The Role of Bound Calcium Ions in Thermostable, Proteolytic Enzymes. II. Studies on Thermolysin, the Thermostable Protease from *Bacillus thermoproteolyticus*†

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ABSTRACT: The functional properties of the four calcium ions, bound by thermolysin, appear to be very similar to those of the single calcium ion bound by thermomycolase (G. Voordouw and R. S. Roche (1975), *Biochemistry*, preceding paper in this issue). Hence when the free calcium ion concentration is varied in the range where the calcium double-site dissociates (G. Voordouw and R. S. Roche (1974), *Biochemistry 13,* 5017), no changes are observed in the sedimentation coefficient or the peptide circular dichroism. Differences in molar ellipticity and molar extinction coefficient occur in the aromatic ultraviolet region, which parallel the occupancy of the calcium binding double site. The difference spectrum, characterized by a main band at 290 nm and a somewhat smaller band at 283 nm, is interpreted as due to the transfer of a partially buried tryptophan residue to the aqueous solvent upon dissociation of the two calcium ions from the double site. This is most likely Trp-186, which is in between Asp-185 and Glu-187, two chelating amino acids of this site. From the calcium dependence of the rate constant for autolytic degradation we conclude, as for thermomycolase, that only conformers devoid of bound calcium ion serve as substrates in the reaction. This rate constant increases about 1000-fold, when the double site dissociates. Hydrogen–tritium exchange studies show the presence of a large stable structural core, comprising about 32% of all the peptide hydrogens present. These do not exchange-in after 24 hr at 25°C, pH 9.0, ionic strength 0.1. The exchange-out of 60 slow hydrogens was found to be independent of the free calcium ion concentration in the range 2.0–8.0 × 10^{-4} *M*, where all four calcium-binding sites are saturated. The calcium dependence of the first-order rate constant for thermal denaturation at 80°C, pH 7.0, indicates that thermolysin is stabilized by only *one* calcium ion under these conditions. These observations are rationalized in terms of a calcium-binding model for thermolysin and the known three-dimensional structure of the enzyme and its calcium-binding sites.

Although the stabilizing role of bound calcium ions in both the thermal denaturation (Feder et al., 1971) and autolysis (Drucker and Borchers, 1971) of thermolysin has been the subject of research in the past, a quantitative and detailed molecular understanding of their role is still lacking. In a previous paper (Voordouw and Roche, 1975) we have developed a calcium-binding model and tested it experimentally for thermomycolase, a thermostable serine protease which binds only one calcium ion. The dependence of the rate constants for autolysis and thermal denaturation on the concentration of calcium ion could be fully rationalized in terms of the calcium-binding constants of the relevant enzyme conformations. Similar models have been used to account for the pH dependence of protein denaturation kinetics (Tanford, 1968, 1970; Laidler and Bunting, 1973). We will demonstrate here that the role of calcium ions in thermolysin can be described in terms of the same concepts. One may expect the equations derived for the model to be somewhat more complicated in the case of thermolysin since it binds four calcium ions. However, we have shown recently for apothermolysin that, in the range where the calcium-binding isotherm is accessible to measurement ($3.0 < pCa^{2+} < 6.0$), two calcium ions dissociate simultaneously with complete positive cooperativity. Hence, in this range, the isotherm can be described by a single constant, $K_{1,2}$ = $2.8 \times 10^9\ M^{-2}$. In view of this cooperativity the observed dissociation was ascribed to the two calcium ions bound at the double-site of thermolysin (Voordouw and Roche, 1974).

We follow the same procedure used in the case of thermomycolase (Voordouw and Roche, 1975) and report here the effect of varying the calcium ion concentration on the conformation of apothermolysin as revealed by the measurement of a number of equilibrium properties (sedimentation coefficient, circular dichroism, and uv difference spectrum) under the same conditions used for the determination of the calcium-binding isotherm. Hence the value of $K_{1,2}$ given above can be used in rationalizing the results.

In order to account for the autolysis results which necessarily have to be obtained with active holoenzyme, the value of $K_{1,2}$ is relaxed in order to find the best fit to the experimental data consistent with the binding model. The description of the thermal denaturation results, obtained under drastically different conditions, requires a binding model in which only *one* calcium ion contributes to the thermal stability.

Experimental Section

Materials. Thermolysin (lot no. 93C-2700) was obtained from Sigma Chem. Co. and TEP[1] from J. T. Baker Chem.

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[1] Abbreviations used are: Me_2SO dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; TEP, tetraethylenepentamine; FAGLA, furylacryloylglycyl-L-leucinamide; $[Ca^{2+}]$ and pCa^{2+}, the free calcium ion concentration and its negative logarithm; $[Ca]_T$, the total calcium ion concentration.

Co. FAGLA was purchased from Cyclo Chem. Co. and Me_2SO from Fisher. Other chemicals used were as described previously (Voordouw and Roche, 1975).

Methods. Since the same methods have been used as in our previous paper, this section will only give modifications of experimental procedures and conditions wherever necessary.

