The Role of Bound Calcium Ions in Thermostable, Proteolytic Enzymes. I. Studies on Thermomycolase, the Thermostable Protease from the Fungus *Malbranchea pulchella*†

Gerrit Voordouw and Rodney S. Roche*

ABSTRACT: Thermomycolase, the thermostable, extracellular, serine protease of the fungus *Malbranchea pulchella* (G. Voordouw, G. M. Gaucher, and R. S. Roche (1974), *Can. J. Biochem. 52,* 981-990), binds one calcium ion with an apparent binding constant of $5 \times 10^5\ M^{-1}$ at 25°C, pH 7.50, and ionic strength 0.1. There is very little change in the overall conformation of thermomycolase upon binding of this calcium ion: no change can be detected, beyond experimental error, in the sedimentation coefficient or the aromatic and peptide circular dichroism of the enzyme. However, binding of calcium changes the absorption spectrum, the ultraviolet difference spectrum being characterized by a strong band at 237 nm and smaller bands at 280 and 295 nm. The difference molar extinction coefficient at 237 nm parallels the calcium-binding isotherm. The small changes in equilibrium properties are contrasted by large calcium-dependent changes in the rates of autolytic degradation and thermal and urea denaturation. The dependence of the second-order rate constant for autolytic degradation on the free calcium ion concentration can be quantitatively accounted for by a model in which *only* conformers with an *unoccupied* calcium binding site serve as substrates in the reaction. The calcium dependence of the first-order rate constant for thermal denaturation at 70°C and pH 7.0 can also be accounted for quantitatively by a model in which the critically activated intermediate has a smaller calcium-binding constant than the native form of the enzyme under these conditions. The same model also accounts for the denaturation in 8 *M* urea at 50°C, pH 7.0. Rates of hydrogen–tritium exchange are shown to decrease when the calcium ion is bound. Irrespective of the occupancy of the calcium-binding site 33% of the backbone peptide hydrogens of thermomycolase do not exchange within 24 hr at 25°C, pH 8.0, and ionic strength 0.1.

Bound calcium ions serve as an important stabilizing influence in the structure of a number of proteolytic enzymes. The well-known proteolytic enzyme trypsin, for example, binds one calcium ion (Epstein et al., 1974) which protects the enzyme against autolytic degradation (Gabel and Kasche, 1973) and stabilizes it against thermal or chemical denaturation (Delaage and Lazdunski, 1967). The latter phenomenon is well known for a variety of thermostable proteins and enzymes (see, for example, Matthews et al., 1974, and the references cited therein). Although this stabilization of proteins is well documented qualitatively, few quantitative relationships have been presented and tested by the appropriate experiments. This situation prevails in spite of the fact that a sound theoretical basis has been laid for an understanding of the effect of ligand binding, on both rate and equilibrium protein denaturation phenomena (Tanford, 1968, 1970). In an attempt to remedy this situation we have developed, therefore, within the latter conceptual framework, a calcium-binding model (see Appendix) which quantitatively accounts for the calcium ion dependence of these phenomena. The equations derived are tested on the data obtained with the thermostable protease thermomycolase, the thermal denaturation of which has previously been shown to be dependent on the free calcium ion concentration (Ong, 1973; Voordouw et al., 1974). To this end the calcium-binding isotherm has been determined under a given set of experimental conditions. The dependence of a number of equilibrium properties and rates of relevant reactions on the free calcium ion concentration were then investigated for the same set of conditions. The calcium dependence of the thermal denaturation of thermomycolase, which necessarily has to be studied at a much higher temperature, has been quantitatively interpreted by relaxing the absolute values of the binding parameters while retaining the binding model. The latter approach also allows a quantitative description of the denaturation of the enzyme in 8 *M* urea at 50°C, pH 7.0, as a function of calcium ion concentration.

Experimental Section

Materials. *N*-Cbz-GlyONph[1] was obtained from Sigma Chem. Co., Dip-F from Calbiochem, and $Na_2EDTA{\cdot}2H_2O$ from Fisher. NTA was purchased from Eastman as the free acid and dried to constant weight in a vacuum oven at 105°C before use. Tritiated water (THO) at a specific activity of 100 mCi/ml was obtained from New England Nuclear and Sephadex G-25 Fine from Pharmacia Fine Chemicals. All other chemicals used were reagent grade.

† From the Biopolymer Research Group, Department of Chemistry, The University of Calgary, Calgary, T2N 1N4, Alberta, Canada. *Received May 5, 1975.* Supported by the National Research Council of Canada (Grant A3608). G.V. acknowledges the award of a National Research Council postgraduate scholarship and an Izaak Walton Killam memorial scholarship.

[1] Abbreviations used are: Dip, diisopropylphosphoryl; Dip-F, diisopropyl fluorophosphate; *N*-Cbz-GlyONph, *N*-carbobenzoxyglycine *p*-nitrophenyl ester; NTA, nitrilotriacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $[Ca^{2+}]$ and pCa^{2+}, the free calcium ion concentration and its negative logarithm; $[Ca]_T$, the total calcium ion concentration. CaL, CaHL, and H_nL are respectively the calcium complexes and protonated forms of the calcium chelators NTA and EDTA.

Enzyme Preparation. Thermomycolase was purified as described previously (Ong, 1973; Voordouw et al., 1974). The purified enzyme was homogeneous in gel filtration on Sephadex G-100 and sodium dodecyl sulfate gel electrophoresis. Both the active enzyme and the inactive Dip derivative (0.1–0.01% residual activity) were stored as ammonium sulfate precipitates at −20°C.

