca^{2+}} < 1.8$ does not allow a more detailed analysis. These constants could well differ by several orders of magnitude. It should be stressed that the binding constants in Equation 3 are *site* binding constants, and, as such, $K_{1,2}$ implies an identification of the double site, S(1)-S(2), with the locus of the cooperative binding. Although there is no *direct* evidence for the latter identification, plausible arguments, based on the known structure of the double site and spectral perturbation data, have been reported[11,24] and are reviewed below.

If one assumes an independent-site binding model, the expression for the corresponding isotherm becomes:

$$\bar{\nu}_{Ca^{2+}} = \sum_{i=1}^{4} \frac{K_i C}{1 + K_i C} \quad (4)$$

It is seen that this model cannot account for *all* the binding data obtained at 25°C even if one makes the unrealistic assumption that all calcium-binding sites, which have been shown to be structurally distinct,[6,7,12] have the same intrinsic association constant, $K_i = 1.5 \times 10^5$ M^{-1} (Figure 6, Curve b). Significant deviations from the experimental data occur both at $pCa^{2+} < 4.8$ and $pCa^{2+} > 5.1$ for the latter model. Allowing different values for the constants K_i in order to be consistent with the known structural differences between the sites tends to spread the binding isotherm along the pCa^{2+} axis and further increases the deviations between experiment and theory. It can thus be concluded that at 25°C, the binding of two calcium ions in the range $4.0 < pCa^{2+} < 5.0$ is best described by a model in which they bind with complete positive cooperativity. Elsewhere,[11,24] we have proposed that cooperative binding occurs at the calcium-binding double site (Figure 2B). This proposal is consistent with the spectral perturbations observed (Figure 9), which suggest that the binding of the two calcium ions perturbs a tryptophan residue.[24] The molecule contains only three Trp residues, one of which (Trp 186) is flanked by two calcium-chelating groups (Asp 185 and Glu 187) of the S(1)-S(2) double site. Therefore, it seems reasonable to infer that this site binds its two calcium ions with complete positive cooperativity. The observed cooperativity is thus likely to be caused by the possibility that the double site is not stable at pH 9.0 with just a single calcium ion bound at site S(1).

The binding data obtained at 6.0°C[42] clearly show a shift along the pCa^{2+} axis from those obtained at 25°C[11] (Figure 6). The binding isotherm is shifted to lower pCa^{2+} values, indicating that the affinity decreases with decreasing temperature. The enthalpy of calcium binding thus appears to be positive in this temperature range. The data at 6°C have been fitted satisfactorily to an independent-binding site model[42] (Equation 4, all $K_i = 2.0 \times 10^4$ M^{-1}) as shown in Figure 6, Curve d. The data can, however, be fitted equally well to Equation 3 using $K_{1,2} = 1.6 \times 10^8$ M^{-2} and $K_3 = K_4 = 5.0 \times 10^4$ M^{-1} (Figure 6, Curve c). Unfortunately, accurate data in the range $3.0 < pCa^{2+} < 4.0$, which would determine whether the cooperative-binding model is also to be preferred at this temperature, are lacking.

Further insights into the relative affinities of the sites have been obtained qualitatively from studies in which thermolysin crystals were soaked in solutions varying in EDTA concentration at pH 7.2[25] in the presence of the reversible inhibitor phosphoramidon.[26] Difference electron density maps obtained under conditions in which calcium dissociates from the protein suggest that Ca(2) dissociates first, followed by Ca(4) and Ca(3). No dissociation was observed from site S(1). These results suggest the following order for the calcium binding constants: $K_1 >> K_3 > K_4 > K_2$. These observations lead to the suggestion[25] that the observed cooperative dissociation[11] comprises Ca(2) and Ca(4) rather than Ca(2) and Ca(1).

However, in view of the rather different experimental conditions under which the two sets of results were obtained (crystal vs. solution, holo- vs. apoenzyme, pH 7.2 vs. pH 9.0), they may not

necessarily be inconsistent with each other. Two possibilities should be considered. First there may be a significant pH dependence of the calcium affinity at each binding site. Second, the co-operativity may also be pH dependent. One can envisage, for example, that in order for the double site to be stable with just a single ion bound at site S(1), the residual electrostatic repulsive forces between the charged carboxyl groups in the site have to be eliminated. This can be accomplished by: (1) increasing the charge on the single ion in site S(1) – the double site is, in fact, stable with a single *trivalent* lanthanide bound at S(1);[12] or (2) binding of a proton to a carboxyl group in the double site not participating in the coordination of the ion at S(1) – this may provide stabilization when a single *divalent* ion is bound at this site. The latter possibility will obviously depend on the pKa of the given carboxyl group, and the pH of the solution and suggests a pH-dependent co-operativity.

It is clear that much more work on the calcium-binding isotherm of thermolysin is required to distinguish definitively between the various possibilities outlined above. The greatest challenge undoubtedly lies in developing new methods to stabilize the enzyme against self-degradation, which would then allow studies to be carried out in a wider range of temperature and pH. Apart from the obvious possibilities of reversible or irreversible covalent inhibition or the preparation of apoenzyme (which most probably will never be completely satisfactory), the immobilization of the enzyme on a solid support may provide a useful alternative approach to the stability problem.