Sedimentation Measurements. The sedimentation coefficient of thermolysin was measured as a function of $[Ca^{2+}]$ using an enzyme concentration of 8.4×10^{-5} *M* in 0.1 *M* NaCl-0.01 *M* TEP (pH 9.00) and $[Ca]_T = 5.38 \times 10^{-4}$ *M*. Variations in $[Ca^{2+}]$ were achieved by varying the concentration of EDTA (see below) in the range $4.0 \leq pCa^{2+} \leq 5.0$ or by addition of $CaCl_2$ in the range $3.0 \leq pCA^{2+} \leq 4.0$. Measurements were performed at 25°C and 52,000 rpm, using a cell with sapphire windows and a filled Epon double sector centerpiece. The apothermolysin stock solution used was prepared by dialysis against 0.1 *M* NaCl-0.01 *M* TEP (pH 9.00) and 2.0×10^{-4} *M* $CaCl_2$ for at least 12 hr. These preparations did not contain more than 0.04 mol of zinc ion/mol of enzyme, as judged by atomic absorption spectroscopy (Voordouw and Roche, 1974). When assayed against FAGLA in the above buffer they showed not more than 1% residual activity. Concentrations of stock solutions were determined from optical density measurements, usually in triplicate, using a molar extinction coefficient ϵ 52,400 at 280 nm (Voordouw and Roche, 1974). They were adjusted to the desired values by gravimetric dilution with dialysis buffer.

Difference Spectroscopy and Circular Dichroism (CD). Measurements were made with apothermolysin solutions similar to those described above. For difference spectroscopy use was made of 10 mm × 10 mm cylindrical tandem cells (Pyrocell, no. 6016) when scanning a spectrum. The dependence of the difference spectral peak at 290 nm on $[Ca^{2+}]$ was studied with regular 10-mm rectangular cells (one compartment; Hellma, 112-QS). The variable contribution made by EDTA to ΔA_{290} was measured separately and subtracted from the values obtained. This correction amounted to not more than 15% of the measured values. Measurements were performed at 23°C with an enzyme concentration of 3.76×10^{-5} *M* and a $[Ca]_T = 2.5 \times 10^{-4}$ *M*. For the CD studies in both the peptide and the aromatic uv regions these concentrations were 3.5×10^{-5} and 2.4×10^{-4} *M*, respectively, using cells (Opticell Co.) with a path length of 0.1 mm in the peptide and 5.0 mm in the aromatic uv region at 25°C.

Autolysis and Thermal Denaturation Studies. The second-order rate constant for autolytic degradation was measured as a function of $[Ca^{2+}]$ in 0.1 *M* NaCl-0.015 *M* Tris (pH 9.00) at 25°C. TEP was substituted by Tris in these studies in order to have normally active enzyme. Also no chelating agents were used for the variation of $[Ca^{2+}]$ since this invariably results in the partial inhibition of this zinc metalloenzyme. We therefore diluted a stock solution of 10^{-4} *M* thermolysin in 1 *M* NaCl-0.015 *M* Tris (pH 9.00) and $[Ca]_T = 10^{-3}$ *M* either 100-fold with 0.09 *M* NaCl-0.015 *M* Tris (pH 9.00) and variable $[Ca^{2+}]$ or tenfold with 0.015 *M* Tris and a variable $[Ca^{2+}]$, to obtain thermolysin concentrations of 10^{-5} or 10^{-6} *M* in 0.1 *M* NaCl-0.015 *M* Tris (pH 9.00) and $4.0 < pCa^{2+} < 5.5$. The remaining active enzyme concentation was determined by assaying periodically with FAGLA according to Feder (1968). The value of $[Ca^{2+}]$ was calculated from the known total enzyme concentration and the known $[Ca]_T$ with the aid of eq 2 and 3, as shown below. Initially $K_{1,2} = 2.8 \times 10^9\ M^{-2}$, determined for apothermolysin, was used in this calculation. $K_{1,2}$ was then adjusted to get an optimal fit with the experimental data and finally the $[Ca^{2+}]$ was recalculated using this new value for $K_{1,2}$. Since $[Ca]_T$ was at least eightfold larger than the thermolysin concentration, the adjustments in pCa^{2+} did not exceed 0.02 pCa^{2+} unit for 10^{-6} *M* and 0.10 unit for 10^{-5} *M* thermolysin when this new value for $K_{1,2}$ was used.

Thermal denaturation studies were performed at 80°C in 0.05 *M* Tris (pH 7.0 *at* 80°C) and a variable concentration of NaCl and $CaCl_2$, such that their combined ionic strength was 0.05. The thermolysin concentration in the final incubation mixture was 3.0×10^{-6} *M* and the experimental procedure exactly as previously described (Voordouw and Roche, 1975).