Calcium-Binding Studies. The amount of bound calcium ions was determined by the gel filtration technique of Hummel and Dreyer (1962), as described previously (Voordouw and Roche, 1974). A K16/70 column with flow adapters and thermostat jacket, packed with Sephadex G-25 Fine, was equilibrated with 0.01 *M* Tris (pH 7.50 ± 0.05) containing a given concentration of $CaCl_2$ in the range $4.0 \leq pCa^{2+} \leq 5.0$ and NaCl to give an ionic strength of 0.1 at 25.0°C. Buffers with $pCa^{2+} > 5.0$ were prepared by adding the required amount of NTA at constant $[Ca]_T = 5.0 \times 10^{-5}$ *M*. Samples of Dip-thermomycolase (1–2 ml containing 8–12 mg of protein) were applied to the gel after previous dialysis for 12 hr against 0.1 *M* NaCl, 0.01 *M* Tris (pH 7.5), and 10^{-3} *M* $CaCl_2$. This dialysis step was necessary in order to remove the excess of sulfate ions present in the sample. When a freshly dissolved ammonium sulfate precipitate was directly chromatographed, anomalously high calcium ion concentrations were found to elute at the void volume of the column. The inactive Dip derivative was used in these studies and in all others, where applicable, in order to slow down the calcium-dependent autolysis of the enzyme. Enzyme and calcium ion concentrations were determined by uv spectroscopy and atomic absorption spectroscopy, respectively (Voordouw and Roche, 1974). A molar extinction coefficient at 280 nm of $\epsilon = 43{,}900 \pm 800$ M^{-1} cm^{-1} was used (Voordouw et al., 1974).

Sedimentation Measurements. The sedimentation coefficient of Dip-thermomycolase was measured as a function of $[Ca^{2+}]$ with a Beckman Model E analytical ultracentrifuge, equipped with a RTIC temperature control unit and electronic speed control, using schlieren optics. The enzyme (1.34×10^{-4} *M*) was centrifuged at 60,000 rpm and 25°C in 0.1 *M* NaCl–0.01 *M* Tris (pH 7.50), $[Ca]_T = 3.15 \times 10^{-4}$ *M*, and a varying concentration of EDTA to produce $4.0 \leq pCa^{2+} \leq 8.0$. The schlieren patterns were recorded on Kodak Type 11-6 glass plates and analyzed with a Nikon Model 6C microcomparator. Sedimentation coefficients were calculated according to Schachman (1957).

Circular Dichroism (CD). CD measurements were made with a Durrum Jasco Model ORD/UV-5 recording spectropolarimeter, with the SS-20 modification (Sproul Co.) at 25.0°C (Voordouw et al., 1974). The Dip-thermomycolase solutions (3.5×10^{-5} *M*) had a $[Ca]_T = 1.35 \times 10^{-4}$ *M* and were otherwise as for the sedimentation measurements. A thermostated 10.00-mm cell was used in the aromatic uv region and a 0.1-mm cell in the peptide uv region (Opticell Co.).

Difference Spectroscopy. Difference spectra were obtained with a Cary Model 15 spectrophotometer at room temperature (23°C), using the experimental procedures as described by Donovan (1969) for this instrument. Dip-thermomycolase solutions (3.5×10^{-5} *M*) were at $[Ca^{2+}] = 1.00 \times 10^{-4}$ *M* in the reference beam, while this quantity was varied in the sample beam by changing the concentration of EDTA. The variable EDTA contribution was eliminated from the spectra by using 10 mm × 10 mm cylindrical tandem cells (Pyrocell, no. 6016) (Herskovits, 1967).

Autolysis and Thermal Denaturation Studies. Calcium-dependent autolysis was studied at 25.0°C at a concentration of thermomycolase of 1.5×10^{-6} *M* in 0.01 *M* Tris (pH 7.50) with NaCl added to give an ionic strength 0.1. The $[Ca^{2+}]$ was varied at constant $[Ca]_T = 5.0 \times 10^{-5}$ *M* by addition of NTA ($5.0 \leq pCa^{2+} \leq 6.5$) or EDTA ($pCa^{2+} > 6.5$). In the range $pCa^{2+} < 5.0$ the $[Ca^{2+}]$ was varied by direct addition of stock $CaCl_2$. The concentration of active enzyme remaining, [E], was determined by assaying periodically with *N*-Cbz-GlyONph as substrate (Voordouw et al., 1974) over a period of time up to 75 hr. The second-order rate constant for autoproteolysis (k_{obsd}) was obtained as the slope of linear 1/[E] vs. time plots.

Thermal denaturation studies were performed at 70°C. The reaction was initiated by adding 1 ml of a stock solution of active thermomycolase (9.2×10^{-5} *M*) to 29 ml of 0.05 *M* Tris (pH 7.0) adjusted at 70°C (pH 8.0 at 25°C) and a variable concentration of NaCl and $CaCl_2$ such that their combined ionic strength was 0.05. The latter solution was first equilibrated at (70.0 ± 0.2°C) in a Colora circulating water bath. Measurements were made in the range $2.0 \leq pCa^{2+} \leq 5.0$. The decrease in [E] was monitored by withdrawing 1-ml samples, stopping the reaction by dilution with 1 ml of 0.05 *M* Tris (pH 8.0), 0.05 *M* NaCl, and 1.0×10^{-4} *M* $CaCl_2$ at 25°C and assaying 0.2 ml of this mixture. First-order rate constants for thermal denaturation (k_{obsd}) were obtained from linear plots of ln ($[E_0]/[E]$) vs. time, where $[E_0]$ is the enzyme concentration at zero time.

Similarly the calcium dependence of the denaturation in 8 *M* urea at 50°C and pH 7.0 was studied by diluting 1 ml of the stock enzyme solution into 29 ml of 8.3 *M* urea–0.05 *M* Tris, adjusted to pH 7.0 at 50°C, containing variable NaCl and $CaCl_2$ concentrations to give a final ionic strength of 0.05.

