Biophysical Chemistry 23 (1986) 183–199
Elsevier

BPC 01037

THE THERMODYNAMICS OF CALCIUM BINDING TO THERMOLYSIN

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Received 22nd July 1985
Accepted 22nd October 1985

Key words: Calcium binding; Thermodynamics; Thermolysin; pH dependence

Calcium binding isotherms were determined for thermolysin in the range pH 5.6–10.5, and from 5 to 45°C. An extensive statistical analysis of the binding data suggests that at least two of the four binding sites bind Ca^{2+} with complete positive cooperativity and independently of the other two. Nonlinear regression analysis of the binding data was used to calculate cooperative (K_1) and independent (K_2) binding constants for the four calcium sites. Thermodynamic parameters obtained from a van't Hoff analysis indicate that calcium binding to both cooperative and independent sites is an entropy-driven process. At pH 7.0, $\Delta H_1 = 90.4$ kJ/mol; $\Delta H_2 = 97.5$ kJ/mol; $\Delta S_1 = 456$ J K^{-1} mol^{-1}; $\Delta S_2 = 262$ J K^{-1} mol^{-1}. These results are compared to those obtained for other calcium-binding proteins. An analysis of the pH dependence of the calcium binding constants indicates that the binding of four protons at the cooperative site and one to two protons at the independent sites, modulates the calcium affinity. This confirms an earlier structural assignment of the double-site as the locus of the two cooperatively binding Ca^{2+}. Calcium binding to thermolysin is enhanced in the presence of an active site directed inhibitor, suggesting that there may be positive cooperativity between substrate and calcium binding.

Introduction

Our knowledge of the structure of thermolysin (EC 3.4.24.4), the calcium-binding, zinc metalloendopeptidase excreted by *Bacillus thermoproteolyticus* is now highly developed. The crystallographic studies of Matthews' group [13,52] have defined the three-dimensional structure of the molecule to a resolution of 1.5 Å, including the detailed structures of the single zinc-binding site and the four calcium-binding sites. We are, therefore, in a unique position to explore the relationship between the thermodynamics of calcium binding and the structure of this protein.

Many of the general aspects of the structural and functional roles of metal ions in thermolysin have been reviewed [16] and a framework for a discussion of the modulation of the structure and stability of the protein by bound metal ions has been formulated in terms of the general theory of ligand binding [16,51].

In this paper, we report the temperature dependence of calcium binding to thermolysin in the range 5–45°C at pH 7 and 9. The most probable binding models were selected on the basis of a thorough statistical analysis of several possibilities and the corresponding binding constants were determined by least-squares fitting to the data. A

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Abbreviations: FAGLA, 3-(2-furylacryloyl)glycyl-L-leucinamide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Taps, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid; TEP, tetraethylenepentamine; Vit D CaBP, vitamin D-dependent calcium-binding protein.

van't Hoff analysis of the temperature dependence of the binding constants is presented which demonstrates that calcium binding to thermolysin is entropy-driven. In addition, the pH dependence at 25°C has been measured and used to estimate the number of ionizable groups which modulate the calcium affinity. Measurements of calcium binding to thermolysin inhibited with a chloromethyl ketone derivative are also reported and discussed.

Calcium-binding proteins are normally grouped according to their functional role, or on the basis of similarities in the geometry of their calcium-binding sites [1–3]. For instance, several proteins such as parvalbumin, troponin C, calmodulin, and vitamin D-dependent calcium-binding protein are thought to have calcium-binding sites of similar geometry [4]. In addition, the biological activity of all of these proteins is regulated by calcium-induced conformational changes [4]. Calcium binding also induces a conformational change in concanavalin A which enhances the ability of this protein to bind saccharides [5]. Alternatively, calcium may bind at the active site of an enzyme and play a direct role in substrate binding and catalysis; staphylococcal nuclease [6] and phospholipase A [7] are examples of this class. For other proteins, such as trypsin [8] and thermolysin [9], the bound calcium appears to function solely in the stabilization of the native structure against autolysis or thermal denaturation.

At present, it is not possible to quantitatively account for the free energy of the calcium-protein interaction either theoretically or on the basis of a comparison to the thermodynamics of calcium binding to small molecules [10–12]. However, it is already well established that the free energy of binding is 8–12 kJ/mol higher for intracellular calcium-binding proteins relative to extracellular calcium-binding proteins [2,10]. It is not known whether this trend can be attributed to differences in ΔH or ΔS. Proteins which undergo activation via a calcium-triggered conformational change may have a ΔS for calcium binding which reflects the contribution of the conformational change of the protein upon calcium binding and which differs from the values for other proteins which bind calcium without major conformational rearrangements. In order to test the validity of these suggestions, and to broaden our understanding of the calcium-protein interaction at the molecular level, it is necessary to increase the data base of thermodynamic parameters for those proteins in which calcium-binding plays different functional roles.

We consider thermolysin to be representative of that group of proteins for which calcium binding plays solely a stabilizing role. The four calcium-binding sites of thermolysin are remote from the active-site region [13]. It has been shown that the concentration of calcium present does not affect the peptidase activity of thermolysin towards synthetic substrates [14,15]. However, bound calcium does stabilize thermolysin against proteolytic degradation or unfolding by organic denaturants or at high temperatures [16,17]. Spectroscopic measurements suggest that the removal of calcium does not induce major conformational changes in thermolysin [9,15]. Recently, it has been shown that calcium bound at sites 1 and 2 protects thermolysin against autolytic or tryptic peptide hydrolysis in an inter-domain region [15,17]. The role of these Ca^{2+} may be to stabilize domain-domain interactions in the native protein.

2. Experimental

2.1. Materials

Thermolysin was obtained from Sigma and used without further purification for the measurement of calcium binding isotherms. The buffers Hepes, Mes, Pipes and Tris were also obtained from Sigma. The synthetic substrate FAGLA was obtained from Vega-Fox or Chemical Dynamics. Dithiazone, 1,10-phenanthroline, granular zinc, calcium carbonate (chelometric standard) and sodium chloride were all obtained from Fisher. Standard buffers for pH meter calibration were obtained from Beckman. Technical grade TEP was obtained from BDH and either used directly or further purified as the chloride salt; the state of purity of TEP had no measureable effect on calcium binding to thermolysin. Calcium binding measurements were performed on columns packed with Sephadex G-25 (Pharmacia). The active-site directed inhibitor *N*-chloroacetyl-DL-hydroxyleucine

methyl ester was provided by Dr. J.C. Powers (School of Chemistry, Georgia Institute of Technology, Atlanta, GA) and used without further purification.

In-house distilled water was further purified by passage down columns for removal of organic material and ions (Barnstead). Water, freshly prepared in this manner, had a resistance of greater than 2 MΩ/cm. To avoid metal ion contamination, polyethylene test tubes and bottles (Nalgene) were used to contain fractions whenever possible.

Measurements of pH were performed using either a Beckman Expandomatic SS-2 pH meter equipped with a Radiometer semi-micro electrode, or a Metrohm 605 pH meter and Metrohm semi-microelectrode. All measurements of weight were made on either a Mettler type H-15 analytical balance or a Mettler P-100 top loading balance.