Hydrogen-Tritium Exchange Studies. Exchange-in of apothermolysin was carried out in 0.1 *M* NaCl, 0.01 *M* TEP (pH 9.0), 1.0×10^{-4} *M* $CaCl_2$, and 1–5 mCi of tritiated water, THO, per ml, at 25°C for 24 hr. From one column run experiments (Englander and Englander, 1972) exchange-out data were extrapolated back to zero time under very slow exchange-out conditions in 0.01 *M* $CaCl_2$–0.08 *M* NaOAc (pH 5.0) at 2°C. Equation 1 from Voordouw and Roche (1975) was used in the extrapolation, using for the free peptide hydrogen exchange rate constant $k_p = 0.275\ min^{-1}$ and for the free primary amide hydrogen exchange rate constant $k_a = 0.0257\ min^{-1}$ (Englander and Staley, 1969; Englander and Poulsen, 1969). The thermolysin concentration was about 6×10^{-5} *M* during exchange-in for these one column runs. For two column run experiments a much higher thermolysin concentration is required in the exchange-in step. Since the solubility of thermolysin is limited ($\sim 10^{-4}$ *M* or 3.5 mg/ml under our experimental conditions) we labeled crystalline suspensions (50 mg/ml) under the conditions indicated above. We then dissolved all of the protein by mixing 0.5 ml of the suspension quickly with 0.5 ml of Me_2SO. Exchange-out was initiated by chromatographing immediately over the first Sephadex G-25 column (1.5 × 12 cm) equilibrated with 0.1 *M* NaCl-0.01 *M* TEP (pH 9.0) and a variable $[Ca^{2+}]$. The protein was collected free of Me_2SO at the void volume of the column in a 5-ml fraction, which was incubated at 25°C. The number of hydrogens remaining unexchanged was determined from second column runs. One column run controls in which the Me_2SO addition was omitted did not indicate any influence of Me_2SO on the exchange behavior of the slow peptide hydrogens studied in these experiments.

Equations for Calculation of $[Ca^{2+}]$ and Data Analysis. The calcium-binding isotherm for apothermolysin, assuming two sites to be independent and two to be completely positively cooperative, is given by:

$$\bar{\nu}_{Ca^{2+}} = \frac{2K_{1,2}c^2}{(1 + K_{1,2}c^2)} + \frac{K_{3}c}{(1 + K_{3}c)} + \frac{K_{4}c}{(1 + K_{4}c)} \quad (1)$$

where we have replaced $[Ca^{2+}]$ by the symbol c. It has been shown (Voordouw and Roche, 1974) that, in the range $3.0 \leq pCa^{2+} \leq 5.0$, $K_3c \gg 1$ and $K_4c \gg 1$ and hence (1) reduces to:

$$\bar{\nu}_{Ca^{2+}} = 2 + \frac{2K_{1,2}c^2}{(1 + K_{1,2}c^2)} \quad (2)$$

The symbol $\bar{\nu}_{Ca^{2+}}$ is the amount of calcium ions bound per mole of apothermolysin at a given $[Ca^{2+}]$ and $K_{1,2} = 2.8 \times 10^9\ M^{-2}$. The relation between $[Ca]_T$, $[Ca^{2+}]$, and the total

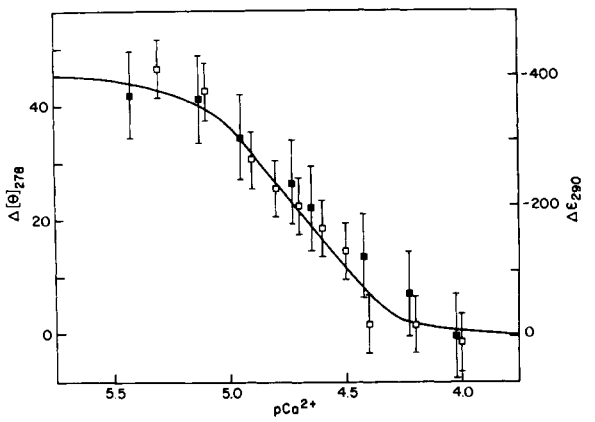

FIGURE 1: Dependence of the difference molar ellipticity at 278 nm, $\Delta[\theta]_{278}$ in deg cm^2 dmol^{-1} (□), and the difference molar extinction coefficient at 290 nm, $\Delta\epsilon_{290}$ in M^{-1} cm^{-1} (■), on pCa^{2+}. Differences are for apothermolysin with $pCa^{2+} = 4.0$ as reference in 0.1 M NaCl-0.01 M TEP (pH 9.00). The solid line is calculated from eq 6, using $\Delta[\theta]_{278,max} = 45$ deg cm^2 dmol^{-1} and $\Delta\epsilon_{290,max} = -400\ M^{-1}$ cm^{-1}.

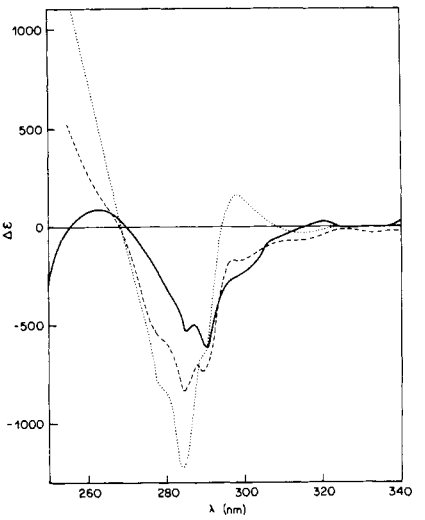

FIGURE 2: Difference spectrum of apothermolysin at 23°C. The enzyme solution (3.76×10^{-5} M) in 0.1 M NaCl-0.01 M TEP (pH 9.00) was at $pCa^{2+} = 4.00$ in the reference and at $pCa^{2+} = 5.43$ in the sample beam. Spectra were recorded directly (—), 20 min (- - -), and 40 min (· · ·) after mixing of stock enzyme and EDTA solutions.

thermolysin concentration $[E]_T$ is given by:

$$[Ca]_T = [Ca^{2+}] + (\bar{\nu}_{Ca^{2+}})[E]_T \quad (3)$$

When EDTA is added in order to vary $[Ca^{2+}]$ at constant $[Ca]_T$ and $[E]_T$, it binds calcium ions stoichiometrically under our experimental conditions ($[EDTA]_T = [CaEDTA]$); we can therefore write:

$$[Ca]_T = [Ca^{2+}] + [EDTA]_T + \bar{\nu}_{Ca^{2+}}[E]_T \quad (4)$$

From (2), (3), and (4), the $[Ca^{2+}]$ can be calculated for every set of experimental conditions used in this study.