Hydrogen–Tritium Exchange Studies. Difference hydrogen–tritium exchange was carried out according to Englander and Rolfe (1973). To two 1.5-ml portions, (a) and (b), of a solution of Dip-thermomycolase ($\sim 6 \times 10^{-4}$ *M*) in 0.1 *M* NaCl, 0.01 *M* Tris (pH 8.0), and 1.0×10^{-4} *M* $CaCl_2$ (dialysis buffer) was added respectively 100 μl of tritiated water, THO, at 100 mCi/ml and 0.5 ml of dialysis buffer to (a) and 100 μl of THO and 0.5 ml of dialysis buffer containing 5×10^{-3} *M* EDTA to (b). Exchange-in was allowed to proceed under these two different conditions, which we will refer to as (a), "slow" and (b), "fast", respectively, for 24 hr at 25.0°C after which exchange-in was stopped by adding 5 mg of $CaCl_2$ and 15 μl of 2 *M* acetic acid to (a) and (b), bringing the pH down to approximately pH 5. Exchange-out was then initiated by chromatographing 1 ml of either (a) or (b), in about 3 min over a column (1.5 × 12 cm) packed with Sephadex G-25 Fine and equilibrated with dialysis buffer. A fraction of 4 ml was collected at the void volume of the column, to which either 1 ml of dialysis buffer (c) or 1 ml of dialysis buffer containing 5×10^{-3} *M* EDTA was added (d). We will refer to the exchange-out conditions (c) and (d) as "slow" and "fast", respectively. The 5 ml of stock solution obtained were incubated in a Colora circulating water bath at 25.00 ± 0.07°C. Samples of 0.5 ml were withdrawn periodically and chromatographed over a second column (1.5 × 6 cm) in order to measure the number of unexchanged hydrogens remaining (Englander and Englander, 1972). The fractions collected (0.2 ml) were diluted with 0.3 ml of dialysis buffer and analyzed for protein by measurement of OD_{280}. From the peak fractions, 0.2 ml was added to 10 ml of scintillation fluid (Bray, 1960) and counted with a Nuclear Chicago Mark II

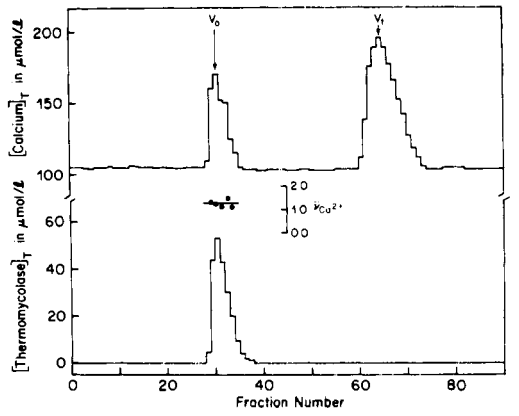

FIGURE 1: Elution profile of Dip-thermomycolase on a column (1.6 × 50 cm) packed with Sephadex G-25 Fine and equilibrated at 25°C with 1.05×10^{-4} *M* $CaCl_2$, 0.01 *M* Tris (pH 7.50), and NaCl to ionic strength 0.1. The sample (1 ml ca. 10 mg/ml of thermomycolase and 10^{-3} *M* $CaCl_2$) was eluted at a flow rate of 0.3 ml/min, fractions of 1.5 ml being collected. The total calcium and Dip-thermomycolase concentrations, represented on the ordinate, were determined as described in the Experimental Section.

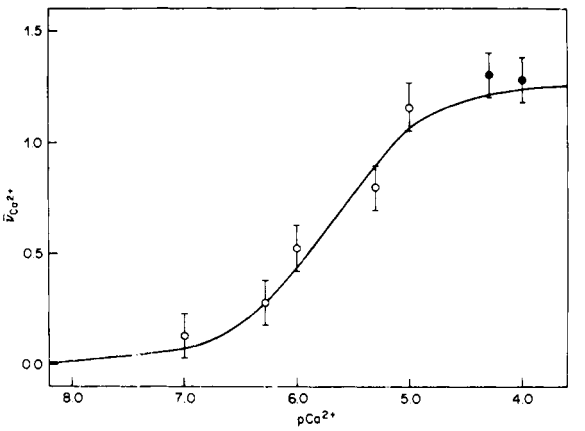

FIGURE 2: Calcium-binding isotherm of Dip-thermomycolase at 25°C in 0.01 *M* Tris (pH 7.50) and NaCl to ionic strength 0.1. The $[Ca^{2+}]$ was varied either by direct addition of $CaCl_2$ to the buffer (●) or by varying the concentration of NTA at constant $[Ca]_T = 5.0 \times 10^{-5}$ *M* (○). The solid line is calculated for $\bar{\nu}_{Ca^{2+}} = nK_{ECa}[Ca^{2+}]/(1 + K_{ECa} \cdot [Ca^{2+}])$, with $n = 1.20$ and $K_{ECa} = 5 \times 10^5\ M^{-1}$.

liquid scintillation counter.

The number of hydrogens per molecule exchanged-in under conditions (b) was determined by extrapolation of one-column exchange-out data to zero time at 2°C in 0.08 *M* sodium acetate (pH 4.50) and 0.01 *M* $CaCl_2$. Under these conditions exchange-out is very slow, allowing a reliable extrapolation to zero time; performed by computer fitting of the data to

$$H(t) = H_p e^{-k_p t} + H_a e^{-k_a t} + C \qquad (1)$$

where $H(t)$ is the number of hydrogens not yet exchanged-out at time t, H_p and H_a the numbers of *free* (non-hydrogen bonded) peptide and primary amide hydrogens exchanged in at zero time, and C the number of hydrogen-bonded peptide and primary amide hydrogens exchanged-in at zero time. For the very early exchange-out part of the hydrogen exchange curve (0–10 min), C can be regarded as a constant under the experimental conditions described above, whereas k_p and k_a are known from model compound studies: $k_p = 0.087\ min^{-1}$ ($t_{1/2} = 7.95$ min) and $k_a = 0.0248\ min^{-1}$ ($t_{1/2} = 28.0$ min),[2] as given by Englander and Staley (1969) and Englander and Poulsen (1969). The number of hydrogens that remain unexchanged during exchange-in (H_{unex}) can then be calculated from

$$H_{unex} = H_{total} - H(0)$$

where H_{total} is the total number of peptide and primary amide hydrogens in the thermomycolase molecule and $H(0)$ the extrapolated value of $H(t)$.

