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Interactions of calcium and other metal ions with caldolysin, the thermostable proteinase from *Thermus aquaticus* strain T351

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Caldolysin, the extracellular proteinase from the extreme thermophile *Thermus aquaticus* strain T351, is stabilized by Ca^{2+}. A variety of metal ions were able to substitute for Ca^{2+}. Most were unable to confer as much stability as Ca^{2+}, with the exception of the lanthanide ions, which increased the half-life at 95°C from 1 h to more than 4 h. Results from a variety of separation methods indicated that caldolysin binds 6 Ca^{2+} ions/molecule of enzyme. The presence of non-linear Ca^{2+} titration plots, and the removal of 4 Ca^{2+} ions/molecule by treatment with a cationic ion-exchange gel suggested that caldolysin possesses at least two different types of Ca^{2+}-binding sites, with different affinities. Average binding constants of the two types of binding sites were $2.8 \times 10^4 M^{-1}$ (for the low-affinity sites) and $7.5 \times 10^5 M^{-1}$ (for the high-affinity sites). The total Ca^{2+}-binding free energy for caldolysin was shown to be greater than for either thermolysin or *Bacillus subtilis* neutral proteinase. It appears that the higher thermostability of caldolysin is due to the presence of 6 Ca^{2+} ions rather than 4 Ca^{2+} ions/molecule.

The biochemistry of metal ions in proteins has always been the subject of considerable research interest. In particular, the characteristics of metal ions in hydrolytic enzymes have been studied in depth (e.g., Vallee *et al.*, 1959; Hsiu *et al.*, 1964; Russin *et al.*, 1974; Voordouw & Roche, 1975*a*; Heinen & Lauwers, 1976; Tajima *et al.*, 1976; Voordouw *et al.*, 1976*a*,*b*; Epstein *et al.*, 1977; Roche & Voordouw, 1978; Frommel & Hohne, 1981). Detailed characterization of the binding of Ca^{2+} and Zn^{2+} by thermolysin has shown that functional metal ion–protein interactions rely on the precise conformational orientations of specific molecular groups (Voordouw *et al.*, 1976*a*; Roche & Voordouw, 1978).

Caldolysin, the extracellular proteinase from *Thermus aquaticus* strain T351, is considerably more thermostable than thermolysin. Like many proteinases, it chelates Ca^{2+} in order to maintain protein stability (Cowan & Daniel, 1982). However, unlike true metalloproteinases such as thermolysin, caldolysin seems to possess no catalytically active metal ion. It has been clearly demonstrated that Ca^{2+} has no effect on its proteinase activity, but only on its stability (Cowan & Daniel, 1982). In the present study, the specificity, stoichiometry and some thermodynamic constants of the Ca^{2+}-binding sites in caldolysin are presented.

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Methods

Enzyme purification

Caldolysin was produced and purified as previously described (Cowan & Daniel, 1982). Apo-caldolysin was prepared by exhaustive dialysis of caldolysin against 10mM-Tris/acetate buffer, pH 7.1, containing 5mM-EDTA, and then against EDTA-free buffer. [Although this preparation is referred to as 'apo-caldolysin' and is Ca^{2+}-free (see below), a tightly bound Zn^{2+} ion is retained (Cowan & Daniel, 1982).] Dialysis was performed with Spectropor tubing (Spectrum Medical Industries) with a nominal M_r-exclusion limit of 3500.

Proteinase assays

Proteinase activity was determined by the modified Kunitz method described in Cowan & Daniel (1982).

Protein concentration

Purified enzyme protein was determined by using a molar absorption coefficient of $37000 M^{-1} \cdot cm^{-1}$ (D. A. Cowan, unpublished work). For other protein samples a modified

Folin–Ciocalteu procedure (Stauffer, 1975; Peterson, 1977) was used.

Determination of thermostability

Where required, apo-caldolysin was equilibrated at room temperature after addition of a known concentration of metal salt. Samples (approx. 5ml) were then incubated in sealed tubes at a specified temperature. Samples were removed at intervals and stored on ice for subsequent assay. The data in Table 1 (below) were obtained by removal of samples for assay after incubation at 85°C for 90min. At temperatures of 100°C and above, the sealed-capillary-tube method of Barach & Adams (1977) was used.