For the description of the dependence of equilibrium properties of apothermolysin on $[Ca^{2+}]$ in the range $3.0 \leq pCa^{2+} \leq 5.0$, we write for the fractional occupancy F ($0 \leq F \leq 1$) of the calcium binding double site ($[Ca^{2+}] = c$):

$$F = \frac{K_{1,2}c^2}{(1 + K_{1,2}c^2)} \quad (5)$$

when a given property has the value $Y(F)$ at fractional occupancy F then the difference $\Delta Y = Y(F) - Y(1)$ can be expressed in terms of the maximal difference, $\Delta Y_{max} = Y(0) - Y(1)$, and c, with $K_{1,2} = 2.8 \times 10^9\ M^{-2}$ as:

$$\Delta Y = \frac{\Delta Y_{max}}{(1 + K_{1,2}c^2)} \quad (6)$$

This equation will be used to express observed differences in molar extinction coefficient and molar ellipticity as a function of $[Ca^{2+}]$.

The dependence of the second-order rate constant k_{obsd} for autolytic degradation on $[Ca^{2+}]$ in a model in which only conformers which have *no* bound calcium ions serve as substrates in the reaction (Voordouw and Roche, 1975, Appendix) is given for isotherm (1) by:

$$k_{obsd} = \frac{k}{(1 + K_{1,2}c^2)(1 + K_3c)(1 + K_4c)} \quad (7)$$

Taking the logarithm of both sides, with $K_3c \gg 1$, $K_4c \gg 1$ we obtain:

$$\log k_{obsd} = \log k' - 2 \log c - \log (1 + K_{1,2}c^2) \quad (8)$$

where we have defined: $k' = k(K_3K_4)^{-1}$. Equation 8 will properly describe the experimental results provided that the model underlying the derivation is correct. This can readily be tested by examining the limiting slopes of the lines observed near $F = 0$ and $F = 1$. For $F = 0$ ($K_{1,2}c^2 \ll 1$) we get a slope of -2 and for $F = 1$ a slope of -4.

Results

Sedimentation Coefficient. Nine measurements of s_{25} for apothermolysin in the range $3.0 \leq pCa^{2+} \leq 5.0$ yielded an average $s_{25} = 3.50 \pm 0.10$ S for the uncorrected sedimentation coefficient thus indicating no significant change in molecular shape upon dissociation of the double-site.

Circular Dichroism. Similarly there are no changes beyond experimental error in the peptide CD in the range $4.0 \leq pCa^{2+} \leq 5.3$. From six measurements of the two CD minima at 210 and 220 nm we find $[\theta]_{210} = 11{,}400 \pm 800$ and $[\theta]_{220} = 10{,}800 \pm 140$ deg cm^2 dmol^{-1}. This finding indicates that there are only minor changes in secondary structure associated with the occupancy of the double site. In the aromatic uv region, however, clear calcium-dependent changes are observed. The largest change occurs in the CD minimum at 278 nm; the magnitude of $[\theta]_{278}$ decreases from -198 to -153 deg cm^2 dmol^{-1}. It is proportional to the occupancy of the double site within experimental error for all the measurements and follows eq 6 (see Experimental Section) (Figure 1). The decrease in absolute value of the CD at this wavelength indicates an increase in overall rotational freedom of one or more aromatic side chains upon dissociation of calcium from the double site.

Difference Spectroscopy. Calcium-dependent conformational changes can also clearly be shown by difference spectroscopy. The difference spectrum in the aromatic uv region, characterized by two minima, one at 290 nm and a somewhat smaller one at 283 nm (Figure 2; solid line), is a typical tryptophan perturbation spectrum (Donovan, 1969). Our interpretation of this spectrum is that a tryptophan residue, partially buried in a hydrophobic area, is exposed to the aqueous solvent upon dissociation of the double site. For the transfer of a completely buried tryptophan from the interior of a protein to the aqueous solvent a much larger change, $\Delta\epsilon = 1700\ M^{-1}$ cm^{-1}, would be expected (Donovan, 1969) than observed here. This result is in agreement with the finding by Colman et al. (1972) that all three tryptophan residues, present in thermolysin, are located on the

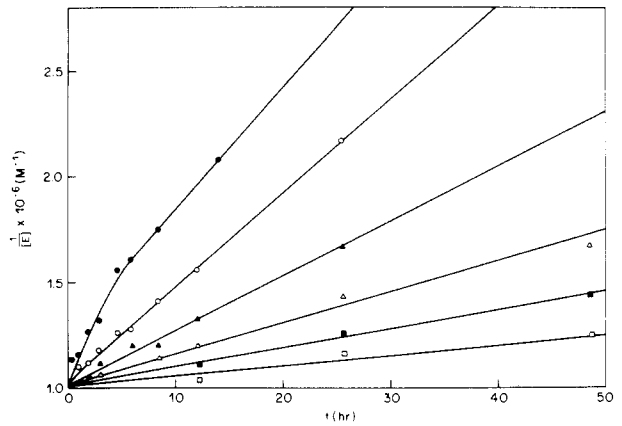

FIGURE 3: Autolytic degradation of thermolysin (1.0×10^{-6} M) at 25°C in 0.1 M NaCl–0.015 M Tris (pH 9.00) and pCa^{2+} as follows: 5.10 (●), 4.89 (○), 4.75 (▲), 4.64 (△), 4.56 (■), 4.48 (□). The reciprocal of the remaining active enzyme concentration is plotted against time. The slopes of the lines are the second-order rate constants k_{obsd}.