Variation of the Free Calcium Ion Concentration. The $[Ca^{2+}]$ was varied by addition of variable amounts of NTA or EDTA at constant $[Ca]_T$. This method was used for $pCa^{2+} > 5.0$, since direct preparation of such low $[Ca^{2+}]$ leads to serious errors. Values for $[Ca^{2+}]$ were then calculated from the known $[Ca]_T$ and the known total NTA or EDTA concentration. The following values for the logarithms of the stability constants of the relevant complexes at pH 7.50, 25°C, and ionic strength 0.1 (Sillen and Martell, 1964) were used: for NTA, CaL (6.33) and HL (9.65), where we have neglected complexes H_2L and H_3L, and for EDTA, CaL (10.57), HL (10.27), H_2L (6.16) with neglect of complexes H_3L, H_4L, and CaHL. A recent book by Perrin and Dempsey (1974) may be consulted for the calculations involved.

Miscellaneous. Stock solutions of thermomycolase in a given buffer were prepared by dialysis for 12 hr against repeated changes of the dialysis buffer. The dialyzed stock solution was then filtered through a 0.22-μ Millipore filter, its absorbance at 280 nm was measured and the solution was diluted gravimetrically with filtered dialysis buffer to the desired concentration. Most of the absorbance measurements were made with a Beckman DB-G grating spectrophotometer. All pH measurements were performed to within 0.05 pH unit of the reported values using a Fisher Accumet Model 320 or a Radiometer Model 26 pH meter. Reported errors and error bars always indicate standard deviations of the mean value.

Results

Calcium-Binding Studies. When Dip-thermomycolase is chromatographed on a Sephadex G-25 column under the conditions indicated in the legend of Figure 1, it is clearly seen that this enzyme binds calcium ions. The elution of the protein peak at the void volume (v_0) of the column is completely paralleled by a changing $[Ca]_T$ of the fractions. When the number of calcium ions bound per mole of enzyme is calculated for each fraction (Figure 1) we obtain an average value $\bar{\nu}_{Ca^{2+}} = (1.20 \pm 0.10)$ under these conditions. When the $[Ca^{2+}]$ of the buffer is varied the binding isotherm shown in Figure 2 is obtained. A Scatchard plot analysis (Scatchard, 1949) of the binding data indicates the class size n to be 1.20 ± 0.12 Ca^{2+} ions bound per mole of enzyme with a binding constant $K_{ECa} = (5.0 \pm 3.0) \times 10^5\ M^{-1}$. The solid line in Figure 2 has been calculated using these values for n and K_{ECa} and fits the data within experimental error. Although the class size n deviates systematically from the nearest integer value, $n = 1$, we will interpret these data as meaning that maximally one calcium ion is bound with high affinity and that the value of 1.20 may reflect some low affinity binding at high calcium concentrations.

[2] This value is actually reported for 0°C and pH 4.7 (Englander and Staley, 1969). Errors arising from using this value for our conditions are likely to be small, since the pH dependence of k_a is at a minimum in this pH range and the contribution of the primary amide term to the time dependence of $H(t)$ is very small in the time interval studied.

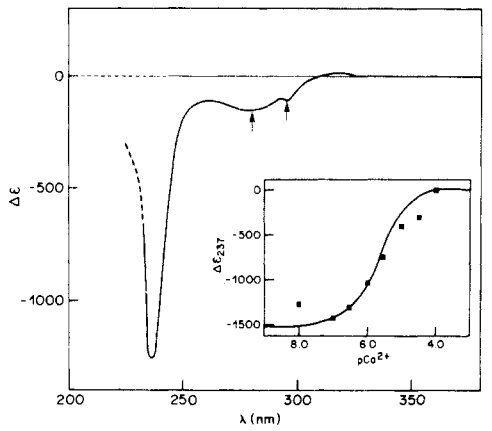

FIGURE 3: Difference spectrum of Dip-thermomycolase. The enzyme (3.5×10^{-5} M) in 0.1 M NaCl, and 0.01 M Tris (pH 7.50) was at $[Ca^{2+}] = 10^{-4}$ M in the reference beam and at 10^{-8} M in the sample beam by addition of EDTA (see Experimental Section). Below 233 nm the optical density of the sample was too high for an accurate recording of the spectrum (- - -). Insert: Values for $\Delta\epsilon_{237}$ (M^{-1} cm^{-1}) plotted against pCa^{2+}. The solid line is calculated for $\Delta\epsilon = \Delta\epsilon_{max}/(1 + K_{ECa}[Ca^{2+}])$, with $\Delta\epsilon_{max} = -1500$ M^{-1} cm^{-1} and $K_{ECa} = 5 \times 10^5$ M^{-1}.

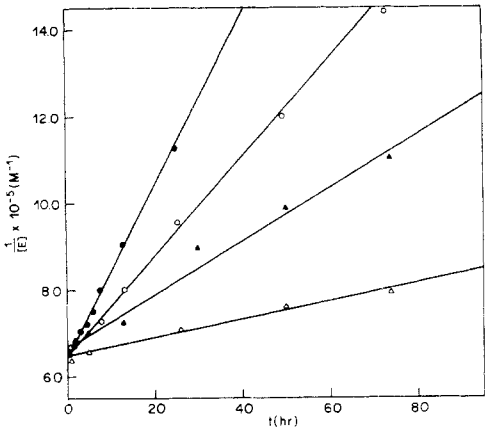

FIGURE 4: Autolytic degradation of thermomycolase (1.5×10^{-6} M) at 25°C, in 0.01 M Tris (pH 7.50), NaCl to ionic strength 0.1, and pCa^{2+} as follows: 7.00 (●), 5.73 (○), 5.05 (▲), and 4.30 (△). The reciprocal of the remaining active enzyme concentration is plotted against time. The slopes of the lines are the second-order rate constants, k_{obsd}.

Sedimentation Coefficient. Five measurements of the sedimentation coefficient in the range $4 \leq pCa^{2+} \leq 8$ at intervals of 1 pCa^{2+} unit do not show any variation beyond experimental error. An average value $s_{25} = 3.18 \pm 0.05$ S is obtained for the uncorrected sedimentation coefficient. The overall shape and dimensions of the enzyme forms E and ECa are hence very similar.