4.3. Replacement of Calcium by Other Ions

X-Ray crystallographic studies have shown that all four of the calcium ions bound by thermolysin can be replaced by a variety of other ions. The alkaline earth ions Ba^{2+} and Sr^{2+} displace all four calcium ions at least partly when they are present at sufficiently high concentrations. Difference electron density maps suggest that these ions bind in identical positions at sites S(1), S(3), and S(4), but that they bind at site S(2) in a position about 0.5 Å further away from site S(1) than Ca^{2+} does. The rare earth ions replace calcium at sites S(1), S(3), and S(4) with high occupancy, but the calcium bound at site S(2) is displaced without substitution.[6,7,12] It is interesting to note that the single europium ion at site S(1) must bind with very high affinity since it cannot be removed with EDTA.[27] The affinity of site S(1) for lanthanides is apparently much higher than the affinity of the double site for both Ca(1) and Ca(2). This leads to displacement of calcium even when the free lanthanide concentration is low relative to the free calcium concentration. The situation is reversed in the case of sites S(3) and S(4), where a high excess of free lanthanide ions is required for replacement. These differences allow the calcium ions of the double site to be specifically replaced by a single lanthanide at site S(1) to yield a thermolysin molecule binding one Zn^{2+}, two Ca^{2+}, and one Y^{3+} (Eu^{3+}, Tb^{3+}) per mole[27-29] (i.e., Zn-Eu(1)-Ca(3)-Ca(4)-thermolysin). The rate of displacement of Ca(1) and Ca(2) from the double site by a single Tb^{3+}, which can be followed by fluorescence spectroscopy (Section 5), suggests that the rate of dissociation of the double site is on the minute time scale. One interesting replacement study that remains to be done is to determine whether *bivalent* lanthanides such as Eu^{2+}, which is thought to be an excellent isomorph for Ca^{2+},[36] do indeed occupy both sites S(1) and S(2) and not, like the trivalent lanthanides, just site S(1).

4.4. Stabilization of Macromolecular Conformation by Ligand Binding

It is clear from the previous sections that a wide variety of studies can provide insights into the structural and functional roles played by various ions binding at the different metal-binding sites in thermolysin. In particular, the stabilizing role of calcium in the structure of native thermolysin is of considerable interest.[24,27,33] The role played by bound ligand in the stability of macromolecular structure is most usefully discussed within the rationale of the general theory of macromolecular binding. In this section, we will reformulate the theory for thermolysin.

In any ligand-macromolecule equilibrium, an important quantity to consider is the binding polynomial Σ.[30] This is the summation of the concentrations of all the macromolecular species present in solution which are expressed as products of the binding constants and free concentrations of the ligands participating in the formation of the ligand-macromolecular complex species, and divided by the concentration of the unliganded form.* For independent sites, the

*$P = \sum_{C=0}^{n} Pi = Po(1 + K_1 L + K_2 L^2 \ldots) = Po\Sigma$[30]

polynomial can be factorized, the number of irreducible factors being equal to the number of linkage groups present.[30]

For holothermolysin, assuming no interaction for all site-site pairs except for the double site,* S(1)-S(2), the five metal-binding sites provide four linkage groups, and the ion-binding polynomial is given by:

$$\Sigma = (1 + K_{ZnE}\, C_{Zn})\,(1 + K_{1,2}\, C_{Ca}^2)\,(1 + K_3\, C_{Ca})\,(1 + K_4\, C_{Ca}) \quad (5)$$

where it has been assumed that concentrations are equal to activities, and C_{Zn} and C_{Ca} are the free zinc and calcium ion concentrations, respectively. When ions other than zinc and calcium are present in solution which compete for specific sites, the factor of Σ corresponding to the linkage group for a given site is expanded to include terms associated with the competitive-binding process. For instance, when cobalt ions are also present in solution, the factor $(1 + K_{ZnE}\, C_{Zn})$ is replaced by $(1 + K_{ZnE}\, C_{Zn} + K_{CoE}\, C_{Co})$.

The finding that the double site S(1)-S(2) can bind a single lanthanide ion at S(1) with complete saturation ($K_{EuE}\, C_{Eu} \gg K_{1,2}\, C_{Ca}^2$) without substitution at sites S(3) and S(4)[12] is extremely interesting and suggests that under these conditions, the ion-binding polynomial can be approximated by

$$\Sigma = (1 + K_{ZnE}\, C_{Zn})\,(1 + K_{EuE}\, C_{Eu})\,(1 + K_3\, C_{Ca})\,(1 + K_4\, C_{Ca}) \quad (6)$$

The binding polynomial Σ is the central function from which many important quantities can be derived; for example, the average number of moles of ion i binding per mole of thermolysin, $\bar{\nu}_i$, at constant (or zero) concentration of all other ions j, is generally given by

$$\bar{\nu}_i = \left(\frac{\partial \ln \Sigma}{\partial \ln c_i}\right)_{c_j,\, j \neq i} \quad (7)$$

where we assume again that all activities are equal to concentrations c_i, c_j. Thus, differentiation of Equation 5 with respect to C_{Ca} leads to Equation 3 (Section 4.2) for the calcium-binding isotherm. Provided the zinc and calcium sites are truly independent and, hence, comprise different linkage groups as suggested in Equation 5, the differentiation does not have to be performed at constant C_{Zn} to obtain Equation 3. Similar differentiation of Equation 6 leads to

$$\bar{\nu}_{Ca^{2+}} = \frac{K_3 C_{Ca}}{1 + K_3 C_{Ca}} + \frac{K_4 C_{Ca}}{1 + K_4 C_{Ca}} \quad (8)$$

indicating that the binding of calcium to sites S(3) and S(4) may be investigated specifically in the absence of calcium binding to the double site under appropriate experimental conditions. This could well be exploited in order to further resolve the different sites with respect to their calcium affinity.