Metal salts and buffers

Laboratory-reagent-grade metal salts (BDH Chemicals) were used in all cases. Lanthanide ions were prepared from their respective oxides by dissolving in hot conc. HCl. The oxovanadium ion (VO^{2+}) was prepared by dissolving $VOSO_4,H_2O$ in distilled water under an O_2-free N_2 atmosphere. Double-glass-distilled water was used throughout. All buffers were passed through a metal-ion-chelating column (Chelex 100; Dow Chemical Co.) before use.

Quantitative determination of Ca^{2+}

Ca^{2+} was determined either by atomic-absorption spectroscopy with an ISCO 1270 spectrometer, or spectrophotometrically by using the metallochromic indicators murexide and Calcon/Solochrome Dark Blue (Schwarzenbach & Flaschka, 1969; Abbott *et al.*, 1975).

Ultrafiltration

Free Ca^{2+} was separated from enzyme-bound Ca^{2+} by using the Amicon Centrifree micropartition method (*Amicon Technical Bulletin I-216*; Hughes & Klotz, 1956; Abbott *et al.*, 1975).

Gel filtration

Gel filtration on Sephadex G-75 (40–120μm pore size) and Sephadex G-25 (20–80μm pore size) (Feder *et al.*, 1971) was used to separate free Ca^{2+} from enzyme-bound Ca^{2+}. Gel columns (24cm × 1.6cm; Pharmacia) were equilibrated with 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH8, before being loaded with 1–2ml of a caldolysin solution.

Ion exchange

The cationic ion-exchanger SP- (sulphopropyl-)Sephadex C-25 was used in a manner analogous to gel filtration (above). The ion-exchanger was pre-equilibrated with Ca^{2+}-free 50mM-Hepes/NaOH buffer, pH8. Caldolysin was eluted without retention.

Equilibrium dialysis

Dialysis of samples of caldolysin in the presence of a known Ca^{2+} concentration against a Ca^{2+}-free buffer (Hughes & Klotz, 1956; Switzer, 1978) was carried out for 24h. The Ca^{2+} concentration was then determined by EDTA titration, with Calcon/Solochrome Dark Blue and murexide as indicators.

Determination of binding constants

Ca^{2+}-occupancy titration curves for caldolysin were obtained spectrophotometrically by the titration of a murexide-containing Hepes buffer solution of caldolysin with Ca^{2+} (Harnach & Coolidge, 1963; Ohnishi *et al.*, 1972; Kwak & Joshi, 1981; Dorogi & Neumann, 1981), and comparison with a similar titration curve in the absence of caldolysin. In both cases plots of ΔA (the absorbance difference between 507nm and 535nm) versus added Ca^{2+} were obtained.

A Scatchard plot was generated from the titration curves, and the two partial plots were derived as described by Ohnishi *et al.* (1972). The best partial plots were derived by a process of mathematical iteration. The slopes and x-axis intercepts provided the Ca^{2+}-binding constants (k_a and k_b) and the Ca^{2+}-occupancy values (n_a and n_b) respectively, for the strong and weak binding sites.

Results and discussion

Stabilization of apo-caldolysin by metal ions

Substitution of a range of metal ions for Ca^{2+} (Table 1) showed that the lanthanide ions, in particular, could provide equal or greater stabilization. Proportional regeneration of the Ca^{2+}-holoenzyme stability by substitution with another metal ion presumably indicates the effectiveness of that ion in fulfilling the binding interactions within the Ca^{2+}-binding sites. The ability of any metal ion to satisfy the binding interactions of the metal-ion-binding sites depends in part on the atomic size, expressed as the effective ionic radius of the hydrated metal ion. That a suitable ionic radius is an important criterion in Ca^{2+}-substitution is shown in a comparison of data for various metal ions (Table 1). The co-ordination number of Ca^{2+} in proteins is usually six or seven (Kretsinger & Nelson, 1976), and thus the selection of suitable lanthanide-ion co-ordination states (between six and nine) could provide all the lanthanide ions used with an effective ionic radius similar to that of hexa-co-ordinate (or hepta-co-ordinate) Ca^{2+}. The limited stabilization provided by Mg^{2+}, Co^{2+},