Circular Dichroism. Six measurements in the range $4 \leq pCa^{2+} \leq 8$ at 25°C gave the following average values for the molar ellipticity at the observed extrema in the CD spectrum: $[\theta]_{210} = -7700 \pm 500$; $[\theta]_{222} = -11{,}500 \pm 700$; $[\theta]_{262} = 10.5 \pm 0.5$; $[\theta]_{274} = -10.3 \pm 0.6$; $[\theta]_{290} = 42.8 \pm 0.5$ deg cm^2 dmol^{-1}. There are, therefore, no changes detectable by CD in either the secondary structure or asymmetry of the aromatic side chain chromophores upon binding of the calcium ion.

Difference Spectroscopy. That there are calcium-dependent changes in the environment of at least some chromophores can clearly be shown by difference spectroscopy.

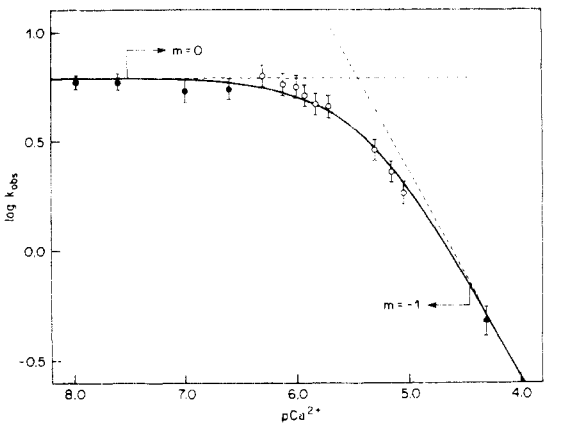

FIGURE 5: Dependence of the second-order rate constant for autolytic degradation, k_{obsd}, on $[Ca^{2+}]$. Determined values for k_{obsd} (M^{-1} sec^{-1}) are plotted as log k_{obsd} against pCa^{2+}. The different $[Ca^{2+}]$ were prepared either by direct addition of $CaCl_2$ (▲), or by addition of NTA (○) or EDTA (●) at constant $[Ca]_T = 5.0 \times 10^{-5}$ M. The solid line is calculated from eq 9 with $k = 6.2$ M^{-1} sec^{-1}, and $K_{ECa} = 2.2 \times 10^5$ M^{-1} (see Appendix).

From Figure 3, under the conditions described in the legend, we see that a few small peaks appear in the aromatic uv region (295 nm, $\Delta\epsilon/\epsilon = 0.6\%$; 280 nm, $\Delta\epsilon/\epsilon = 0.4\%$), while a much larger change is observed in the peptide uv region (237 nm, $\Delta\epsilon/\epsilon = 3.2\%$). The change at the latter wavelength parallels the calcium-binding isotherm (Figure 3, insert), showing that at least small conformational differences, probably confined to the direct environment of the calcium-binding site, exist between the enzyme forms E and ECa. From the observed ratio, $\Delta\epsilon_{237}/\Delta\epsilon_{295} = 10$, we conclude that the change we observe at 237 nm is largely attributable to perturbation of tryptophan (Donovan, 1969).

Autolysis Studies. When the reciprocal of the concentration of the remaining active thermomycolase is plotted against time straight lines are observed as expected for a second-order reaction (Figure 4). Deviations from linearity do not occur until more than 50% of the active enzyme has autolyzed. The second-order rate constant (k_{obsd}) increases with decreasing $[Ca^{2+}]$. When the logarithm of k_{obsd} is plotted against pCa^{2+} (Figure 5) the data obtained appear to fit eq 9 (see Appendix). The value $K_{ECa} = 2.2 \times 10^5$ M^{-1} used in the fitting lies within experimental error of the one determined by gel filtration. The use of either NTA or EDTA for the regulation of $[Ca^{2+}]$ does not seem to have any other effects on the enzyme since the data for these two chelators agree well with the theoretical relationship. This satisfactory fit indicates that the model underlying the derivation is correct: only conformers from the population E, with an *unoccupied* calcium-binding site, serve as substrates in the autolytic degradation reaction (see Appendix). Hence at $pCa^{2+} > 7$, where the calcium-binding site is known to be unoccupied (Figure 2), no change of k_{obsd} with $[Ca^{2+}]$ is found, whereas at $pCa^{2+} < 5$, where the site is practically saturated, k_{obsd} is inversely proportional to $[Ca^{2+}]$.

Thermal and Urea Denaturation Studies. The thermal denaturation reaction of thermomycolase is first order under the conditions used (see Experimental Section). As with the autolysis process, the rate constant clearly increases with decreasing $[Ca^{2+}]$. The data, when plotted as log k_{obsd} vs. pCa^{2+}, cannot be fitted by a model in which we only take into account the binding of a calcium ion to the

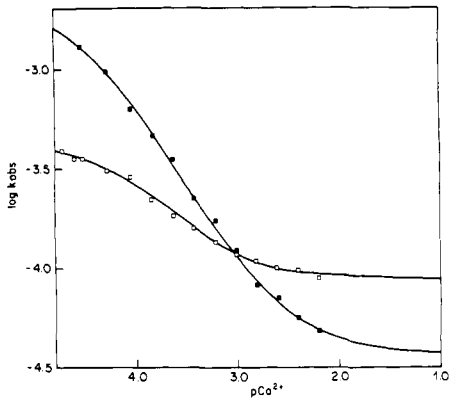

FIGURE 6: Dependence of the first-order rate constant, k_{obsd} in sec^{-1}, for thermal denaturation of thermomycolase (3.1×10^{-6} M) at 70°C (■) in 0.05 M Tris (pH 7.0) at 70° and various concentrations of NaCl and $CaCl_2$ such that their combined ionic strength is 0.05. The solid line is calculated from eq 11, with $k = 2.39 \times 10^{-3}$ sec^{-1}; $K_1 = 3.0 \times 10^4$ M^{-1}; $K_1^* = 4.6 \times 10^2$ M^{-1}. For denaturation in 8 M urea at 50°, pH 7.0 (□), the parameters are $k = 4.37 \times 10^{-4}$ sec^{-1}; $K_1 = 10^4$ M^{-1}, $K_1^* = 2 \times 10^3$ M^{-1}.