The contribution of ion i to the binding free energy** is given by

$$\Delta G_i = -RT \int_{c=0}^{c_i} \bar{\nu}_i \, d \ln c_i \quad (9)$$

which can be evaluated by plotting the data ($\bar{\nu}_i$, ln c_i) and determining the area under the resulting curve. The total binding free energy ΔG_b, for the different ions is obtained by summation of the various ΔG_i contributions. Thus, Equations 7 and 9 offer the opportunity to investigate the thermodynamic contributions of a specific ion even in a mixture of many others. The role of specific ions in reactions involving a change in the conformation of thermolysin can also be investigated.

Consider two conformations, the native (N) and some denatured (D) conformation, respectively, both of which have their own characteristic binding polynomial, Σ_N and Σ_D. The average numbers of moles of i bound per mole of N or D, $\bar{\nu}_{i,N}$ or $\bar{\nu}_{i,D}$, as well as the binding free energy contributions of ion i to the chemical potentials of N and D, $\Delta G_{i,N}$ and $\Delta G_{i,D}$, can readily be written in a manner analogous to Equations 7 and 9. The apparent equilibrium constant, K, in the N $\Leftrightarrow$ D conversion can be expressed in terms of the intrinsic constant, in the absence of ion binding at

*Kinetic studies suggest a small positive interaction free energy between the active site zinc ion and the calcium-binding double site.[24] This possibility will be ignored for the remainder of the discussion.

**The binding free energy is given by $\Delta G_b = -RT \ln \Sigma$.[30]

low free ion concentration, K_I, and the binding polynomials, Σ_N and Σ_D, as:

$$K = K_I \frac{\Sigma_D}{\Sigma_N} \tag{10}$$

The question of whether a given ion i influences the N ⇔ D conversion can be answered by measuring K as a function of the concentration of ion i at constant concentration of all the other ions. One uses the fundamental relation (Equation 11):[30-33]

$$\left(\frac{\partial \ln K}{\partial \ln c_i}\right)_{c_j,\, j\neq i} = m = \bar{\nu}_{i,D} - \bar{\nu}_{i,N} = \Delta\bar{\nu}_i \tag{11}$$

which indicates that the slope (m) of a (ln K vs. ln c_i plot is equal to the difference $\Delta\bar{\nu}_i$) in the average number of moles of i bound per mole of N and D. The size of the cooperative unit can thus be estimated by measuring the ion dependence of reactions involving conformational changes.

In the case of holothermolysin, for example, a general expression for K is obtained by combining Equations 5 and 10

$$K = K_I \frac{(1 + K_{D,Zn} C_{Zn})(1 + K_{D,1,2} C_{Ca}^2)(1 + K_{D,3} C_{Ca})(1 + K_{D,4} C_{Ca})}{(1 + K_{N,Zn} C_{Zn})(1 + K_{N,1,2} C_{Ca}^2)(1 + K_{N,3} C_{Ca})(1 + K_{N,4} C_{Ca})} \tag{12}$$

where $K_{D,Zn}$, $K_{N,Zn}$, etc. are the association constants for the conformation D and N respectively. If the cooperative unit is small, affecting, for instance, only site S(3), while the affinities of all the other sites remain unchanged (i.e., $K_{D,Zn} = K_{N,Zn}$, etc.), Equation 12 reduces to

$$K = K_I \frac{(1 + K_{D,3} C_{Ca})}{(1 + K_{N,3} C_{Ca})} \tag{13}$$

Equations 10 to 13 can also be applied to rates of denaturation reactions, provided that one can regard the reaction as an equilibrium between N and the activated intermediate (X) as suggested by absolute reaction rate theory.[34] In the latter application, Equations 10 to 13 are replaced by Equations 14 to 18.

$$k_{obs} = k_I \frac{\Sigma_X}{\Sigma_N} \tag{14}$$

$$\left(\frac{\partial \ln k_{obs}}{\partial \ln c_i}\right)_{c_j,\, j\neq i} = m = \Delta\bar{\nu}_i \tag{15}$$

$$k_{obs} = k_I \frac{(1 + K_{X,Zn} C_{Zn})(1 + K_{X,1,2} C_{Ca}^2)(1 + K_{X,3} C_{Ca})(1 + K_{X,4} C_{Ca})}{(1 + K_{N,Zn} C_{Zn})(1 + K_{N,1,2} C_{Ca}^2)(1 + K_{N,3} C_{Ca})(1 + K_{N,4} C_{Ca})} \tag{16}$$

$$k_{obs} = k_I \frac{(1 + K_{X,3} C_{Ca})}{(1 + K_{N,3} C_{Ca})} \tag{17}$$

where k_{obs} and k_I are the rate constants for a given rate process in the presence and absence of bound ions, respectively.