Table 1. *Thermostability of metal-ion-substituted caldolysins in relation to metal ionic radii*
Ionic radii are taken from Shannon (1976), except those in square brackets, which are from Hanzlik (1976). Relative stabilization is defined in terms of the percentage of proteinase activity retained after incubation of apo-caldolysin at 85°C for 90min in presence of 10mM metal-ion concentration. Activity values have been rounded off to the nearest 5%. Standard errors of duplicate assays were between 2% and 6% in all cases.

	Ionic radius (nm)			
Metal ion	Co-ordination number ... 6	8	9	Relative stabilization by metal ions (%)
None				0
Mg^{2+}	0.066			45
Ca^{2+}	0.100 [0.099]	0.112	0.118	100
Mn^{2+}	0.080			20
Co^{2+}	0.072			35
Cu^{2+}	0.069			20
Sr^{2+}	0.112 [0.113]			80
Cd^{2+}	0.097			25
Ba^{2+}	0.134			35
VO^{2+}	—			75
La^{3+}	0.103 [0.115]	0.116	0.122	100
Pr^{3+}	0.099 [0.109]	0.113	0.118	100
Nd^{3+}	0.098 [0.128]	0.111	0.116	100
Eu^{3+}	0.096 [0.106]	0.108	0.113	100
Gd^{3+}	0.094 [0.102]	0.105	0.111	100
Tb^{3+}	0.092 [0.100]	0.104	0.110	100
Dy^{3+}	0.091 [0.099]	0.103	0.108	100
Ho^{3+}	0.090 [0.097]	0.102	0.107	100
Lu^{3+}	0.086 [0.093]	0.098	0.103	100

Cu^{2+} and Mn^{2+} might be attributed, in part, to the fact that each has an effective ionic radius considerably less than that of Ca^{2+}. The other major factor in metal-ion binding in proteins is directionality. Ca^{2+}, Sr^{2+} and the lanthanide ions possess a low directional specificity and are thus readily able to partake in protein–metal-ion interactions. Conversely Mn^{2+}, Cu^{2+} and Cd^{2+} all possess a high degree of directional specificity (a requirement for ligands corresponding to the set configurations of the metal-ion molecular orbitals). This may explain the apparent inability of these ions to substitute effectively for Ca^{2+}. Cd^{2+}, for example, has an ionic radius very similar to that of Ca^{2+}, but provides little stabilization.

The stability provided by the oxovanadium ion VO^{2+} is much greater than would be expected from the vanadium ionic radius [0.06nm (0.6Å)]. Clearly, the oxygen atom must be significant in the orientation and co-ordination of VO^{2+} in caldolysin. As with many other Ca^{2+}-binding proteins (see, e.g., Kretsinger & Nelson, 1976; Roche & Voordouw, 1978), carboxylate groups have been implicated in Ca^{2+}-binding in caldolysin (D. A. Cowan, unpublished work). VO^{2+} is known to bind to ligands with oxygen donor atoms (Chasteen, 1981). Thus the ability of VO^{2+} to accept carboxylate oxygen atoms as electron donors may offer an explanation for the relatively high thermostability of VO^{2+}–caldolysin.

Thermostability of caldolysin derivatives at high temperatures

At 95°C and higher temperatures, activity loss in Ca^{2+}–caldolysin is largely the result of thermal denaturation (Cowan & Daniel, 1982). Denaturation becomes very rapid above about 105°C (Fig. 1). Unlike thermolysin (Barach & Adams, 1977) and *Pseudomonas aeruginosa* proteinase (Stepaniak *et al.*, 1982), caldolysin shows little recovery of thermostability at temperatures in excess of 100°C. The recovery of thermostability at 110°C shown by thermolysin (Stepaniak *et al.*, 1982) may be the result of protein renaturation after cooling. Attempts to demonstrate recovery of proteinase activity after cooling samples of Ca^{2+}–caldolysin incubated for 7min at 120°C were unsuccessful. We speculate that the failure of caldolysin to undergo renaturation may be a consequence of its high molecular stability in the native state. The energetic requirements for conversion from a native into a denatured conformation may be sufficiently high so as to effectively preclude the reverse reaction under the mild conditions used in such experiments.