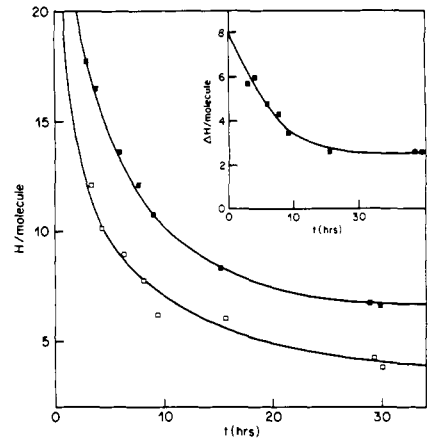

FIGURE 7: Difference hydrogen-tritium exchange of Dip-thermomycolase. The number of H atoms/molecule remaining unexchanged after exchange-in for 24 hr is plotted vs. the exchange-out time. The enzyme was exchanged-in "fast" and exchanged out "slow" (■) or exchanged-in "slow" and out "slow" (□), as described in the Experimental Section. Insert: Difference hydrogen exchange curve.

native enzyme (Figure 6). In such a model a constant slope $m = -1$ would be expected at high calcium ion concentration. The decrease in the absolute value of the slope with decreasing pCa^{2+}, observed in the region $pCa^{2+} < 3$, can be explained by assuming that the activated intermediate also binds a calcium ion with lower affinity (eq 11, Appendix). Using this as the model, the solid line in Figure 6 was obtained after systematic variation of the constants to obtain an optimal fit. The optimal fitting of the experimental data to the latter model (Figure 6) yields the following values for the constants of eq 11 (Appendix): the intrinsic first-order rate constant for the thermal denaturation process, $k = 2.39 \times 10^{-3}$ sec^{-1}; the calcium-binding constant of the native enzyme at 70°C, pH 7.0, $K_1 = 3.0 \times 10^4$ M^{-1}, and the calcium-binding constant for the activated intermediate (X), $K_1^* = 4.6 \times 10^2$ M^{-1}. As expected the value for K_1 is less than the value obtained by direct measurement at 25°C ($K_1 = (5.0 \pm 3.0) \times 10^5$ M^{-1}).

It is seen that the theory can indeed accurately account for the experimental data. Recognizing that in many cases, the products of thermal denaturation of globular proteins are far from complete random coils (Tanford, 1968) and that the activated intermediates are therefore also likely to be still largely globular and structured, the most appealing interpretation of the two constants is that there is a shift in binding constant from the value K_1 to K_1^* of the *same site,* when the native enzyme form (N) is transformed to the activated intermediate (X).

Similarly, the dependence of the first-order k_{obsd} on $[Ca^{2+}]$ in 8 M urea at 50°C and pH 7.0 agrees well with eq 11 using $k = 4.37 \times 10^{-4}$ sec^{-1}, $K_1 = 10^4$ M^{-1}, and $K_1^* = 2 \times 10^3$ M^{-1} (Figure 6).

Hydrogen-Tritium Exchange. The data obtained from a difference hydrogen exchange experiment are shown in Figure 7. Here two identical samples of Dip-thermomycolase have been exchanged-in under (a): slow conditions, calcium-binding site saturated; and (b): fast conditions, calcium-binding site vacant, respectively, as described in the Experimental Section. When exchange-out of these two samples is measured at high $[Ca^{2+}]$ (slow, c), it is seen that fast exchange-in does indeed label a few more hydrogens. Subtraction of the smoothed background curve ("in-slow", "out-slow") from the experimental data ("in-fast", "out-slow") gives the difference hydrogen exchange curve, shown in Figure 7 (insert). Apparently, the exchange of approximately eight hydrogens is affected by the presence of the bound calcium ion. About six of these exchange with a measurable half-life under slow conditions ($t_{1/2} \sim 12$ hr), whereas 2-3 exchange with $t_{1/2} > 30$ hr. When this same experiment was repeated with fast exchange-out the difference exchange curve showed only one hydrogen over the entire course of the exchange-out curve. Hence most of the hydrogens in the difference exchange curve of Figure 7 have a much smaller half-life under fast exchange conditions. The total number of hydrogens that exchange calcium ion dependently may obviously be much larger than shown in the insert of Figure 7, since we see the exchange of only 20 out of a total of about 347 hydrogens in this experiment (see below).

We also determined the number of hydrogens that have actually exchanged-in after 24 hr of fast exchange-in (b) by the extrapolation procedure described in the Experimental Section. The data in Table I, when fitted to eq 1, give $H(0) = 243$. This extrapolated value differs considerably from the sum of peptide and primary amide hydrogens in thermomycolase (H_{total}). From the known molecular weight (32,500) and mean residue molecular weight (102.2) reported before (Voordouw et al., 1974) we calculate the total number of peptide hydrogens to be 317. From the known total number of Asx and Glx (60), the isoelectric point pI = 6.0 of thermomycolase (Ong, 1973) and some preliminary sequence data on the enzyme (Dorian, V. and Stevenson, K. J., personal communication) the total sum of Asn and Gln is estimated as 30 ± 5. Hence we obtain for H_{total} 347 ± 5 and conclude that 104 ± 5 hydrogens do not exchange-in under conditions (b). This large class of very slowly exchanging hydrogens probably only exchange after a major denaturation reaction (Tanford, 1970, pp 88 and 89). Assuming that the loss of the calcium binding site accompanies such a reaction, the dependence of the exchange rate constant (k_{obsd}) of this slow class on $[Ca^{2+}]$ is expected to follow eq 8. The order of magnitude of k_{obsd} under our experimental conditions is too small, however, for this relation to be accessible to experimental evaluation.