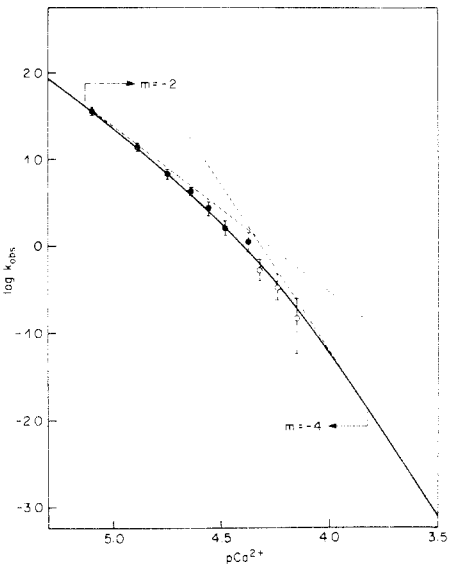

FIGURE 4: Dependence of the second-order rate constant for autolytic degradation k_{obsd}, on $[Ca^{2+}]$. Determined values for k_{obsd} (M^{-1} sec^{-1}) are plotted as log k_{obsd} against pCa^{2+} for 10^{-6} M (●) and 10^{-5} M (○) thermolysin. The solid line is calculated from eq 8 with log $k' = -8.64$ and $K_{1,2} = 3.0 \times 10^8$ M^{-2}.

outside of the molecule.

When the $[Ca^{2+}]$ in the sample cell was smaller than 10^{-5} M a clear time dependence of the spectrum was observed (Figure 2, dotted lines). This is caused by autolysis. Although the residual activity of the apothermolysin samples used does not exceed 1%, the second-order rate constant for autoproteolysis increases more than 1000-fold over this range of $[Ca^{2+}]$ (see below). The increase in $\Delta\epsilon_{283}$ and the appearance of a shoulder at 277 nm strongly indicate an increasing tyrosine contribution to the difference spectrum. This is the expected result for an enzyme containing 28 tyrosines and only 3 tryptophans (Titani et al., 1972) in an autolytic process in which most of the chromophores become exposed to solvent. Although for $[Ca^{2+}] > 10^{-5}$ M the time dependence of the spectra was much smaller than for those in Figure 2, we decided to eliminate this potential source of error by measuring ΔA_{290} directly after mixing the stock enzyme solution with the various EDTA solutions in one compartment cells as described in the Experimental Section. The results obtained, plotted as $\Delta\epsilon_{290}$ vs. pCa^{2+} (Figure 1), agree well with theoretical expectations: the conformational change observed by difference spectroscopy parallels the calcium-binding isotherm (eq 6, see Experimental Section). We may add here that the autolysis effect, as discussed above, could not be demonstrated within 0–20 min after mixing by measurement of either peptide or aromatic CD. In sedimentation velocity experiments the formation of small peptides was apparent only at $pCa^{2+} > 5.5$ and after long centrifugation times (45 min), as an asymmetric boundary spreading and a decreasing sedimentation velocity with time. The difference spectrum seems therefore to be particularly sensitive to small amounts of autolyzed enzyme.

Autolysis Studies. The autolytic degradation of thermolysin is second order in enzyme concentration with respect to time, as seen from the straight lines in Figure 3. The deviations from linearity for the lowest $[Ca^{2+}]$ studied ($pCa^{2+} = 5.10$) are caused by the substantial increase in $[Ca^{2+}]$ as autolysis proceeds. The rate constant has been obtained from the initial slope at this concentration. We see a clear decrease in k_{obsd} with increasing $[Ca^{2+}]$. Figure 4 shows that when the data are plotted as log k_{obsd} vs. pCa^{2+}, they can be fitted to eq 8. A very good fit is obtained using $K_{1,2} = 3.0 \times 10^8$ M^{-2}; the solid line in Figure 4 has been calculated for this value of K and fits the data within experimental error. The increase in error bars in Figure 4 with decreasing pCa^{2+} is due to the fact that the magnitude of k_{obsd} approaches the experimental error in the range $pCa^{2+} \leq 4.3$. Assuming the value of K found in the fitting procedure to be the association constant for the two calcium ions bound to the double site of holothermolysin, we note that this value is about tenfold smaller than the $K_{1,2}$ for apothermolysin. One of the factors contributing to this decrease could be an electrostatic repulsion between the active site zinc ion and the calcium ions in holothermolysin. According to the coordinates given by Matthews et al. (1974) the distances between Ca(1) and the zinc ion and Ca(2) and the zinc ion are 13.7 and 17.2 Å, respectively.