The lanthanide ions are capable of conferring greater thermostability on apo-caldolysin than Ca^{2+} can (Table 2). With a half-life of longer than 4h at 95°C, lanthanide ion–caldolysin is one of the most stable enzymes known.

Table 2. *Stability of caldolysin derivatives at very high temperatures*

	$t_{\frac{1}{2}}$ (min)	
Temperature ...	95°C	105°C
Apo-caldolysin + Ca^{2+}	60	1.3
Apo-caldolysin + VO^{2+}	10	—
Apo-caldolysin + Sr^{2+}	20	—
Apo-caldolysin + La^{2+}, Pr^{2+}, Nd^{2+}, Eu^{2+}, Gd^{2+}, Tb^{2+}, Dy^{2+}, Ho^{2+} or Lu^{2+}	>250	~1

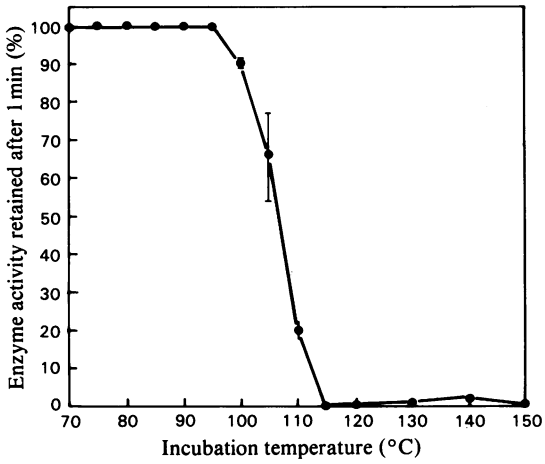

Fig. 1. *Thermostability of caldolysin*
The thermostability of caldolysin (15 μg/ml in 0.1 M-Tris/acetic acid buffer, pH 8.1, with 10 mM-$CaCl_2$ added) at high temperatures was measured. Half-lives: 30 min at 95°C; 4.2 min at 100°C; 1.3 min at 105°C; 0.3 min at 110°C.

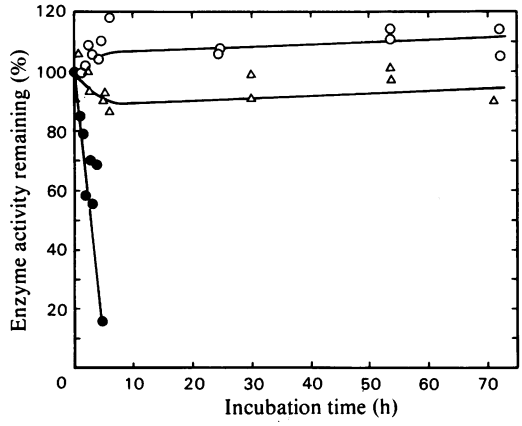

Fig. 2. *Autolysis of caldolysin at high enzyme concentrations*
Solutions of caldolysin (in 0.1 M-Tris/acetic acid buffer, pH 8.1, with 1 mM-$CaCl_2$ added) were incubated at the temperature specified. Samples were assayed at intervals: △, 5.0 mg of enzyme/ml, 20°C; ○, 27 mg of enzyme/ml, 20°C; ●, 6.4 mg of enzyme/ml, 75°C.

Autolysis

At room temperature, little loss of activity was observed with enzyme protein concentrations of 5 mg/ml and 27 mg/ml (Fig. 2). At 75°C and a concentration of 6.4 mg/ml, an activity half-life of about 3 h was observed. Surprisingly, less activity loss occurred at the higher enzyme concentration. This may be the result of substrate inhibition, which is significant at protein substrate concentrations of 5 mg/ml and above (Cowan, 1980).