Discussion

In an earlier study (Voordouw et al., 1974), it was established that thermomycolase is a typical globular protein.[3] At the present time the amino acid sequence and three-dimensional structure of the enzyme are unknown. Although the ultimate objective of studies of the kind reported here is to provide a detailed molecular explanation of the role played by bound calcium ions in the stabilization of calcium-binding proteins, the lack of structural detail in the case of thermomycolase does not vitiate in any way a discussion of the results obtained in the present study in terms of the binding model which we have proposed. Indeed, even when the sequence and three-dimensional structure of a thermostable protein are known in detail, as they are for example in the case of thermolysin, an understanding of the calcium ion dependent autolysis and thermal denaturation behavior in terms of the binding model elaborated here is an essential prerequisite to a molecular explanation of the role played by calcium ion in stabilizing the molecule (Voordouw and Roche, 1975).

The results reported here clearly demonstrate that the binding of a single calcium ion to thermomycolase (Figure 2) causes only *local* perturbation (Figure 3) of a binding site which exists independently of bound calcium ion. If this site were formed by a *major* refolding of the protein chain in response to the presence of Ca^{2+} ions more significant changes would be expected in the sedimentation coefficient and uv circular dichroism than those actually observed. The magnitude of the binding constant, $K_1 = (5.0 \pm 3.0) \times 10^5$ M^{-1} at 25°C, suggests that the site is specific and therefore structurally quite specialized. From the latter discussion it is clear that the loss of calcium-binding capacity would require an unfolding of the chain from the native conformation. Without the detailed structure and a knowledge of the folding–unfolding pathway for thermomycolase it is impossible to say exactly how far the chain would have to unfold before the calcium-binding site is lost.

The binding model (see Appendix), which quantitatively accounts for the observed dependence of the rate constant for autolysis on pCa^{2+} (Figure 5), requires only that those molecules *without* bound calcium ions will serve as substrate. It cannot specify the extent to which the chain unfolds before the rate of autolysis becomes appreciable nor can it specify that the calcium binding site be lost before autolysis ensues. The latter possibility is, however, highly probable.

In order to rationalize the thermal denaturation data (Figure 6) the binding model requires that the critically activated intermediate on the unfolding pathway still bind calcium, probably at the *same* site, but with a lower affinity than the native form. The binding constants derived from the optimal fitting of the data to the binding model (Figure 6) (see Results) suggest that the calcium-binding site remains at least partially intact in the activated intermediate and that the unfolding of the native conformation and loss of calcium-binding capacity are concerted processes.

With the possible exception of the case of staphylococcal nuclease [Anfinsen et al., 1971; Anfinsen, 1973; and Jardetzky et al., 1971] our understanding of protein folding–unfolding is almost totally lacking in *molecular* detail. However, a number of essential features are already well established. They are the following. The overall process is highly cooperative. Although a two-state model for the folding–unfolding process, involving the native and denatured forms, often provides a useful phenomenological description it is now apparent that the folding process should be regarded as an ordered sequence of separate processes involving nucleation of secondary structures (α helices; β bends) and stabilization of long-range cooperative interactions (Scheraga, 1974).

In general terms, the role of calcium in stabilizing the resulting fold against autolysis and thermal denaturation in the case of thermostable proteins seems clear, when it is viewed within the present rationale of protein folding (Anfinsen, 1973). We postulate that the stabilizing role of bound calcium ions is due largely to their effect on the normal conformational fluctuations of the tertiary structure (see Appendix) and to the fact that the integrity of the calcium-binding site and the initial stages of the unfolding sequence are intimately linked. Thus the more cooperative the unfolding process the more stabilizing will be the effect of the "calcium lock" if it is near the beginning of the unfolding pathway. Conceivably, it is for this reason that only one bound calcium is required in the case of thermomycolase. It is interesting to note that the calcium binding site in staphylococcal nuclease [Cotton et al., 1971] is near the amino terminal of the sequence from which it is suggested [Jardetzky et al., 1971] the molecule unfolds. In our proposed rationalization of the stabilizing role played by calcium, there is no a priori distinction made between surface-bound and buried calcium. The important question to determine, when full structural detail is available, is whether or not the calcium ion is bound at a *critical* point in a conceivable unfolding pathway.

The calcium-binding model can also be used to describe the calcium dependence of denaturation reactions, other than pure thermal denaturation, by the appropriate adjustment of the parameters k, K_1, and K_1^* of eq 11. For instance, the dependence of the first-order k_{obsd} on $[Ca^{2+}]$ in 8 M urea at 50°C, pH 7.0, agrees excellently with eq 11 with $k = 4.37 \times 10^{-4}$ sec^{-1}, $K_1 = 10^4$ M^{-1}, and $K_1^* = 2 \times 10^3$ M^{-1} (Figure 6). An interesting feature of the urea denaturation data is the relative stabilities of the calcium complexes in the native and critically activated states in the presence of urea at 50°C in comparison to the situation, in its absence, at 70°C. Thus $K_1/K_1^*(50°C) \simeq 5$ whereas $K_1/K_1^*(70°C) \simeq 70$. This is reflected in the crossing of the two curves in Figure 6. This is largely due to the preferential destabilization of the calcium complex of the native form by urea and suggests that there is a significant contribution by hydrophobic bonding to the stabilization of the calcium-binding site in the native fold of the protein.

Our hydrogen–tritium exchange data (see Results) (Figure 7) confirm that there are calcium-dependent conformational fluctuations in thermomycolase, their being fewer exchangeable hydrogens when the calcium site is occupied. The presence of a large "core", comprising 104 ± 5 slowly exchanging hydrogens (Table I, see Results), also suggests that there may be important structural features other than calcium binding which contribute to the stability of thermostable proteins.

Appendix

We will derive here equations for the dependence of the rate constant k_{obsd} for autolysis and thermal denaturation reactions on $[Ca^{2+}]$. In the case of the autolysis of a proteolytic enzyme, consider a polypeptide chain binding a single

[3] Thermomycolase has a molecular weight of 32,000–33,000; $s_{20,w}^0$ = 2.97 S; $[\eta]$ = 3 cm^3/g.