The stabilization of N relative to D or X by ion i is given by

$$\Delta(\Delta G_i) = \Delta G_{i,N} - \Delta G_{i,D} = RT \int_{c=o}^{c_i} \Delta\bar{\nu}_i \, d \ln c_i \tag{18}$$

4.5. The Stabilizing Role of Bound Calcium Ions

From the previous section, it is seen that a quantitative delineation of the roles of the various metal ions bound by thermolysin is possible by measuring the ion dependence of reactions which involve changes in protein conformation and an associated change in the affinity of one or more of the protein metal-binding sites. As an example, consider two reactions: the mechanisms of which are thought to involve similar changes in the conformation of the protein: (a) hydrogen exchange and (b) proteolysis. For both types of reactions, a given section of the polypeptide chain is thought to unfold before it can react.[45,46] The overall rate constant, k, is generally written as the product of the equilibrium constant governing the unfolding, K, and the actual rate constant, k_3, for the exchange or proteolysis step.

$$k = Kk_3 \tag{19}$$

The calcium dependencies of the rates of hydrogen

exchange and autoproteolysis of thermolysin have been investigated and show very different results.[24] Whereas the hydrogen exchange rate shows little or no dependence on pCa^{2+}, it is found that the rate of autoproteolysis depends strongly on pCa^{2+}, in a manner suggesting that all the constants $K_{D,1,2}$, $K_{D,3}$, and $K_{D,4}$ are small and that sites S(1)-S(2), S(3), and S(4) are of low affinity in the autolysis substrate (see Equation 12 and Reference 24). Hence, small conformational fluctuations suffice to expose exchangeable hydrogens, but large conformational changes, to which large decreases in calcium affinity are coupled, are necessary to transform the thermolysin molecule into a conformation which is susceptible to proteolysis. We can, therefore, suggest that while the bound calcium ions "allow" small conformational fluctuations, i.e., those required for hydrogen exchange, they "lock" the native fold by lowering the probability of highly unfolded structures. The degree of flexibility allowed in a given region of the structure of thermolysin could, in principle, be estimated by measuring the pCa^{2+} dependence of reactions in which specific residues of that region are modified with a set of reagents of increasing bulkiness. Since the conformational change necessary to make a given residue available for reaction depends on both the location of the residue and the size of the reagent, there will be a critical size beyond which reaction via calcium-independent fluctuations is no longer possible, but has to be facilitated by large, cooperative, and calcium-dependent conformational changes. The larger the critical size of the reagent, the greater the flexibility of the given sector of the protein in terms of possible solvent exposure or reagent penetration. Finally, we should point out that we do not doubt that there are amide hydrogens; for instance, those which are deeply buried in the protein matrix which show calcium-dependent exchange kinetics. However, they are undetectable on the time scale of the exchange experiments which we have conducted.[24] To study their exchange rates on a reasonably accessible time scale, one would have to conduct the hydrogen-exchange experiments under conditions close to those favoring denaturation of the molecule. However, such experiments cannot conveniently be done for thermolysin in solution without considerable autolysis of the molecule.

The fact that the various metal ions bind to thermolysin in different regions of the molecule makes a study of the ion dependence of denaturation rates and equilibria extremely interesting, since each bound ion serves as an indicator of the conformational integrity of its binding site and the associated region of the molecule. Equilibrium denaturation studies are difficult to perform in the case of thermolysin since they are invariably hampered by the autolytic degradation of the molecule.[27] Studies have been performed, however, on the ion dependence of the rate of purely thermal denaturation of thermolysin by monitoring the first-order, irreversible decrease in the enzymatic activity.[24] The dependence of the rate on the free calcium ion concentration is shown in Figure 10, which follows Equation 17, and shows that the native conformation is protected from inactivation by only a single ion binding in excess to N since $m = \Delta\bar{\nu}_{Ca^{2+}} = -1$.[24] This single calcium ion contributes considerably to the stabilization of the native structure: $\Delta(\Delta G_{Ca^{2+}})$ (Equation 18) and has been estimated to be -8.1 kcal/mol.[33] Values for $\Delta(\Delta G_{Ca^{2+}})$ for a number of other calcium-binding proteolytic enzymes were found to be on the average threefold smaller.[33] Since the intrinsic stability of the polypeptide chain of thermolysin (measured at very low C_{Ca} where there is no stabilization due to bound calcium) is lower than any of the other calcium-binding proteases studied, one may attribute the well-known thermal stability of thermolysin to this particular bound calcium ion.[33] It is not known at the present time whether the same results are obtained when some other property such as absorbance is used to monitor the isothermal rate of the denaturation reaction; however, from experiments employing a continuous increase in temperature while monitoring the absorbance at 286 nm, the dependence of the "melting" temperature, T_M, on the free calcium ion concentration indicates a $\Delta\bar{\nu}_{Ca^{2+}}$ of -1 to -2 for the observed denaturation reaction.[27] The free zinc ion concentration does not seem to influence the rate of inactivation ($\Delta\bar{\nu}_{Zn^{2+}} = 0$), since apo- and holothermolysin are inactivated at the same rate.[14,33] Also, when the calcium ions at sites S(1) and S(2) are replaced by a single europium binding at S(1), the melting profile of the resulting [Zn-Eu(1)-Ca(3)-Ca(4)]-thermolysin is the same as that of holothermolysin.[27] These results indicate that the single critical calcium which provides the dominant stabilizing influence against purely thermal unfolding of thermolysin (Figure 10) may