Stoichiometry of Ca^{2+} binding in caldolysin

$K_{obs.}$ values (the second-order rate constants for autolytic loss of enzyme activity) were obtained from activity profiles of caldolysin samples incubated at 85°C [plotted as the reciprocal of the concentration of remaining active enzyme versus time (Voordouw & Roche, 1974)]. The relationship between the rate of autolysis and Ca^{2+} concentration (Fig. 3) is not linear throughout. The presence two intersecting gradients implies that more than one type of Ca^{2+}-binding interaction exists. That is, up to a Ca^{2+} concentration of about 77 μM, some or possibly all of the Ca^{2+}-binding sites are probably unoccupied. Above this concentration, some (high-affinity?) sites will be completely occupied. A similar dependence of $K_{obs.}$ on Ca^{2+} concentration was observed for thermolysin (Voordouw & Roche, 1975*b*).

A similar plot was seen when the thermostability of Ca^{2+}–caldolysin was determined after titration with EDTA. $K_{obs.}$ values obtained from the initial linear portions of 1/(enzyme activity)-versus-time plots (not shown) were plotted against EDTA concentration (Fig. 4). Since the initial Ca^{2+} concentration was known, and since the Ca^{2+}-binding affinity of EDTA is very high ($\log K = 10.6$; Dawson *et al.*, 1969) and the Ca^{2+}/EDTA binding stoichiometry is 1:1, it is reasonable to assume that EDTA is fully saturated at all times. Thus the 'free' Ca^{2+} concentration can

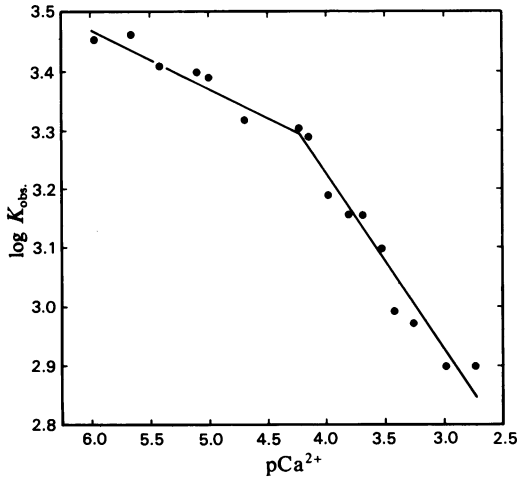

Fig. 3. *Relationship between Ca^{2+} concentration and the rate of autolytic loss of caldolysin activity*
The rate of autolytic activity loss ($K_{obs.}$, the second-order rate constant) at 85°C in solutions of caldolysin (0.14mg/ml in 0.1M-Hepes buffer, pH7.2) in the presence of various concentrations of Ca^{2+} was measured. Plots were obtained by linear-regression analysis.

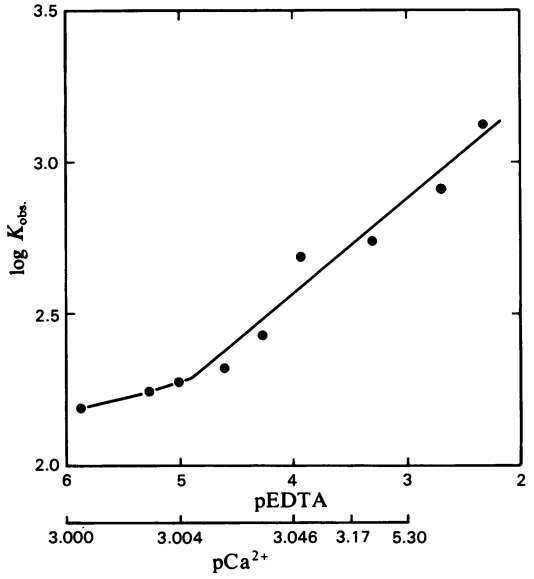

Fig. 4. *Relationship between the concentration of added EDTA and the rate of autolytic loss of caldolysin activity*
The rate of autolytic activity loss in solutions of caldolysin (0.14mg/ml in 0.1M-Hepes buffer, pH7.2, with 1mM-$CaCl_2$ added) in the presence of various concentrations of added EDTA was measured. Plots were obtained by linear-regression analysis.

be calculated (Fig. 4, lower scale). The points are a reasonable fit to a biphasic plot, suggesting the presence of dual Ca^{2+}-binding characteristics in caldolysin.