2.2. Measurement of calcium binding isotherms

Metal ion binding data were obtained using the method of Hummel and Dryer [21]. This method has been reviewed by Ackers [22] and applied to the study of calcium-binding proteins by Voordouw and Roche [19]. Experiments were performed using jacketed K16-100 Pharmacia columns containing Sephadex G-25 which had been pretreated as suggested by Reiland [18]. These columns were equilibrated with 3–4 bed volumes (600–800 ml) of a solution containing 0.1 M NaCl, 0.01 M buffer (Pipes, Mes, Hepes, Tris, or Taps, depending on the pH), and the metal ion concentration of interest. These solutions were routinely prefiltered through a 0.45 μm Nylon 66 membrane (Rainin Instrument Co.). Stock 0.1 M calcium solutions were prepared by dissolving accurately weighed amounts of calcium carbonate in a minimum volume of concentrated 1 M HCl and were diluted to prepare other standard solutions using calibrated volumetric glassware and pipettes. Columns and column elutants were maintained at constant temperature using a Colora circulating water bath. Column flow rates were maintained at 25 ml/h using an LKB Ultra Rac 7000 fraction collector. An Isco-UV 5 absorbance monitor coupled to the column effluent was used to locate fractions containing protein. Thermolysin concentrations in the eluting fractions were determined by measuring their absorbance ($\epsilon_{280} = 52\,400\ cm^{-1}\ M^{-1}$ [19]).

To reduce contaminating zinc levels, solutions used for calcium binding experiments were extracted with a 0.01% dithiazone solution, dissolved in chloroform or carbon tetrachloride. Further studies showed that it was more convenient to simply include 0.01 M TEP in these solutions. Even at contaminating zinc levels as high as 10^{-7} M, this concentration of TEP should be capable of reducing the free zinc levels to less than 10^{-15} M ($K_{Zn}(TEP) = 1.6 \times 10^9\ M^{-1}$ at pH 7.0, 25°C [23]).

For most binding measurements commercial thermolysin powder was dissolved in a solution composed of 0.1 M NaCl, 0.01 M Pipes, p*Ca* 3.0, p*Zn* 6.0, pH 7.0, and either 2 mM phenanthroline or 10 mM TEP at 23°C. Of these two zinc chelators, TEP is preferred since phenanthroline tends to be retarded on Sephadex and requires extensive washing to be completely removed from a column. In order to avoid autolysis, it is important to preincubate thermolysin in TEP for at least 20 min before application to a column equilibrated at low calcium concentrations. The protein was applied as a 10 mg/ml solution, adjusted to pH 10.5 in order to increase protein solubility. For experiments performed with inhibited thermolysin approx. 8 mg of the inhibitor was dissolved in 0.5 ml of solution containing 0.1 M NaCl, 0.01 M Pipes, p*Ca* 2.0, p*Zn* 6.0, and 10% dimethyl formamide to increase solubility. This solution was the same as that described above, except that the solution was adjusted to pH 7.0 before mixing with the inhibitor. Thermolysin was preincubated for approx. 15 min in the presence of the inhibitor before calcium binding measurements were initiated.

The metal ion concentrations of eluting fractions containing thermolysin were measured using a Varian 1200 atomic absorption spectrophotometer. Calcium and zinc determinations were made using the absorption mode with the emission monochromator set at 422.7 and 213.7 nm, respectively. An air-acetylene flame and 10 cm burner head path length, adjusted to stoichiometric conditions, was used for the analysis. All atomic absorption and emission calibration standards for metal ions contained the same concentrations of NaCl

and buffer as the samples.

As a check for irreversible denaturation or autolysis, column fractions were periodically measured for enzyme activity. The activity of thermolysin was determined by measuring the rate of hydrolysis of the synthetic substrate FAGLA. The hydrolysis of FAGLA was quantitated by following the decrease in absorbance of the substrate upon peptide bond hydrolysis ($\Delta\epsilon_{345} = -348\ M^{-1}\ cm^{-1}$). Assay solutions contained 0.1 M NaCl, 0.01 M Pipes, p*Ca* 3.0, p*Zn* 6.0, pH 7.0. In a typical assay, the absorbance at 345 nm was recorded at 0.25-min intervals for 4–5 min. FAGLA concentrations were determined by measuring absorbance at 345 nm ($\epsilon_{345} = 766\ M^{-1}\ cm^{-1}$ [20]).

Knowing the total metal ion concentration, $[M_t]$, and the protein concentration [P] for each of the fractions eluting from the column, the number of metal ions bound per molecule of protein, ($\bar{\nu}_m$) may be calculated using the following equation:

$$[M_t]_f = \bar{\nu}_m[P]_f + [M]_0 \quad (1)$$

where $[M_t]_f$ is the metal ion concentration of fraction f and $[M]_0$ that in the absence of protein. A plot of $[M_t]$ vs. [P] gives a slope equal to $\bar{\nu}_m$ and intercept equal to $[M]_0$. If metal ion equilibrium between the protein and the buffer has been achieved on the column, the latter plot should be linear and the intercept $[M]_0$ should equal the metal ion concentration of the elutant buffer used to equilibrate the column.

2.3. Analysis of calcium binding data

In this study we have used both graphical methods of analysis and computer-assisted nonlinear least-squares or regression analysis [24–26]. Many of the technical details of the nonlinear regression procedure have been considered by Jennrich and Ralston [27] and Duggleby [28]. We have used the subprogram NONLINEAR of the SPSS package developed by Robinson [29]. Computations were performed either on a CDC Cyber 170 or 172 computer system. As suggested by Robinson [29], scaling factors were introduced into the binding equations so that all parameter values could be started at the same numerical value. The standard error for the binding constants was estimated by matrix inversion, which has been shown by Duggleby [30] to give reliable results when used in the analysis of binding data. In order to ensure that the nonlinear least-squares analysis had not converged at a local minimum, the analysis was started at several different initial values of the adjustable parameters (binding constants).

To obtain the best fit of a given theoretical binding model to the observed binding data $(\bar{\nu}_{exp})_i$ measured at various ligand concentrations $[L]_i$, a residual sum of squares (RSS) is calculated according to eq. 2:

$$RSS = \sum_{i=1}^{N} (\bar{\nu}_{exp} - \bar{\nu}_{theor})_i^2 \quad (2)$$

where N is the number of data points and $(\bar{\nu}_{theor})_i$ the theoretical value of $\bar{\nu}$ calculated from the given binding model for the ligand concentration $[L]_i$.

In addition to the residual sum of squares, the χ^2 and F tests were used as criteria of the statistical significance of a given binding model. The latter criteria have been successfully applied in the analysis of binding data [24,28]. Tables of χ^2 and F values are listed by Burington and May [31]. The latter tests were applied in this study in the following way.

The χ^2 statistic is defined as:

$$\chi^2_{exp} = \sum_{i=1}^{N} (\bar{\nu}_{exp} - \bar{\nu}_{theor})_i^2 / C_i^2 \quad (3)$$

where N is the number of data points and C_i the experimentally determined confidence limit for $\bar{\nu}_{exp}$.

If the χ^2 value calculated from eq. 3 is less than the tabulated χ^2 value corresponding to the degrees of freedom allowed by the theoretical binding model and the designated confidence level, then the model is considered to be a good fit to the data.

The F test for the significance of extra parameters is defined as:

$$F(p-q, n-p) = \frac{(n-p)(RSS_q - RSS_p)}{(p-q)RSS_p}; p > q \quad (4)$$

where p and q refer to the total number of param-

eters (binding constants) in two models which have, respectively, RSS_p and RSS_q as the residual sum of squares for the best fit to a given data set. If the F value calculated according to eq. 4 is greater than the tabulated value for the degrees of freedom and the designated confidence limit of the data, then the model with p parameters fits the data significantly better than that with q parameters. This test has been used to choose between binding models which have similar functional form but with different numbers of binding constants (see sections 3 and 4).

3. Results

3.1. The effect of temperature on calcium binding

Some typical experimental data, obtained by the method described in section 2.2 for the determination of calcium binding isotherms and plotted according to eq. 1, are shown in fig. 1. The data show the total calcium and thermolysin concentrations present in fractions eluting from the void volume of the column. The data follow a linear relationship with an intercept equal to the column calcium concentrations. This equality confirms that an equilibrium has been obtained between bound and unbound calcium during the time course of the column experiment.