Hydrogen–Tritium Exchange. Exchange-out data for extrapolation to zero exchange-out time are presented in Table I. From this table it is clearly seen that the data do not extrapolate to anywhere near the total number of peptide (315) and primary amide hydrogens (32) of thermolysin (347). Computer fitting of the experimental data to the theoretical exchange curve (Voordouw and Roche, 1975, eq 1) gives a value of 247 for $H(0)$. Hence we conclude that 100 hydrogens do not exchange-in within 24 hr at 25°C in 0.1 M NaCl–0.01 M TEP(pH 9.00) and $[Ca^{2+}] = 10^{-4}$ M. Assuming all of these to be peptide hydrogens, this represents 32% of the entire polypeptide backbone. Raising the pH, the pCa^{2+} or the temperature does not increase the number of hydrogens that are exchanged-in: the 100 very slow hydrogens found are therefore located in a number of extremely tight structural elements, whose peptide hydrogens can make solvent contacts only after a major denaturation reaction has occurred. In view of this one may expect the dependence on $[Ca^{2+}]$ of the exchange rate constant k_{obsd} for these core hydrogens to be given by eq 7. This relationship cannot be tested for this class of hydrogens because they are too slow. The hydrogens that do exchange-in under the above conditions exchange-out independently of the $[Ca^{2+}]$, as shown in Figure 5. The $[Ca^{2+}]$ used in this experiment were always at saturating levels. Difference hy-

Table I: Exchange-out Data for Apothermolysin Obtained from One Column Exchange-out Runs in 0.08 *M* NaOAc (pH 5.00)–0.01 *M* $CaCl_2$ at 2°C after 24 hr of Exchange-in at 25°C in 0.1 *M* NaCl–0.01 *M* TEP (pH 9.0)–10^{-4} *M* $CaCl_2$.

Time (min)	*H*(*t*)	Time (min)	*H*(*t*)
0	247[a]	3.20	215
0.87	226	7.75	186
1.14	223	15.60	164
2.07	220		

[a] Obtained by computer fitting of the data to eq 1 of Voordouw and Roche (1975); see Experimental Section.

drogen exchange techniques as used in the study of thermomycolase (Voordouw and Roche, 1975) cannot be used even with apothermolysin due to the autolysis complications reported above.

The lack of any dependence of hydrogen exchange rates on pCa^{2+} is rather surprising in view of the strong dependence of autolysis on pCa^{2+} under the same conditions. The marked pH dependence of exchange (Figure 5, insert) suggests that an EX_2 mechanism (Hvidt and Nielsen, 1966) is operating. Analyzed in terms of the latter model, our results suggest that the exchanging protons are being exposed to the solvent as a result of conformational isomerizations or fluctuations involving standard free energy changes of 8–10 kcal mol^{-1}. These values seem rather high for local conformational fluctuations and one would normally interpret them in terms of an unfolding of the chain. The latter interpretation would be hard to reconcile with the fact that the exchange is independent of pCa^{2+} and we conclude therefore that these large free energy fluctuations must be occurring in molecules with all four calcium sites occupied.

Thermal Denaturation Studies. The thermal denaturation of thermolysin is first order in enzyme concentration with respect to time. The data are presented in Figure 6 as log k_{obsd} vs. pCa^{2+} and show a rather surprising result: the slope of the linear part of the curve at $pCa^{2+} > 3.5$ is 0.92 ± 0.03 as determined by linear regression analysis. The obvious conclusion is that the thermostability of thermolysin at 80°C and pH 7.0 is due to only one calcium ion. As for thermomycolase, the decrease in absolute value of the slope for $pCa^{2+} < 3.5$ can be explained by assuming a calcium-binding site of lower affinity in the activated intermediate ($K_1^* = 600\ M^{-1}$). The equation derived for this case (Voordouw and Roche, 1975, eq 11) accounts well for the thermolysin thermal denaturation data. The intrinsic rate constant k and the binding constant of the native enzyme conformation K_1 under these conditions cannot be estimated separately from our data, since no decrease in slope is observed in the region $pCa^{2+} > 4.0$ in which the rates of thermal denaturation are too fast to be measured with accuracy using the technique described here.

Discussion

Thermolysin offers an unique opportunity to study in molecular detail the role of bound calcium ions in protein structure. Its amino acid sequence and three-dimensional structure are known (Titani et al., 1972; Matthews et al., 1972a,b; Colman et al., 1972), a detailed crystallographic study of its calcium-binding sites has been made by lanthanide substitution (Matthews and Weaver, 1974) and preliminary atomic coordinates have recently been published (Matthews et al., 1974). Two of the four calcium ions

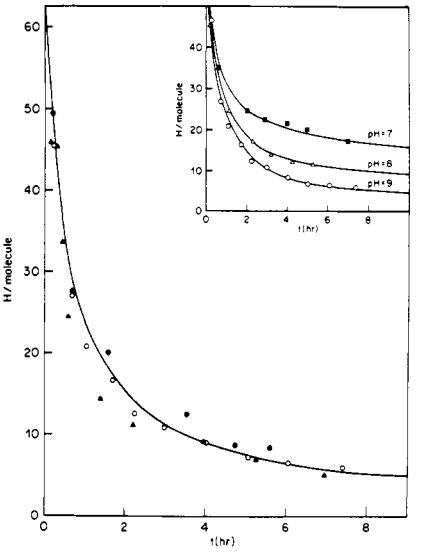

FIGURE 5: Hydrogen–tritium exchange of apothermolysin. The number of hydrogens remaining unexchanged is represented on the ordinate as a function of exchange-out time. The enzyme was exchanged-in for 24 hr in 0.1 *M* NaCl–0.01 *M* TEP (pH 9.0), $[Ca^{2+}] = 10^{-4}$ *M* at 25°C and exchanged-out under identical conditions with $[Ca^{2+}]$: 2.0×10^{-4} *M* (▲), 4.0×10^{-4} *M* (○), and 8.0×10^{-4} *M* (●). Insert: pH dependence of exchange-out at $[Ca^{2+}] = 4.0 \times 10^{-4}$ *M*.