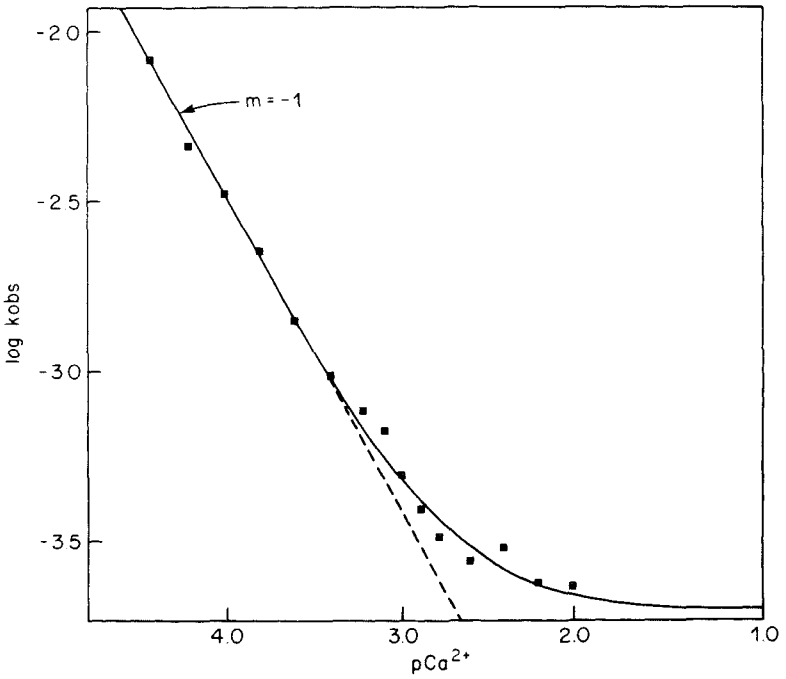


FIGURE 10. Thermal denaturation of thermolysin (3.0×10^{-6} *M*) at 80°C in 0.05 *M* tris (pH 7.0 *at* 80°C) and various concentrations of NaCl and $CaCl_2$ such that their combined ionic strength is 0.05. The logarithm[24] of the first-order rate constant (per second) log k_{obs}, is plotted against pCa^{2+}. The solid line has been obtained from the relation $k_{obs} = k_I \frac{(1 + K_X C)}{(1 + K_N C)}$ (cf. Equation 16) using the condition $K_N C >> 1$; $(K_I/K_N) = 3.09 \times 10^{-7}$ sec^{-1} M^{-1} and $K_X = 600\ M^{-1}$. K_N and K_X are the binding constants for the calcium ion to the critical site in the native conformation (N) and the activated intermediate rate (X), respectively. The conditions $K_N C >> 1$ simply implies that the site in N is saturated in the entire range for which data are presented. This site[33] has an estimated K_N (80°C) $\cong 10^7$ to $10^8\ M^{-1}$; hence, no dissociation is likely in the range $[Ca^{2+}] = 10^{-4}$ to 10^{-5} *M*.

be quite remote from both the active site zinc ion and the double site [S(1)-S(2)]. The calcium binding at S(3) near the amino terminal of the molecule is, therefore, a likely candidate. In the case of purely thermal denaturation, the unfolding of thermolysin may initiate at the N-terminal, with a large coupled change in the calcium affinity at site S(3)[24,27] before or as the process passes through the activated intermediate stage. In contrast, the thermal denaturation rates in 8 M urea (pH 7.0, 50°C) were found to depend on changes in the affinities of *two* calcium ions.[44] The latter conclusion is based on the fact that the slope of the first-order rate constant for thermal denaturation in 8 M urea vs. pCa^{2+} approaches the value m = -2 (Figure 11). A numerical fitting of the experimental data to Equations 16 and 17 yields values for the calcium-binding constants K_N and K_X. However, no conclusions can be drawn from these values concerning the identity of the calcium sites which stabilize the molecule against denaturation under these conditions.

In concluding this discussion of the stabilizing role played by bound metal ions, it is worth emphasizing that only a beginning has been made in establishing a deeper understanding of the roles played by individual ions at their specific sites in the thermolysin molecule. The numbers of calcium ions playing a stabilizing role vary considerably with the type of rate process: none are involved in regulating the rate of exchange of many of the exchangeable hydrogens,[24] all four stabilize against autolysis, two protect the native structure from thermal denaturation in urea,[44] and only a