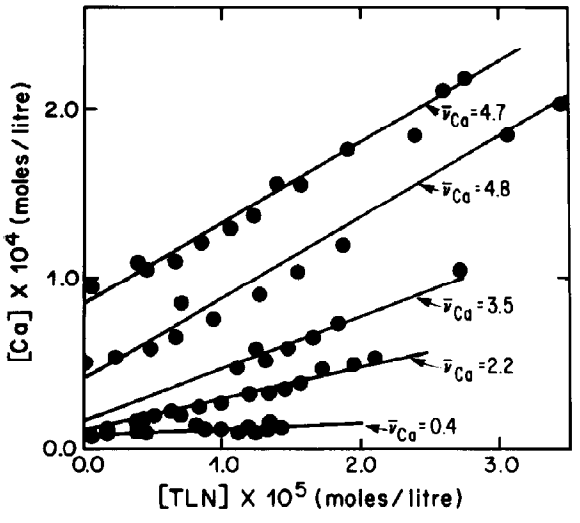

Fig. 1. Calcium binding to thermolysin at pH 9.0, 30°C. The total calcium concentration is plotted against the thermolysin concentration present in fractions collected from columns equilibrated with 0.10 M NaCl, 0.01 M TEP, and different calcium concentrations. See section 2.2 for more details of the experimental techniques used. The plots shown give slopes equal to $\bar{\nu}_{Ca}$ and an intercept equal to the column calcium concentration.

Calcium binding isotherms for thermolysin at pH 9.0 and temperatures from 6 to 35°C are shown in fig. 2. It is clear that the affinity of calcium for thermolysin increases in the temperature range from 6 to 25°C. Hill plots of the binding data (fig 2; insets) give a Hill coefficient with a value of 1.4–1.3, suggesting that there is no significant change in the cooperativity of calcium binding from 6 to 25°C. In contrast, at 30 and 35°C there is a significant increase in cooperativity. The binding isotherm obtained at 35°C suggests that there may be five Ca^{2+} binding to the protein under the conditions of this experiment. The Hill coefficient for the latter data set suggests that at least three calcium-binding sites must be interacting cooperatively.

Calcium binding isotherms for thermolysin at pH 7.0 and temperatures from 16 to 45°C are shown in fig. 3. As with the pH 9.0 data, an increase in calcium affinity with temperature was observed. However, at higher temperatures, the cooperativity of calcium binding does not increase significantly, as judged from the Hill coefficient. At 45°C and higher, column runs at low calcium concentrations showed significant decreases in the concentration of thermolysin eluting from the column at the void volume. For these runs, the amount of low molecular weight compounds eluting shows a concomitant increase, suggesting that an extensive degree of autoproteolysis has occurred during the column run. Hence, calcium binding measurements at pH 7.0 and temperatures higher than 45°C are not reliable using the procedure outlined in section 2.2.

In order to put these results in a more quantitative perspective, the binding data were analyzed using the nonlinear regression procedure outlined in section 2.3. The binding models, listed in table 1, plus other models of similar form, were fitted to the data shown in figs. 2 and 3. The residual sum

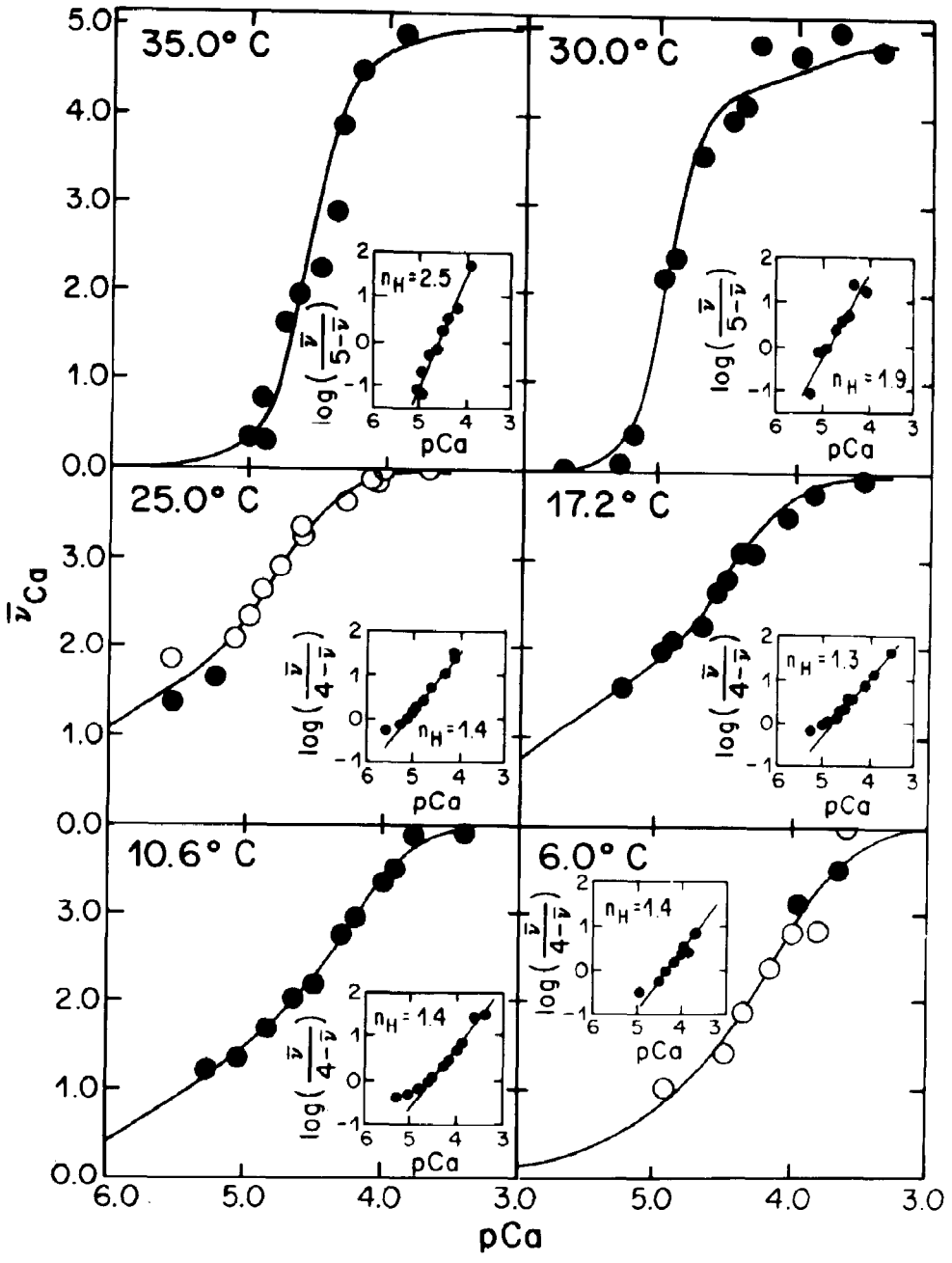

Fig. 2. The effect of temperature on the binding of calcium to thermolysin at pH 9.0. Experiments were performed in solutions containing 0.10 M NaCl and 0.01 M TEP, at the temperatures indicated in the figure. The insets show Hill plots and the value of the Hill coefficients (n_H) for these binding data. Open circles show the binding data reported by Voordouw and Roche [19] at 25°C, and Tajima et al. [34] at 6°C. The solid curves were generated using the binding constants and models described in table 3.