Results obtained from the separation of enzyme-bound Ca^{2+} from free Ca^{2+} by ultrafiltration, gel-filtration chromatography and equilibrium dialysis are shown in Table 3. Despite the reported variability of the ultrafiltration method (Heyde, 1973; Kurz *et al.*, 1977), the data for all three techniques are consistent with the presence of 6 bound Ca^{2+} ions/molecule in caldolysin [mean $\pm$ S.D., excluding upper and lower data points: 6.06 ± 0.49 ($n = 17$)]. Data obtained from the Folin protein-determination method are generally thought to be more precise and reliable than those obtained from u.v. absorption at 280nm (Peterson, 1983). We found the mean $\pm$ S.D. of the Ca^{2+}/enzyme ratios to be 6.46 ± 1.01 ($n = 7$) by using the A_{280} determination but 5.74 ± 0.26 ($n = 5$) by using the Folin method.

Separation of Ca^{2+} by cation-exchange chromatography yielded enzyme containing only 2 bound Ca^{2+} ions/molecule (Table 4), suggesting that these bind with higher affinity (lower k_b) than the other four ions. The sulphopropyl groups of SP-Sephadex are strongly cationic and are presumably able to compete successfully for the more weakly bound enzyme–Ca^{2+} ions. A determination of the stability of $2Ca^{2+}$–caldolysin and $6Ca^{2+}$–caldolysin at 85°C showed that $2Ca^{2+}$–caldolysin lost activity at more than double the rate of $6Ca^{2+}$–caldolysin (75% and 33% respectively of initial activity lost after incubation at 85°C at pH8 for 150min). Nevertheless, Ca^{2+} ions 1 and 2 (defined, for convenience, as the two more tightly bound Ca^{2+} ions) are evidently also critical for enzyme stability, since Ca^{2+}-free caldolysin loses almost 100% of its activity under the same conditions.

Binding constants of Ca^{2+}-binding sites in caldolysin

Spectrophotometric analysis of the Ca^{2+}-binding sites in caldolysin by the method of Ohnishi *et al.* (1972) yielded the titration curves shown in Fig. 5. Partial plots derived from the resulting Scatchard plot yielded values of 2 and 4 respectively for n_a and n_b, and values of $k_a = 7.5 \times 10^5 M^{-1}$ and $k_b = 2.8 \times 10^4 M^{-1}$ (i.e. 2 strongly bound Ca^{2+} ions and 4 weakly bound Ca^{2+} ions per molecule).

With the use of these values, the equilibrium constants for a series of Ca^{2+}-binding equilibria were calculated by using the equation of Tajima *et al.* (1976):

$$K_j = V - (j-1) \cdot K/j$$

Table 3. *Stoichiometry of Ca^{2+}-binding in caldolysin, as determined by ultrafiltration, gel filtration, and equilibrium dialysis*
For experimental details see the text. Abbreviations: $[Ca^{2+}]_T$, [total Ca^{2+}]; $[Ca^{2+}]_{bd.}$, [bound Ca^{2+}]; $[Ca^{2+}]_{ext.}$, [external Ca^{2+}]; $[Ca^{2+}]_{int.}$, [internal Ca^{2+}]; N.D., not determined.

Ultrafiltration

Sample	$[Ca^{2+}]_T$ (μM)	$[Ca]_{free}$ (μM)	[Enzyme] (μM)	$[Ca^{2+}]_{bd.}$ (μM)	$[Ca]_{bd.}$/[Enzyme]
1	312.8	262.5	6.92	50.3	7.27
2	199.5	174.0	4.49	25.5	5.68
3	1230.0	1200.0	8.51	30.0	3.53
4	1230.0	1177.5	8.51	52.5	6.17

Gel-filtration chromatography (Sephadex G-25 and G-75)

Sample	$[Ca^{2+}]_{bd.}$ (μM)	[Enzyme] (μM)		$[Ca^{2+}]_{bd.}$/[Enzyme]
		By A_{280}	By Folin method	
(G-25) 1	12.5	1.89	(2.28)	6.61 (5.48)
2	20.7	3.78	(3.68)	5.48 (5.63)
3	15.0	2.43	(2.60)	6.17 (5.77)
4*	12.5	2.16	(2.21)	5.79 (5.66)
5*	20.7	2.43	(3.36)	8.52 (6.16)
(G-75) 1	29.9	5.00	N.D.	5.98 N.D.
2	29.9	4.44	N.D.	6.73 N.D.