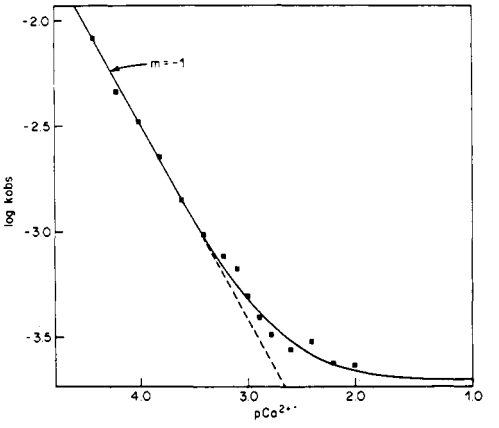

FIGURE 6: Thermal denaturation of thermolysin (3.0×10^{-6} *M*) at 80°C in 0.05 *M* Tris (pH 7.0 *at* 80°C) and various concentrations of NaCl and $CaCl_2$, such that their combined ionic strength is 0.05. The logarithm of the first-order rate constant (sec^{-1}), log k_{obsd}, is plotted against pCa^{2+}. The solid line is calculated for log k_{obsd} = log (k/K_1) – log $[Ca^{2+}]$ + log $(1 + K_1^*[Ca^{2+}])$ with log $(k/K) = -6.51$ and $K_1^* = 600\ M^{-1}$.

bound by thermolysin, Ca(1) and Ca(2), form a double site in which their interatomic distance is only 3.8 Å. The distances between Ca(3) and Ca(4) and the double site are a factor of 3–8 larger. The finding that apothermolysin binds two of its calcium ions with complete positive cooperativity (Voordouw and Roche, 1974) was therefore interpreted as the binding of Ca(1) and Ca(2) to the double site. This conclusion can be further substantiated by the difference spectroscopy data presented in this paper.

The sedimentation velocity and peptide CD (see "Results") indicate that no major conformational change is associated with the binding of calcium ion in the range $3.0 < pCa^{2+} < 6.0$ in which it has been shown earlier (Voordouw and Roche, 1974) that two of the four Ca^{2+} bound by apothermolysin dissociate. The uv difference spectrum and the aromatic CD reveal, on the other hand, a subtle confor-

mational change which parallels the binding isotherm according to eq 6 (Figure 1). The latter conformational change is therefore most probably limited to the direct environment of the two calcium ions which dissociate under these conditions. The aromatic CD is consistent with and the uv difference spectra clearly demonstrate the perturbation of a single tryptophan by the two cooperatively binding calcium ions. Since there are only three tryptophan residues in thermolysin one of which, Trp-186, is flanked by Asp-185 and Glu-187, two of the chelating amino acids of the double site, this observation lends further support to our earlier assignment of the two cooperatively binding calcium ions to the double site (Voordouw and Roche, 1974).

In a previous paper (Voordouw and Roche, 1975) we have elaborated a calcium-binding model which quantitatively accounts for the calcium dependence of the rate of autolytic, thermal, and urea denaturation of the thermostable protease thermomycolase. In contrast to thermomycolase, which binds only one calcium ion, thermolysin presents a rather more challenging case for the model since it binds four calcium ions. While there is some simplification of the problem as a result of the cooperative binding of two of the four calcium ions (see introduction), the task of correlating the individual calcium ion in the thermolysin structure with the observed autolytic and thermal denaturation behavior as expressed in terms of our binding model still remains. Although it is tempting to speculate on the role played by each individual calcium ion in thermolysin in terms of its position in the structure, as Matthews and coworkers have done (Matthews et al., 1974), this could be misleading without experimental data on the calcium-binding isotherm and the calcium dependence of the autolysis and thermal denaturation reactions.

The dependence of the second-order rate constant for the autolysis of thermolysin on pCa^{2+} can be accounted for quantitatively (Figure 4) by the calcium-binding model (eq 8). The latter model requires that only those molecules *devoid* of calcium act as substrate in the reaction. All *four* calcium ions contribute therefore to the stabilization of thermolysin against autolysis. Because of the distribution of the four calcium ions in the thermolysin molecule (Matthews et al., 1974; Table IX) we can conclude that the autolysis substrates which, as required by the binding model, are devoid of calcium must also be highly unfolded. This can be demonstrated as follows. Firstly, we note that Ca(3) and Ca(4) bind with higher affinity than do Ca(1) and Ca(2) (Voordouw and Roche, 1974). Therefore, the two remaining calcium ions which stabilize the molecule after the double site has dissociated (Figure 4; $m = -2$ for $pCa^{2+} > 5.0$) must be Ca(3) and Ca(4). Since these are bound in the region of Asp-57 and Asp-200, respectively, it is clear that before both are lost, producing an autolysis substrate molecule, the chain must unfold extensively. Hence the function of all four calcium ions in the protection of thermolysin against autolysis is to limit the structure to nonautolyzable conformations which, nonetheless, have considerable conformational flexibility as revealed by the hydrogen–tritium exchange data (see Results).