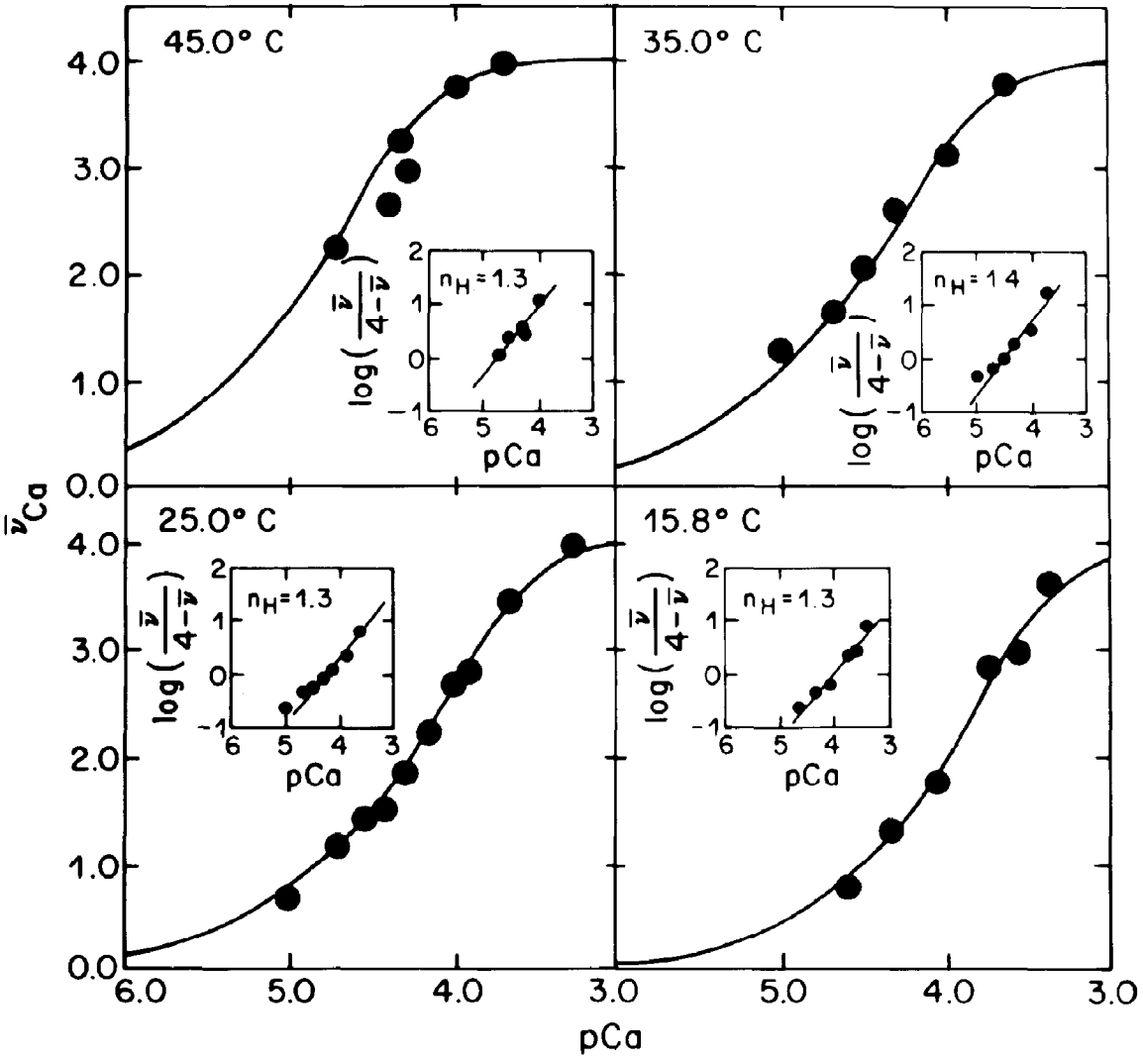

Fig. 3. The effect of temperature on the binding of calcium to thermolysin at pH 7.0. Experiments were performed in 0.10 M NaCl and 0.01 M Pipes, at the temperatures indicated in the figure. The insets show the Hill plots and Hill coefficients (n_H) for these binding data. The solid curves were generated using model 4 and the binding constants listed in table 3.

of squares (RSS) obtained from the best fit was used to calculate χ^2 using eq. 3. These experimental values were then compared to tabulated χ^2 values in order to determine the confidence level at which a particular model may be considered to give a good fit to a set of binding data.

The results of the χ^2 test applied to the best-fit RSS obtained for the binding data are summarized in table 2. The 30 and 35°C data sets, obtained at pH 9, will be considered separately. In all cases, model 1 required a large number of iterations in order to converge or did not converge at all within 100 iterations. When a best fit was obtained with this four-parameter model, the calculated standard deviation for the binding constants was greater than 100%, suggesting that an ill-conditioned solu-

Table 1

Summary of calcium binding models

Model	Functional form [a]	Description [b]
1	$\frac{K_1[Ca]+K_2[Ca]^2+K_3[Ca]^3+K_4[Ca]^4}{1+K_1[Ca]+K_2[Ca]^2+K_3[Ca]^3+K_4[Ca]^4}$	Adair model
2	$\frac{4K_1[Ca]^4}{1+K_1[Ca]^4}$	four strongly cooperative sites
3	$\frac{3K_1[Ca]^3+K_2[Ca]}{1+K_1[Ca]^3+K_2[Ca]}$	three strongly cooperative plus one independent site
4	$\frac{2K_1[Ca]^2+2K_2[Ca]}{1+K_1[Ca]^2+K_2[Ca]}$	two strongly cooperative plus two independent sites
5	$\frac{4K_1[Ca]}{1+K_1[Ca]}$	four independent identical sites

[a] The K values in these models refer to macroscopic or stoichiometric binding constants [35]. Each of the above expressions is equal to the average number of Ca^{2+}, $\bar{\nu}_{Ca}$ bound to the protein.

[b] The term identical refers to sites which may be quite different structurally but which have the same affinity for a ligand.

tion had been obtained. Similar results were obtained for other binding models which contained three or four parameters (e.g., three partially cooperative sites plus one independent site; two partially cooperative plus two independent sites; four independent sites).

Nonlinear regression analysis of the binding data using models 2–5 usually gave well-condi-

Table 2

Comparison of best fits obtained for different models

Data set [a]	n [b]	C_i [c]	CL [d]	χ^2_{ex}/χ^2_T			
				Model 2	Model 3	Model 4	Model 5
pH 9, 25.0°C	16	0.085	99	19.3	8.75	0.65	1.49
pH 9, 17.2°C	11	0.103	99	31.4	8.04	0.69	0.75
pH 9, 10.6°C	11	0.131	99	22.4	10.2	0.16	0.98
pH 9, 6.0°C	9	0.200	90	7.64	2.82	1.02	0.98
pH 7, 45°C	6	0.073	99	30.5	4.48	0.92	3.50
pH 7, 35.0°C	6	0.062	95	53.4	23.8	1.84	0.75
pH 7, 25.0°C	11	0.075	99	35.0	12.7	0.54	1.34
pH 7, 15.8°C	6	0.071	90	32.0	7.55	1.09	0.99

[a] The binding data referred to here are shown in figs. 2 and 3.

[b] n, number of data points.

[c] C_i, average standard deviation found in [Ca].

[d] CL, confidence limit (%) at which the χ^2 test was performed.

[e] The functional forms of models 2–5 are given in table 1.

[f] Tabulated values refer to the ratios of the χ^2_{ex} values calculated using eq. 3, compared to tabulated values (χ^2_T) obtained from statistical tables [31]. If $(\chi^2_{ex}/\chi^2_T) < 1$, then the model is considered to be a good fit to the data at the CL specified.

tioned solutions. However, on the basis of the χ^2 test, models 2 and 3 did not give fits as good as those obtained using models 4 and 5.

Models which assume different combinations of independent sites (e.g., two pairs of independent sites; three identical independent sites plus one independent site) gave best-fit solutions close or equal to that obtained using model 5. Therefore, these models were rejected since they would not pass the F test for the significance of extra parameters (95% confidence level).