Equilibrium dialysis

Sample	$[Ca^{2+}]_{ext.}$ (μM)	$[Ca^{2+}]_{int.}$ (μM)	[Enzyme] (μM)	$[Ca]_{bd.}$ (μM)	$[Ca^{2+}]_{bd.}$/[Enzyme]
1	39.0	62.4	4.05	23.4	5.78
2	53.4	93.6	6.24	40.2	6.44
3	62.4	101.1	6.24	39.0	6.25

* 40mM-Ca^{2+} was added to G-25 fractions 1 and 2, which were then re-run.

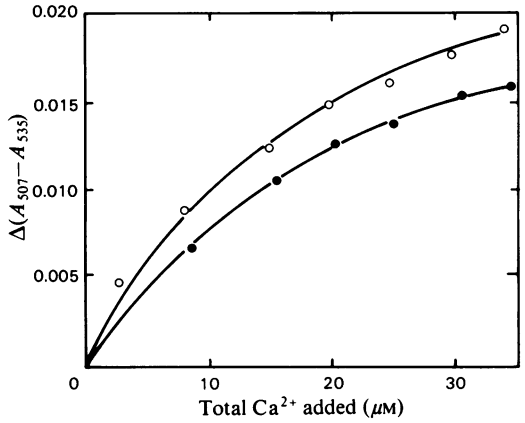

Fig. 5. *Titration curve for the addition of Ca^{2+} to caldolysin*
Titration curves are shown for the addition of Ca^{2+} to 50mM-Hepes buffer, pH8.1 (○), and buffer containing 39μg of caldolysin/ml (●).

Table 4. *Removal of Ca^{2+} from caldolysin by ion-exchange chromatography*
For experimental details see the text.

Sample	$[Ca^{2+}]$ (μM)	[Enzyme] (μM)	$[Ca^{2+}]$/[Enzyme]
1	4.99	2.70	1.85
2	4.99	2.70	1.85
3	4.99	2.43	2.05

where V is the maximum number of Ca^{2+} ions found, and $J = 1 \rightarrow V$. The free-energy changes for each successive equilibrium ($\Delta G_j = RT \cdot \ln K_j$) have also been calculated (Table 5). The sum of the Ca^{2+}-binding free energy for caldolysin at 25°C (ΔG_T) is approx. 170kJ/mol. In comparison, ΔG_T values for thermolysin and *Bacillus subtilis* neutral proteinase are 92 and 96kJ/mol respectively (Tajima *et al.*, 1976). It is evident that the Ca^{2+} contribution to the stability of caldolysin is greater than in either thermolysin or *B. subtilis* proteinase. Since both these proteinases possess only 4 bound Ca^{2+} ions/molecule [with binding constants of $2 \times 10^4 M^{-1}$ for thermolysin, and $1.1 \times 10^5 M^{-1}$ ($3Ca^{2+}$) and $1.5 \times 10^3 M^{-1}$ ($1Ca^{2+}$) for *B. subtilis* proteinase (Tajima *et al.*, 1976)] it is possible that the greater stability of caldolysin results from the binding of two extra Ca^{2+} ions with high affinity. The total free energy of stabilization for the four weakly bound Ca^{2+} ions in caldolysin is 95kJ/mol, almost identical with the values for thermolysin and *B. subtilis* neutral proteinase.

Table 5. *Equilibrium constants and free-energy changes for Ca^{2+}-binding in caldolysin*

	j	K_j (M^{-1})	ΔG_j (at 25°C)
High-affinity sites	1	4.5×10^6	−37.9
	2	1.9×10^6	−35.8
Low-affinity sites	3	3.7×10^4	−26.1
	4	2.1×10^4	−24.6
	5	1.1×10^4	−23.1
	6	0.5×10^4	−21.1

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