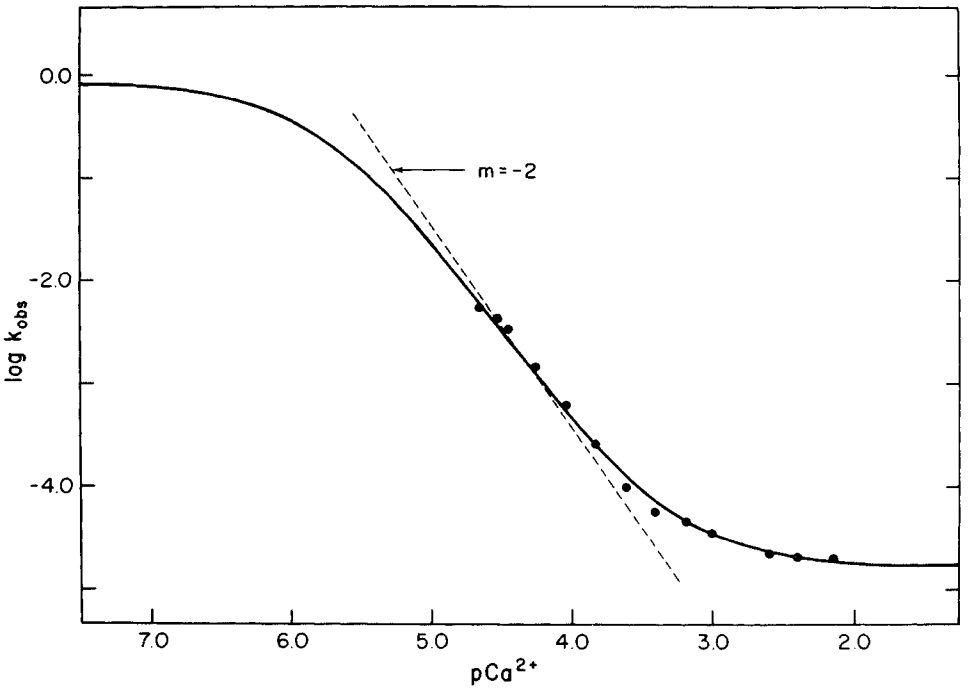


FIGURE 11. Thermal denaturation of thermolysin (3×10^{-6} *M*) at 50°C in 8 *M* urea, 0.05 *M* tris pH 7.0 at 50°C, and various concentrations of NaCl and $CaCl_2$ such that their combined ionic strength is 0.05. The logarithm of the first order rate constant (per second), log k_{obs}, is plotted against pCa^{2+}. The solid line is calculated for $k_{obs} = k_I \frac{(1 + K_X C)^2}{(1 + K_N C)^2}$ with $k_I = 0.82$ sec $^{-1}$, $K_X = 2200\ M^{-1}$, and $K_N = 5 \times 10^5\ M^{-1}$.

single calcium ion reduces the rates of purely thermal denaturation. It will be interesting to see whether the present indications[24] that the double site is *not* the locus that governs purely thermal unfolding will be substantiated in the future. This site, by virtue of its apparently strategic location, was proposed as a most probable stabilizing influence against thermal denaturation of thermolysin.[6,7] If the purely thermal unfolding rates are indeed independent of the occupancies of both the zinc site and the calcium-binding double site, then it follows from the discussion of Section 4.4 (Equations 16 and 17) that the refolding rates have to show a dependence on the occupancy of these sites. The double site and/or zinc site may then act as nucleation centers for the folding of thermolysin into its native conformation. Such a role for these ions would be consistent with the fact that the coordinating ligands of these sites are close together in the amino acid sequence of the protein.

5. STUDIES OF SITE-SITE ENERGY TRANSFER

A specific fluorescent probe can be introduced into the structure of thermolysin by taking advantage of the fact that a single terbium ion binding at site S(1) can replace Ca(1) and Ca(2) without replacement of Ca(3) and Ca(4).[12] The resulting [Zn-Tb(1)-Ca(3)-Ca(4)]-thermolysin derivative exhibits markedly enhanced terbium fluorescence upon excitation at 280 nm due to efficient Trp to Tb(III) energy transfer.[28] The emission spectrum contains four well-resolved bands corresponding to 4f shell transitions of the terbium ion: 489 nm ($^5D_4 \rightarrow {}^7F_6$), 545 nm ($^5D_4 \rightarrow {}^7F_5$), 587 nm ($^5D_4 \rightarrow {}^7F_4$), and 620 nm ($^5D_4 \rightarrow {}^7F_3$). The most intense band is the one centered at 545 nm. When the active site zinc ion is also specifically substituted by a cobalt ion, producing the [Co-Tb(1)-Ca(3)-Ca(4)]-thermolysin derivative, a considerable quenching of the terbium

fluorescence is observed due to the operation of a Trp-Tb(III)-Co(II) energy transfer relay with dipole-dipole radiationless energy transfer between terbium as donor and cobalt as acceptor.[28,29] Analysis of the fluorescence data on the basis of Förster theory yields a Co-S(1) site-site distance of 13.7 Å[29] in good agreement with the Zn-S(1) distance obtained by X-ray crystallography (Table 1). These elegant studies show how subtle interactions between the two groups of ion binding sites present in the thermolysin molecule may be monitored and used as probes of the relative integrity of the various parts of the molecule. The latter possibilities for the further exploration of the structural and functional roles played by metal ions in thermolysin are by no means exhausted, and one can anticipate many interesting results from future work.

ACKNOWLEDGMENTS

The authors acknowledge useful discussions at various times and places with the following: C. Tanford, K. A. Walsh, R. Biltonen, R. Bruce-Martin, R. H. Kretsinger, F. S. Richardson, J. Reuben, S. Blumberg, A. Levitzki, M. Epstein, A. T. Hagler, A. Yonath, M. Werber, A. Warshel, and B. W. Matthews. The authors are particularly grateful to B. W. Matthews for a preview of his papers on the structure of thermolysin-inhibitor complexes. The authors would also like to acknowledge their friends and colleagues at the Weizmann Institute of Science for providing a congenial environment in which to work. Special mention should be made of the hospitality tendered to one of us (RSR) by Professor Henryk Eisenberg in the course of a visit during June 1976 when the first draft of this review was completed. The invaluable contributions of Miss Carol Milo to the experimental work on thermolysin is most gratefully acknowledged. The continued financial support of the NRC of Canada through Grant A3608 to RSR is also gratefully acknowledged.