Model 4, which assumes two strongly cooperative plus two independent identical sites, usually gave residual sums of squares low enough to pass the χ^2 test at confidence levels of 90% or higher. However, three parameter models of similar form (e.g., two partially cooperative sites plus two independent identical sites; two strongly cooperative plus two nonidentical independent sites) gave ill-conditioned solutions and would not pass the F test for the significance of extra parameters (95% confidence level). This result suggests that the inclusion of three or more parameters in a cooperative binding model is not statistically valid given the number and quality of the data shown in figs. 2 and 3. Thus, although more complex models may intuitively seem to be a more realistic representation of the calcium-binding sites than model 4, the experimental data are neither accurate nor extensive enough to validate their use.

In summary, models 4 and 5 gave the best fits to the binding data while still being a statistically valid representation. Of these two models, model 4 gives better and/or equally good fits to all data sets save one: pH 7.0, 35°C. Hence, model 4 was taken to be the model of choice for further analysis of the data (see below). However, it should be kept in mind that there are not very strong statistical grounds for eliminating model 5 as an accurate representation of the binding data.

The 30 and 35°C data sets at pH 9.0 show that the cooperativity of calcium binding has increased markedly at these temperatures. This is reflected in the high Hill coefficient (fig. 2, inset) and low RSS values obtained from the fits of binding models which assume three or four highly cooperative sites. In fact, the best fits were obtained when a model assuming four cooperative plus one independent site was used.

The calcium binding constants and binding free energy obtained from the best fit of model 4 to binding data are summarized in table 3. It is important to note that regardless of which model is chosen, the binding constants from the best fit give, to a first approximation, identical total free energies of binding. This point was first recognized in fitting different binding models to simulated data [15]. For example, using the pH 7.0, 25°C data set, models 2, 3, 4 and 5 give total binding free energies of 97.5, 95.0, 99.6 and 97.9 kJ/mol, respectively. This demonstrates that even if an unrealistic model is chosen to represent the binding data, the binding constants obtained from nonlinear regression analysis can always be used to calculate an accurate total binding free energy.

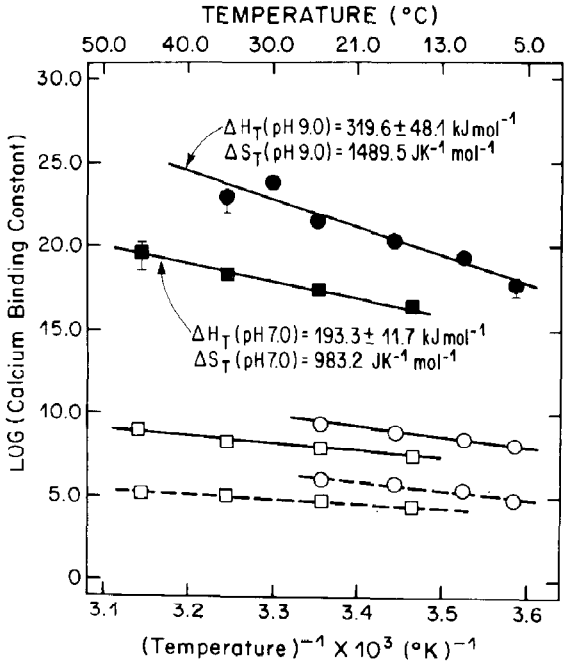

Fig. 4. Van't Hoff plots for calcium binding data collected at pH 7.0 (○, ●) and pH 9.0 (□, ■). Closed symbols show the total enthalpy (ΔH_T) and entropy (ΔS_T) for all four calcium-binding sites. Also shown are van't Hoff plots for the cooperative (○———○, □———□) and independent (○— — —○, □— — —□) binding sites (K_1 and K_2 from model 4), respectively. The thermodynamic parameters obtained from these plots are summarized in table 4.

Table 3

Apparent calcium binding constants for thermolysin at various pH and temperatures

Data set [a]	K_1 [b]	K_2 ($\times 10^{-5}$) [b]
pH 10.3, 25°C	9.77 ±3.32 $M^{-4} \times 10^{17}$	0.0346 ± 0.0495 M^{-1}
pH 9.0, 35.0°C	2.58 ±0.87 $M^{-4} \times 10^{18}$	0.227 ±0.227 M^{-1}
30.0°C	6.72 ±1.53 $M^{-4} \times 10^{11}$	0.127 ±0.072 M^{-1}
25.0°C	31.2 ±4.40 $M^{-2} \times 10^{8}$	10.7 ±2.50 M^{-1}
17.2°C	7.42 ±0.94 $M^{-2} \times 10^{8}$	6.04 ±1.59 M^{-1}
10.6°C	2.86 ±0.23 $M^{-2} \times 10^{8}$	2.58 ±0.25 M^{-1}
6.0°C	1.12 ±0.39 $M^{-2} \times 10^{8}$	0.65 ±0.26 M^{-1}
pH 8.0, 25.0°C	4.57 +0.71 $M^{-2} \times 10^{8}$	1.18 +0.22 M^{-1}
pH 7.0, 45.0°C	11.3 ±3.8 $M^{-2} \times 10^{8}$	2.04 ±1.32 M^{-1}
35.2°C	1.92 ±0.34 $M^{-2} \times 10^{8}$	1.13 ±0.18 M^{-1}
25.0°C	0.766 ±0.087 $M^{-2} \times 10^{8}$	0.585 ±0.59 M^{-1}
15.8°C	0.318 ±0.063 $M^{-2} \times 10^{8}$	0.280 ±0.049 M^{-1}
pH 6.5, 25.0°C	0.635 ±0.172 $M^{-2} \times 10^{8}$	0.299 ±0.068 M^{-1}
pH 6.1, 25.0°C	0.800 ±0.191 $M^{-2} \times 10^{8}$	0.181 ±0.037 M^{-1}
pH 5.6, 25.0°C	0.0463 ± 0.0095 $M^{-2} \times 10^{8}$	0.129 ±0.023 M^{-1}

[a] The calcium binding data collected here are presented in figs. 2, 3, 5 and 6.

[b] These binding constants were obtained from the best fit of model 4 (table 1) to the binding data with the exception of the (pH 10.3, 25°C), (pH 9.0, 35.0°C) and (pH 9.0, 30.0°C) data sets, for which a model assuming four cooperative sites plus one independent site was used.

Table 4

Comparison of apparent thermodynamic parameters for binding of calcium to various proteins

Protein	Site	ΔH/site (kJ mol^{-1})	ΔS/site (J K^{-1} mol^{-1})	ΔG^{298}/site (kJ mol^{-1})	Conditions
Thermolysin [a]	(1,2)	+45.1	+227	−22.7	pH 7.0, 0.1 M NaCl
	(1,2)	+58.5	+286	−26.8	pH 9.0, 0.1 M NaCl
	(3,4)	+51.0	+262	−27.0	pH 7.0, 0.1 M NaCl
	(3,4)	+97.4	+443	−34.6	pH 9.0, 0.1 M NaCl
Phospholipase A_2 [b]	(1)	−16.4	+15.0	−21.0	pH 8.0, 0.1 M NaCl
Concanavalin A [c]	(S2)	+37.2	+177	−15.5	pH 5.3, 0.9 M NaCl
Parvalbumin [d]	(CD,EF)	−37.6	+55.2	−53.9	pH 7.4, 80 mM KCl
Cardiac troponin C [e]	(1,2)	−9.61	+93.6	−37.6	pH 7.0, 0.1 M KCl
Skeletal muscle troponin C	(1,2) [f]	−32.2	+61.4	−50.2	pH 7.0, 0.1 M KCl
	(1) [g]	−56.4	−36.4	−45.6	pH 7.0, 0.1 M KCl
	(2) [g]	+8.78	+161	−39.3	pH 7.0, 0.1 M KCl
	(3,4) [f]	−32.2	+33.4	−42.2	pH 7.0, 0.1 M KCl
	(3,4) [g]	−7.94	+70.2	−28.4	pH 7.0, 0.1 M KCl
Calmodulin [h]	(1,2)	+3.00	+129	−35.5	pH 7.0, 0.1 M KCl
	(3,4)	+7.52	+178	−27.6	pH 7.0, 0.1 M KCl

[a] Results from the present study. [b] Ref. 36. [c] Ref. 37. [d] Ref. 38. [e] Ref. 39. [f] Ref. 40. [g] Ref. 41. [h] Ref. 42; for a critical review of this result see ref. 53.