In contrast to the autolysis reaction, it is found that the rate constant for the first-order thermal denaturation reaction at 80°C and pH 7.0 is dependent on the binding of only *one* calcium to the native conformation and to the critically activated intermediate (Figure 6; $m = -1$). Since it is very unlikely that the relative affinities of the sites [Ca(1)–Ca(2) $\ll$ Ca(3); Ca(4)] (Voordouw and Roche, 1974) will be very sensitive to temperature it can be reasonably inferred that the single calcium ion involved in the rate-determining step for thermal denaturation under these conditions is either Ca(3) or Ca(4).

We can also discuss the role of calcium in mechanistic terms. As in our previous discussion of the stabilizing role of the single calcium ion of thermomycolase (Voordouw and Roche, 1975) we consider the mechanism of the unfolding of globular proteins. Because it is highly cooperative protein unfolding can be viewed as an overall two-state process. The binding of calcium to the native enzyme can thus be regarded as a simple mass-action stabilization of the native fold. However, it is now clear (Anfinsen, 1973; Scheraga, 1974) that the unfolding process is a complex, ordered sequence of events and that most probably there are only a limited number of pathways if not an unique one for a given globular protein. We postulate, within the latter rationale, that calcium binds at strategic positions in the unfolding pathway. By lowering the free energy of its binding site it "blocks" unfolding by raising the local activation energy for unfolding. Since highly unfolded molecules act as autolysis substrates (see above) it is incorrect to suggest that a given calcium bound at its site in the molecule protects that particular region of the molecule. Rather, one must consider the role of a given calcium ion in terms of its position on the unfolding pathway. Because they are bound with the highest affinity, it seems reasonable to infer that Ca(3) and Ca(4) are situated at critical points on the unfolding pathway of thermolysin. If, as seems to be the case for staphylococcal nuclease (Jardetzky, 1971), the chain unfolds from the amino terminal then the Ca(3) binding site provides the key to the calcium lock in thermolysin and Ca(3) could be identified as the single calcium ion determining the rate of unfolding (Figure 6) in thermal denaturation at 80°C. There is at present no general a priori reason to suggest at which end of the chain unfolding is initiated. The latter suggestion, therefore, must remain speculative.

Added in Proof

The lack of any dependence of hydrogen-exchange rates on pCa^{2+} (Figure 5) is probably better explained in terms of the limited solvent accessibility model put forward recently by Ellis et al. (1975), which leads to the suggestion that the accessibility of solvent to the exchangeable hydrogens is independent of pCa^{2+} even when the calcium binding sites are saturated. The model also accounts for the similar number of core hydrogens we have found for thermomycolase and thermolysin, 104 and 100, respectively, in terms of the similarity in the size of the two molecules (molecular weight 32000 and 34500, respectively).

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Crystallization and Partial Characterization of Glutamate Dehydrogenase from Ox Liver Nuclei†

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ABSTRACT: Glutamate dehydrogenase has been obtained in crystalline form from purified ox liver nuclear fractions. The enzyme appeared homogeneous, as judged by several electrophoretic techniques at two pH values. A comparative study with the widely known ox liver mitochondrial glutamate dehydrogenase revealed several common features, such as the allosteric effect of the nucleotides ADP and GTP, the activation at high concentrations of the cofactor NAD^+, and the existence of a concentration-dependent reversible monomer–polymer(s) equilibrium. However, the two enzymes differed in many other respects. Inorganic phosphate activated nuclear glutamate dehydrogenase to a much greater extent than the mitochondrial enzyme; the substrate NH_4^+ showed cooperative homotropic interactions only with nuclear glutamate dehydrogenase; kinetic differences were detected with most of the reaction substrates, as well as different rates of oxidative deamination of other L-amino acids; the nuclear enzyme had a higher anodic mobility and a different chromatographic behavior on anionic exchangers. The latter evidence indicates that the glutamate dehydrogenase activity in liver is associated with two proteins which are structurally different, thus confirming the results of a separate immunological study. Preliminary evidence suggests that the enzyme in nuclei is attached to the nuclear envelope, probably the inner membrane, from which it can be solubilized by the addition of salts.

In a previous report (di Prisco and Garofano, 1974) we have described the partial purification of glutamate dehydrogenase associated with purified ox liver nuclear fractions. Some of its properties, related to the response of activity to pH variations, in the absence and presence of some allosteric modifiers, have also been described. The enzyme has been obtained now in homogeneous and crystalline form. We wish to report its final purification as well as some of its characteristics.

The results of this study show that while some aspects of the mechanism of allosteric activation and inhibition, as well as of the molecular organization of the nuclear enzyme, resemble those of mitochondrial GDH,[1] some others notably differentiate the two enzymes.

Moreover, differences between the two enzymes were found when examining their electrophoretic and chromatographic behavior, suggesting a higher net negative charge of nuclear GDH. The two proteins appear, therefore, to have some structural differences, as indicated also by immunological studies (Casola et al., 1974; di Prisco and Casola, 1975).

Experimental Section

Preparation of Nuclear Fractions. Ox liver nuclei were isolated essentially according to the procedure of Pogo et al.

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[1] Abbreviation used is: GDH, glutamate dehydrogenase.