REFERENCES

1. **Endo, S.,** The protease produced by thermophilic bacteria, *Hakko Kogaku Zashi,* 40, 346, 1962.
2. **Ohta, Y., Ogura, T., and Wada, A.,** Thermostable protease from thermophilic bacteria, *J. Biol. Chem.,* 241, 5919, 1966.
3. **Titani, K., Hermodson, M. A., Ericsson, L. H., Walsh, K. A., and Neurath, H.,** Amino acid sequence of thermolysin, *Nature (London) New Biol.,* 238, 35, 1972.
4. **Matthews, B. W., Jansonius, J. N., Colman, P. M., Schoenborn, B. P., and Dupourque, D.,** Three-dimensional structure of thermolysin, *Nature (London) New Biol.,* 238, 37, 1972.
5. **Matthews, B. W., Colman, P. M., Jansonius, J. N., Titani, K., Walsh, K. A., and Neurath, H.,** Structure of thermolysin, *Nature (London) New Biol.,* 238, 41, 1972.
6. **Colman, P. M., Jansonius, J. N., and Matthews, B. W.,** The structure of thermolysin: an electron density map at 2.3 A resolution, *J. Mol. Biol.,* 70, 701, 1972.
7. **Matthews, B. W., Weaver, L. H., and Kester, W. R.,** The conformation of thermolysin, *J. Biol. Chem.,* 249, 8030, 1974.
8. **Latt, S. A., Holmquist, B., and Valee, B. L.,** Thermolysin: a zinc metalloenzyme, *Biochem. Biophys. Res. Commun.,* 37, 333, 1969.
9. **Pangburn, M. K. and Walsh, K. A.,** Thermolysin and neutral protease: mechanistic considerations, *Biochemistry,* 14, 4050, 1975.
10. **Feder, J., Garrett, L. R.,and Wildi, B. S.,** Studies on the role of calcium in thermolysin, *Biochemistry,* 10, 4552, 1971.
11. **Voordouw, G. and Roche, R. S.,** The cooperative binding of two calcium ions to the double site of apothermolysin, *Biochemistry,* 13, 5017, 1974.
12. **Matthews, B. W. and Weaver, L. H.,** Binding of lanthanides to thermolysin, *Biochemistry,* 13, 1719, 1974.
13. **Matsubara, H. and Feder, J.,** *The Enzymes,* Boyer, P. D., Ed., Academic Press, New York, 1971, 721.
14. **Holmquist, B. and Vallee, B. L.,** Metal substitutions and inhibition of thermolysin: spectra of the cobalt enzyme, *J. Biol. Chem.,* 249, 4601, 1974.
15. **Sillen, L. G. and Martell, A. E.,** *Stability Constants,* Special Publ. No. 17, The Chemical Society, London, 1964.
16. **Sillen, L. G. and Martell, A. E.,** *Stability Constants,* Special Publ. No. 25 (Suppl. No. 1 to No. 17), The Chemical Society, London, 1971.

17. **Voordouw, G., Milo, C., and Roche, R. S.,** The determination of the binding constant of metalloenzymes for their active site metal ion from ligand inhibition data, *Anal. Biochem.*, 70, 313, 1976.
18. **Feder, J., Garrett, L. R., and Kochavi, D.,** Studies on the inhibition of neutral proteases by 1,10-phenanthroline, *Biochem. Biophys. Acta,* 235, 370, 1971.
19. **Coleman, J. E. and Vallee, B. L.,** Metallocarboxypeptidases, *J. Biol. Chem.*, 235, 390, 1960.
20. **Bigbee, W. L. and Dahlquist, F. W.,** Magnetic resonance studies of the active-site region of thermolysin, *Biochemistry,* 13, 3542, 1974.
21. **Navon, G.,** Proton relaxation times and hydration numbers of manganese ions bound to enzymes, *Chem. Phys. Lett.*, 7, 390, 1970.
22. **Kretsinger, R. H.,** Calcium binding proteins, *Annu. Rev. Biochem.*, 45, 239, 1976.
23. **Voordouw, G. and Roche, R. S.,** The role of bound calcium ions in thermostable, proteolytic enzymes. I. Studies on thermomycolase, the thermostable protease from the fungus *malbranchea pulchella, Biochemistry,* 14, 4659, 1975.
24. **Voordouw, G. and Roche, R. S.,** The role of bound calcium ions in thermostable, proteolytic enzymes. II. Studies on thermolysin, the thermostable protease from *bacillus thermoproteolyticus, Biochemistry,* 14, 4667, 1975.
25. **Weaver, L. H., Kester, W. R., Ten Eyck, L. F., and Matthews, B. W.,** Structure and stability of thermolysin, in *Symposium on Enzymes and Proteins from Thermophilic Microorganisms,* Zuber, H., Ed., Birkhauser-Verlag, Basel, 1976, 31.
26. **Suda, H., Aoyagi, T., Takeuchi, T., and Umezawa, H.,** A thermolysin inhibitor produced by actinomycetes: phosphoramidon, *J. Antibiot.*, 26, 621, 1973.
27. **Dahlquist, F. W., Long, J. W., and Bigbee, W. L.,** Role of calcium ions in the thermal stability of thermolysin, *Biochemistry,* 15, 1103, 1976.
28. **Berner, V. G., Darnall, D. W., and Birnbaum, E. R.,** Distance measurements between the metal-binding sites in thermolysin using terbium ion as a fluorescent probe, *Biochem. Biophys. Res. Commun.*, 66, 763, 1975.
29. **Horrocks, W. de W., Holmquist, B., and Vallee, B. L.,** Energy transfer between terbium (III) and cobalt (II) in thermolysin: a new class of metal-metal distance probes, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 4764, 1975.
30. **Schellman, J. A.,** Macromolecular binding, *Biopolymers,* 14, 999, 1975.
31. **Tanford, C.,** Protein denaturation. Part C. Theoretical models for the mechanism of denaturation, *Adv. Protein Chem.*, 24, 1, 1970.
32. **Wyman, J.,** Linked functions and reciprocal effects in hemoglobin: a second look, *Adv. Protein Chem.*, 19, 223, 1964.
33. **Voordouw, G., Milo, C., and Roche, R. S.,** Role of bound calcium ions in thermostable, proteolytic enzymes. Separation of intrinsic and calcium ion contributions to the kinetic thermal stability, *Biochemistry,* 15, 3716, 1976.
34. **Glasstone, S., Laidler, K. J., and Eyring, H.,** *The Theory of Rate Processes,* McGraw-Hill, New York, 1941.
35. **Lipscomb, W. N., Hartsuck, J. A., Quiocho, F. A., and Reeke, G. N.,** The structure of carboxypeptidase A. IX. The X-ray diffraction results in the light of the chemical sequence, *Proc. Natl. Acad. Sci. U.S.A.*, 64, 28, 1969.
36. **Williams, R. J. P.,** The biochemistry of sodium, potassium magnesium and calcium, *Q. Rev. Chem. Soc.* (London), 24, 331, 1970.
37. **Drum, D. E. and Vallee, B. L.,** Differential chemical reactivities of zinc in horse liver alcohol dehydrogenase, *Biochemistry,* 9, 4078, 1970.
38. **Vallee, B. L., Coombs, T. L., and Williams, R. J. P.,** Spectrophotometric evidence for enzyme inhibitor complexation, *J. Am. Chem. Soc.*, 80, 397, 1958.
39. **Blumberg, S., Holmquist, B., and Vallee, B. L.,** Reversible inactivation and superactivation by covalent modification of thermolysin, *Biochem. Biophys. Res. Commun.*, 51, 987, 1973.
40. **Burstein, Y., Walsh, K. A., and Neurath, H.,** Evidence of an essential histidine residue in thermolysin, *Biochemistry,* 13, 205, 1974.
41. **Kaden, T. A., Holmquist, B., and Vallee, B. L.,** Magnetic circular dichroism of cobalt metalloenzyme derivatives, *Biochem. Biophys. Res. Commun.*, 46, 1654, 1972.
42. **Tajima, M., Urabe, I., Yutani, K., and Okada, H.,** Role of calcium ions in the thermostability of thermolysin and *Bacillus subtilis var. amylosaccharitícus* neutral protease, *Eur. J. Biochem.*, 64, 243, 1976.
43. **Drucker, H. and Borchers, S. L.,** The role of calcium in thermolysin: effect on kinetic properties and autodigestion, *Arch. Biochem. Biophys.*, 147, 242, 1971.
44. **Voordouw, G.,** *The Role of Bound Calcium Ions in the Structure of Proteolytic Enzymes,* Ph.D. thesis, University of Calgary, Calgary, Alberta, 1975.
45. **Rupley, J. A.,** Susceptibility to attack by proteolytic enzymes, *Methods Enzymol.*, 11, 905, 1967.
46. **Hvidt, A. and Nielsen, S. O.,** Hydrogen exchange in proteins, *Adv. Protein Chem.*, 21, 287, 1966.
47. **Nemethy, G. and Printz, M. P.,** The γ turn, a possible folded conformation of the polypeptide chain. Comparison with the β turn, *Macromolecules,* 5, 755, 1972.
48. **Matthews, B. W.,** The γ turn. Evidence for a new folded conformation in proteins, *Macromolecules,* 5, 818, 1972.
49. **Werber, M.,** The role of metal ions in the mechanism of action of hydrolytic metalloenzymes: carbonic anhydrase, *J. Theor. Biol.*, 60, 51, 1976.
50. **Nieboer, E.,** The lanthanide ions as structural probes in biological and model systems, *Struct. Bonding* 22, 1, 1975.