The results of a van't Hoff analysis of the binding constants in table 3 are shown in fig. 4. The thermodynamic parameters obtained from them are summarized in table 4.

3.2. The effect of pH on calcium binding

Calcium binding isotherms for thermolysin at pH values from 5.6 to 10.3 at 25°C are shown in

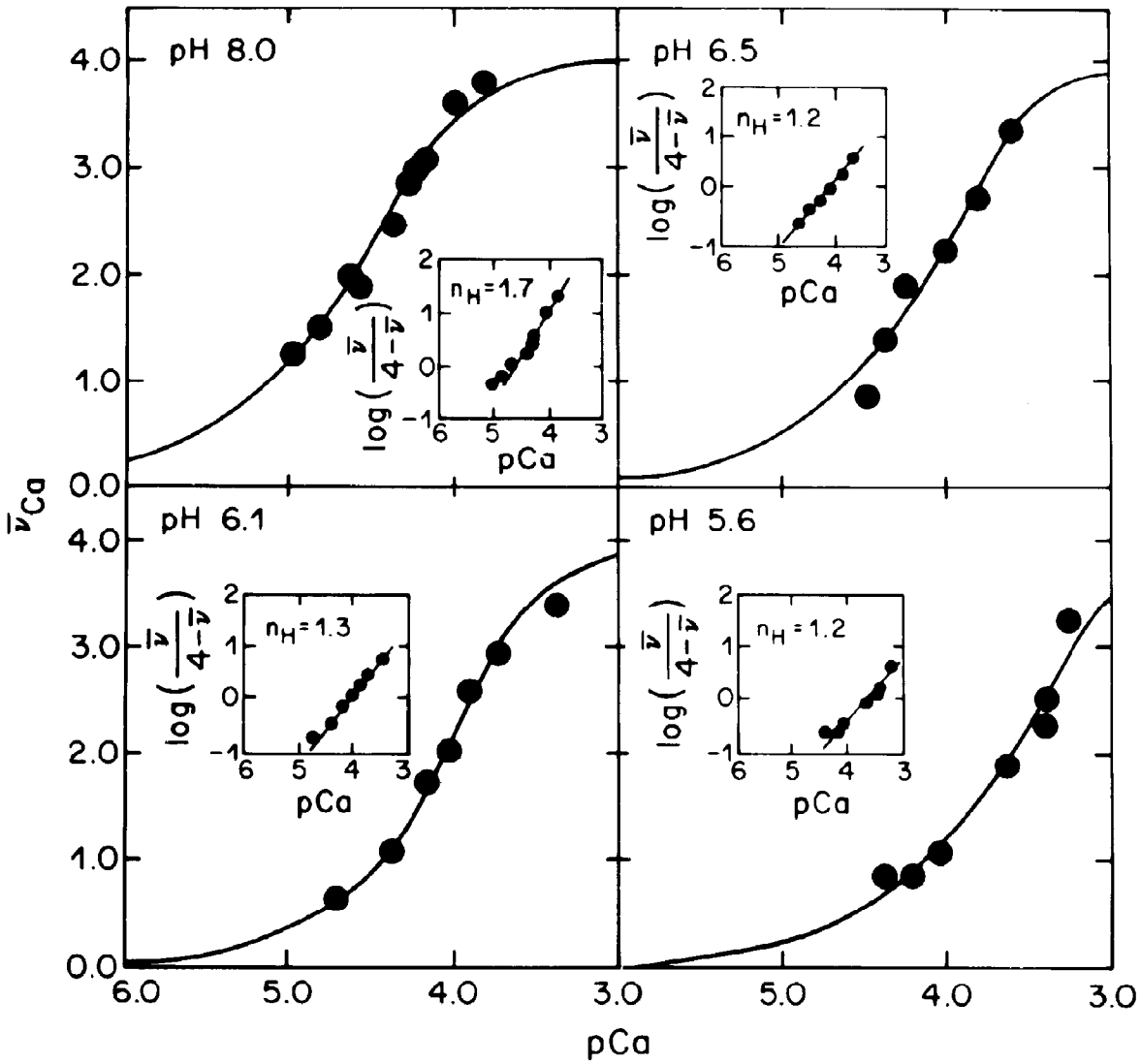

Fig. 5. The binding of calcium to thermolysin at 25°C and various pH values. Experiments were performed in 0.10 M NaCl, plus: 0.01 M Tris or Hepes at pH 8.0, 0.01 M Pipes at pH 6.5 and 6.1, and 0.01 M Mes at pH 5.6. The insets show the Hill plots and Hill coefficients (n_H) for these binding data. Solid curves were generated using the models and binding constants listed in table 5.

figs. 5 and 6. From pH 5.6 to 9.0, there is a significant increase in calcium binding affinity with little or no change in the cooperativity of calcium binding, as judged from Hill plots (fig. 5, insets). In contrast, at pH 10.3 cooperativity increases markedly; at this pH at least three Ca^{2+} must be interacting, since the Hill coefficient is greater than 2 (fig. 6).

The binding data were subjected to the same nonlinear regression procedure and statistical analysis described in section 2.3. The best fit to the binding data collected at pH 8.0, 6.5 and 6.1 was obtained using model 4. Models 4 and 5 fit the binding data collected at pH 5.6 equally well. A model which assumes four cooperative and one independent site gave the best fit to the binding

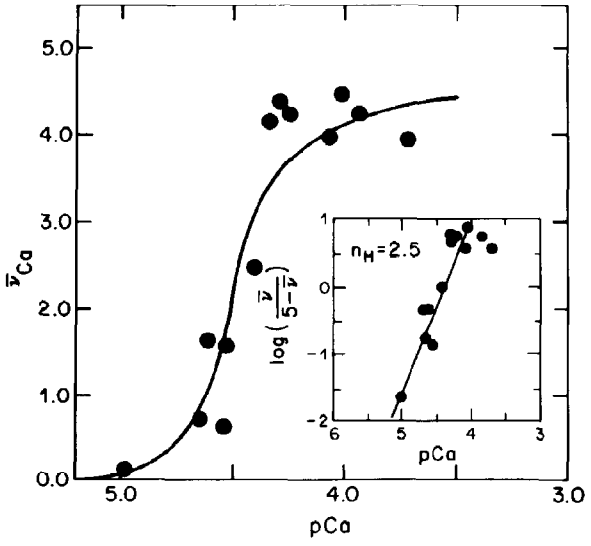

Fig. 6. Calcium binding to thermolysin at pH 10.3, 25°C. Experiments were performed in solutions containing 0.01 M TEP and 0.10 M NaCl. The insets show the Hill plot and Hill coefficient (n_H) for these binding data. Solid curves were generated using the models and binding constants listed in table 5.

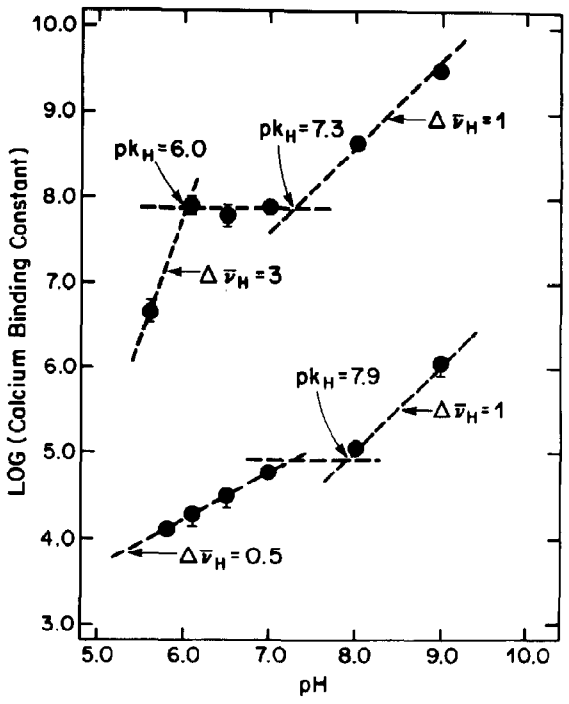

Fig. 7. Dixon plot, illustrating the effect of pH on the cooperative (upper) and independent (lower) binding constants for thermolysin at 25°C. The slopes of the dashed lines refer to the number of ionizable groups ($\Delta\bar{\nu}_H$) which modulate the affinity of calcium for the different binding sites. The pK_H values for these ionizable groups were estimated from the points of intersection of the lines [33].

data collected at pH 10.3. A summary of the binding constants obtained from the best fit of model 4 to the binding data collected in the pH range from 5.6 to 8.0 is listed in table 3.

The binding constants given in table 3 were used to construct a Dixon plot [33] which may be used to determine the number of H^+ which modulate the calcium binding affinity. The slopes from these plots (fig. 7) indicate that for the cooperative calcium-binding sites, one H^+ with an apparent pK_H of 7.3 and at least three H^+ with an apparent pK_H of 6.0 modulate the calcium binding affinity of thermolysin in the range from pH 5.6 to 9.0.

The Dixon plot for the independent binding constant is more complex. At least one H^+ modulates the calcium binding affinity from pH 8.0 to 9.0. From pH 5.6 to 7.0, the Dixon plot gives a fractional slope. This might be expected if one of the two independent calcium-binding sites loses an H^+ while the other site does not. Using the experimentally determined number of H^+ titrated, and knowing from the crystal structure, the number of ionizable groups at each of the calcium sites, it is possible to identify tentatively the cooperative binding sites as sites 1 and 2. This analysis is considered in detail in section 4.

3.3. The effect of an active-site inhibitor on calcium binding

The calcium binding isotherms for thermolysin in the presence and absence of the covalent inhibitor *N*-chloroacetyl-DL-hydroxyleucine methyl ester are compared in fig. 8. The presence of inhibitor does not appear to alter significantly the cooperativity of calcium binding, as judged from the slope of the Hill plot. Nonlinear regression analysis shows that model 4 gave fits with the lowest RSS values to the binding data for both the inhibited and active enzyme. In the presence of the

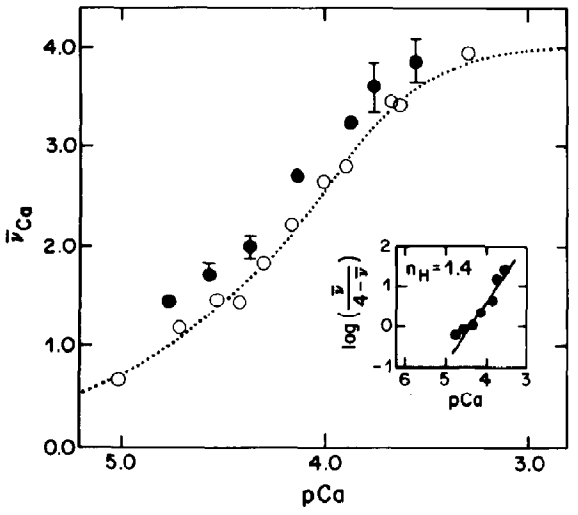

Fig. 8. Comparison of calcium binding isotherms for thermolysin in the presence (●) and absence (○) of a chloromethyl ketone inhibitor at pH 7.0, 25°C. The inset shows the Hill plot and Hill coefficient (n_H) for the inhibited enzyme. The solid curve was generated as described in the legend to fig. 3. Nonlinear regression analysis of the binding for the inhibited enzyme using model 4 (table 1) gives; $K_1 = 1.32 \pm 0.15 \times 10^8$ M^{-2} and $K_2 = 1.24 \pm 0.18 \times 10^5$ M^{-1}.

inhibitor, thermolysin binds calcium with slightly higher affinity. The cooperative and independent binding constants for the inhibited enzyme have both approximately doubled; this corresponds to an increase in calcium binding free energy of 4.6 kJ/mol for these sites.

Discussion

4.1. Calcium binding to thermolysin

On the basis of a Hill plot of their binding data, Voordouw and Roche [19] concluded that at pH 9, 25°C, two Ca^{2+} bind to thermolysin with positive cooperativity. Although the value for the binding constant reported (2.8×10^9 M^{-2}) is within the error limits found for K_1 in the present study (table 3), no detailed statistical analysis of the data was undertaken. It was assumed that the binding constant for the other two sites was greater than 10^6 M^{-1}. No estimate of the binding constants for the other two sites was made.

Tajima and co-workers [34] measured the calcium binding properties of thermolysin at pH 9, 6°C. They analyzed the binding data assuming four identical independent sites ($K = 2.0 \times 10^4$ M^{-1}). A Hill plot of the binding data was not reported and no attempt was made to determine if a cooperative binding model would fit the data better or equally well. The latter data have been critically reviewed [16].

In order to provide an objective basis for evaluating the relative merits of the earlier interpretations of the calcium binding behaviour of thermolysin, a detailed statistical analysis of the binding data was undertaken. In addition, the earlier data have been supplemented in the present study with 13 new calcium binding isotherms from pH 10.3 to 5.6 and from 6.0 to 45°C plus calcium binding measurements in the presence of an active-site inhibitor. Both the new and previous binding data have been subjected to nonlinear regression and statistical analysis procedures described in section 2.3. With these new findings, we are now in a position to discuss in more detail the nature of calcium binding to thermolysin.

The analysis of the data collected at pH 9 using the Hill equation shows that the cooperativity of calcium binding does not change significantly from 25 to 6°C. Therefore, conflicting claims that binding is totally cooperative for two Ca^{2+} at 25°C [19] and independent at 6°C [34] appear to be due to differences in the quality and interpretation of binding data rather than a change in the nature of calcium binding over this temperature range. We have shown (see section 3) that the most statistically valid representation of these binding data, as well as data collected at lower pH values, is that provided by model 4, which assumes two totally cooperative and two independent and identical sites. Another finding from the present study is that the binding constants for the two independent sites are less than 10^6 M^{-1} and in fact can be quantitated.

An important general result demonstrated by the present study is the utility of nonlinear regression and χ^2 and F statistics in the analysis of binding data. Nonlinear regression is a fast and objective procedure which gives one the ability to

test thoroughly a variety of different binding models within a reasonable period of time. Furthermore, nonlinear regression provides an estimate of the error in the calcium binding constants obtained from the best fit of a model to binding data. The most important feature of nonlinear regression analysis is the relative ease in identifying ill-conditioned solutions. Thus, in an objective way, unnecessarily complex models can be avoided.