51. **Reuben, J.,** The lanthanides as spectroscopic and magnetic resonance probes in biological systems, *Naturwissenschaften,* 62, 172, 1975.
52. **Epstein, M.,** *The Calcium Site of Bovine and Porcine Trypsin as Probed by Lanthanides,* Ph.D. thesis, Weizmann Institute of Science, Rehovot, Israel, 1976.
53. **Reuben, J.,** Bioinorganic chemistry: lanthanides as probes in systems of biological interest, in *Handbook on the Physics and Chemistry of the Rare Earths,* Schneider, G., Jr. and Eyring, Le R., Eds., North-Holland, Amsterdam, chap. 39, in press.
54. **Zuber, H., Ed.,** *Enzymes and Proteins from Thermophilic Microorganisms: Structure and Function,* Proc. Int. Symp., Birkhäuser Verlag, Basel, 1976.
55. **Kester, W. R. and Matthews, B. W.,** A crystallographic study of the binding of dipeptide inhibitors to thermolysin: implications for the mechanism of catalysis, *Biochemistry,* 16, 2506, 1977.
56. **Weaver, L. H., Kester, W. R., and Matthews, B. W.,** A crystallographic study of the complex of phosphoramidon with thermolysin; a model for the presumed catalytic transition state and for the binding of extended substrates, *J. Mol. Biol.,* 114, 119, 1977.
57. **Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Jr., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steite, T. A., Muirhead, H., and Coppola, J. C.,** The structure of carboxypeptidase A. VII. The 2.0 Å resolution studies of the enzyme and its complex with glycyltyrosine and mechanistic deductions, *Brookhaven Symp. Biol.,* 21, 24, 1968.
58. **Sundberg, R. J. and Martin, R. B.,** Interactions of histidine and other imidazole derivatives with transition metal ions in chemical and biological systems, *Chem. Rev.,* 74, 471, 1974.
59. **Fontana, A., Boccu, E., and Veronese, F. M.,** Effect of EDTA on the conformational stability of thermolysin, in *Enzymes and Proteins from Thermophilic Microorganisms,* Proc. Int. Symp. Zuber H., Ed., Birkhäuser-Verlag, Basel, 1976, 229.