It is also important to keep in mind that, even though a more complex model may intuitively seem more appealing, its use may not be statistically valid for a given a set of binding data. The quality and quantity of the binding data put constraints on the ability of nonlinear regression analysis to differentiate between different types of models. For instance, given the structural differences among the four calcium-binding sites of thermolysin, it seems unlikely that there are two independent sites with exactly identical affinities for calcium, as assumed by model 4. If, however, we wish to account for these differences, using a model which assumes two cooperative and two independent nonidentical sites, we would need more accurate data or a larger number of data points in order to obtain a significantly improved fit. This point was confirmed using simulated data. For instance, if the two independent binding constants differed by less than an order of magnitude, then [Ca] values with an error of less than ± 0.085 would have to be collected in order to differentiate between model 4 and the expanded model described above. Since the binding data actually collected have errors equal to this order of magnitude (table 2) it seems likely that the independent calcium-binding sites have affinities for calcium within an order of magnitude of each other.

A similar observation can be made concerning the cooperative binding sites. Model 4 assumes that two calcium-binding sites interact with complete positive cooperativity. However, it is plausible that the three sites, 1, 2, and 4, could interact partially to give rise to the observed cooperativity. In fact, the calcium binding data collected at pH 7.0, 25°C, in the presence of 6 M urea, clearly show that three Ca^{2+} which bind to thermolysin under these conditions bind with strong positive cooperativity [15]. However, nonlinear regression analysis of simulated binding data revealed that in order to fit partially cooperative data using a model which assumes three partially interacting sites, extremely accurate data are required. For instance, a simulated data set must have an error of less than ± 0.03 in order for nonlinear regression analysis to differentiate between a model which assumed three partially cooperative sites and a model which assumed two totally cooperative sites plus one independent site.

Another interesting trend revealed by the calcium binding data is the tendency for the cooperativity of calcium binding to increase when thermolysin is placed in extreme environments of pH or temperature. At 25°C there is a significant increase in cooperativity when the pH is increased from 9.0 to 10.3. An increase in temperature at pH 9.0, also results in an increase in cooperativity.

Voordouw and Roche [19] suggested that calcium binds cooperatively to sites 1 and 2 on the basis that the Ca^{2+} bound at these sites share three carboxyl groups as chelating ligands. Furthermore, Voordouw and Roche observed that the dissociation of the cooperative Ca^{2+} results in increased exposure of a Trp residue to the bulk solvent; presumably this reflects a perturbation of Trp 186, which is located near sites 1 and 2. In contrast, Matthews and co-workers [43] suggested that sites 2 and 4 bind calcium cooperatively based on the observed relative order of dissociation of calcium(II) from crystals of thermolysin soaked in EDTA. There are a number of experimental considerations which make Matthews' suggestion questionable. The crystals of thermolysin were reported to dissolve and crack in the presence of EDTA, giving rise to poor-quality data. It was not established that an equilibrium between bound and free calcium had been reached during the course of the X-ray diffraction experiment. To help stabilize the thermolysin crystals experiments were performed in the presence of phosphoramidon, an inhibitor of thermolysin. Unfortunately, the effect that phosphoramidon may have on calcium binding to thermolysin was not assessed.

The results obtained in the present study provide further evidence for the assignment of sites 1 and 2 as the cooperative binding sites. The Dixon plot of the pH dependence of pK_1, corresponding

to the cooperative binding, reveals that there are at least four ionizable groups ($\Delta\bar{\nu}_H = 4$) which affect the affinity of calcium for the cooperative sites. If these ionizable groups correspond to ionizable ligands directly involved in the chelation of calcium, then the value of $\Delta\bar{\nu}_H$ should be equal to the total number of Asp-O^{δ}, Glu-O^{ϵ} or Thr-O^{γ} groups coordinated to calcium(II). For sites 1 and 2, the total number of ionizable groups is four and the assignment of sites 1 and 2 as the locus of cooperative binding is most consistent with the observed total $\Delta\bar{\nu}_H$ for the cooperative binding constant (fig. 7).

In the presence of the chloromethyl ketone inhibitor covalently bound to the active site of thermolysin, it was found that calcium binds with higher affinity to all four sites. However, the increased affinity is only of the order of 4 kJ mol^{-1} and it is difficult to assign it to any specific interaction.

4.2. Calcium binding thermodynamics

The analysis presented in section 4.1 leads to the conclusion that, under nondenaturing conditions, calcium binds to sites 1 and 2 cooperatively, and independently to sites 3 and 4. On the basis of this identification, we will attempt to correlate the thermodynamic parameters determined for calcium binding with the structure of the binding sites of thermolysin and compare these parameters to those obtained for other proteins.

Kretsinger and Nelson [46] have proposed that the affinity of calcium may correlate with the total number of carboxyl groups at the binding site which chelate to calcium. This implies that the electrostatic interaction energy between the Ca^{2+} and the negatively charged atoms at the binding site dominates the free energy of binding. Increasingly strong electrostatic energies should also be reflected in larger, more negative enthalpies of calcium binding. When these suggestions are tested by comparing the ΔH and ΔG of calcium binding for various proteins, it is apparent that the expected correlations are unclear. For instance, site 3 and 4 of thermolysin contain two and one carboxylate groups which chelate calcium [13]. However, the present study has shown that the ΔG values for calcium binding to these sites are indistinguishable. Although the number of different carboxyl groups at sites 1 and 2 totals four, the affinity of calcium at these sites is lower than for sites 3 and 4. In view of the proximity of sites 1 and 2, we suggest that a significant degree of electrostatic repulsion between the two centers accounts for the reduced calcium affinity at these sites. Electrostatic repulsion may also account for the positive enthalpy for calcium binding to sites 1 and 2 of thermolysin as well as site S2 of concanavalin A, which is in close proximity to the manganese-binding site (S1) [47].

There seems to be a great deal of variety in the sign and magnitude of the ΔH values listed in table 4. For instance, while the range of ΔG for calcium binding is not more than 35 kJ mol^{-1} for all of the proteins listed in table 4, the ΔH may differ by as much as 154 kJ mol^{-1}. It is difficult to account for this variability solely on the basis of the number of the carboxylate groups associated with the calcium-binding sites. This point is most strikingly seen in the case of the structurally similar calcium-binding sites found in the calcium-trigger proteins parvalbumin, cardiac and skeletal troponin C and calmodulin which have a large range of calcium binding enthalpies.

The results of this study establish that the binding of calcium to thermolysin is an entropy-driven process. The large positive entropy for calcium binding accounts for the observed thermostability of this protein [9]. Since bound calcium stabilizes the native structure of thermolysin, the increased affinity of calcium at higher temperatures will serve to shift the equilibrium between folded and unfolded conformations towards folded structures which possess calcium-binding sites.

The positive entropy of calcium binding observed in most of the cases summarized in table 4 reflects the fact that dehydration of the Ca^{2+} is an integral part of the binding process. The wide range of values observed arises from the fact that variable numbers of water molecules are found liganded to the bound calcium and the proteins undergo variable extents of conformational accommodation in the binding process. Given that the entropy of solvation of gaseous Ca^{2+} is -254 J K^{-1} mol^{-1} [48], it is clear that in the case of the

calcium-trigger proteins a significant loss of conformational entropy contributes to the observed entropy of calcium binding. In contrast, thermolysin has the largest values for the entropy of calcium binding, an observation fully consistent with spectroscopic evidence [16,19] which suggests that the conformation of the protein is minimally perturbed by calcium binding and the observed stabilization of the protein by bound calcium [9].

That the thermodynamics of calcium dehydration play a key role in determining the observed values for the thermodynamic parameters of the protein-calcium interaction is further emphasized by the fact that the dehydration enthalpy of Ca(II) is 1670 kJ mol^{-1} [48]. The net enthalpy of the protein-calcium interaction which is observed experimentally is thus the difference between two very large enthalpies of opposite sign. Clearly, any attempt to correlate the calcium affinity with a given structural feature of a protein calcium-binding site requires consideration of more than just the number of ionizable groups at a given binding site. A more rigorous approach would require a full calculation of all interactions occurring in the system between protein, metal ion and solvent.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada.

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