Table I: Exchange-out Data for Dip-Thermomycolase Obtained from One Column Exchange-out Runs in 0.08 *M* NaOAc (pH 4.50)–0.01 *M* $CaCl_2$ at 2°C, after 24 hr of Exchange-in under Conditions (b) (Fast Exchange).

Time (min)	$H(t)$	Time (min)	$H(t)$
0	243[a]	3.38	219
1.25	241	4.91	203
1.58	228	6.60	201
2.18	218		

[a] Obtained by computer fitting of the data to eq 1; see Experimental Section.

calcium ion. The chain can exist in a number of conformations both with and without bound calcium ion (cf. Weber, 1972):

$$\begin{array}{ccccccc} E_1 & \overset{k_{1,2}^0}{\rightleftharpoons} & E_2 & \overset{k_{2,3}^0}{\rightleftharpoons} & E_3 & \rightleftharpoons & \dots E_m \dots E_n \\ K_1 \Big\uparrow\Big\downarrow & & K_2 \Big\uparrow\Big\downarrow & & K_3 \Big\uparrow\Big\downarrow & & K_n \Big\uparrow\Big\downarrow \\ E_1Ca & \rightleftharpoons & E_2Ca & \rightleftharpoons & E_3Ca & \rightleftharpoons & \dots E_mCa \end{array}$$

The conformations E_1 and E_1Ca are the lowest free energy conformations of the populations E and ECa, respectively. The conformations E_i ($1 \leqslant i \leqslant n$) and E_iCa ($1 \leqslant i \leqslant m$) are ranked in such a way that all $k_{i,i+1}^0 \geqslant 1$ (all $\Delta F_{i,i+1} \geqslant 0$). Hence the conformers E_n and E_mCa are the highest free energy conformers of the populations E and ECa. Partially or completely randomly coiled conformers, in which the calcium-binding site has been lost, only occur in the population E. One can envisage the presence of the bound calcium ion as limiting the possible conformational isomerizations of the protein chain to conformations not too remote from E_1Ca. It follows then that the number of different conformations in E is much larger than in ECa ($n \gg m$). The total concentrations of the two populations are related as follows (Weber, 1972):

$$K_{ECa} = \frac{\sum_{i=1}^{m}[E_iCa]}{[Ca^{2+}]\sum_{i=1}^{n}[E_i]} \qquad (2)$$

where K_{ECa} is the experimentally determinable calcium-binding constant. The contribution of partially or completely randomly coiled conformers (like E_n) to the total concentration of the population E is negligible under native conditions. Hence they will not make significant contributions to equilibrium properties of the system which are averaged over all molecules at any value of $[Ca^{2+}]$. They can, however, contribute considerably to rate phenomena such as autoproteolysis. We assume that: (a) all the substrates of the autoproteolysis reaction are in the population E not in ECa; (b) the sum of the concentrations of these substrates $\Sigma_{j=m}^{n}[E_j]$ is negligible compared to the sum of the concentrations of the enzymatically active conformers $\Sigma_{i=1}^{m}([E_iCa] + [E_i])$; (c) the latter sum equals therefore the total enzyme concentration c_p; (d) the autoproteolysis reaction is first order with respect to all substrate concentrations; (e) all of the enzymatically active conformers have the same activity (k_{cat}, K_m) toward a given substrate.

With these assumptions we can write for the rate, v, of autoproteolysis:

$$v = \sum_{j=1}^{n} k_j[E_j]c_p = c_p \sum_{j=1}^{n} k_j[E_j] \qquad (3)$$

where k_j is the overall $(k_{cat}/K_m)_j$ for a given substrate E_j. The summation extends over the entire population E. The rate constant k_j is zero for the well-folded conformers of the population ($1 \leqslant j \leqslant m$), then increases continuously with increasing j ($m \ll j \leqslant n$). The $[E_j]$ on the other hand decreases continuously with increasing j. From (3) we see that the conformers making the largest contribution to v are those for which the product $k_j[E_j]$ is maximal. The $[E_j]$ can readily be expressed in terms of $[E_1]$ and the conformational isomerization constants $k_{i,i+1}^0$:

$$[E_j] = \prod_{i=1}^{j-1} k_{i,i+1}^0[E_1]; \quad (1 < j \leqslant n) \qquad (4)$$

Similarly, $[E_1]$ can be expressed in terms of $k_{i,i+1}^0$, K_{ECa}, $[Ca^{2+}]$, and c_p:

$$[E_1] = \frac{c_p}{(1 + K_{ECa}[Ca^{2+}])}\left\{\sum_{j=1}^{n}\prod_{i=1}^{j-1} k_{i,i+1}^0\right\}^{-1} \qquad (5)$$

Combination of (3), (4), and (5) gives:

$$v = \frac{kc_p^2}{(1 + K_{ECa}[Ca^{2+}])} = k_{obsd}\, c_p^2 \qquad (6)$$

where we have defined the number average intrinsic rate constant k:

$$k = \frac{\sum_{j=1}^{n} k_j \prod_{i=1}^{j-1} k_{i,i+1}^0}{\sum_{j=1}^{n}\prod_{i=1}^{j-1} k_{i,i+1}^0} \qquad (7)$$

and the experimentally measurable k_{obsd}:

$$k_{obsd} = k/(1 + K_{ECa}[Ca^{2+}]) \qquad (8)$$

Equation 8 can be conveniently tested by taking the logarithm of both sides:

$$\log k_{obsd} = \log k - \log(1 + K_{ECa}[Ca^{2+}]) \qquad (9)$$

Hence for $K_{ECa}[Ca^{2+}] \ll 1$ the equation reduces to: $\log k_{obsd} = \log k$, and a plot of $\log k_{obsd}$ vs. pCa^{2+} will have a slope $m = 0$. For $K_{ECa}[Ca^{2+}] \gg 1$ the equation reduces to: $\log k_{obsd} = \log (k/K_{ECa}) - \log [Ca^{2+}]$, and the plot will have a slope $m = -1$.

It can be shown that, when the native conformation of the enzyme binds more than one calcium ion with binding constants $K_1, K_2, \dots K_p$ and the autolysis substrates bind also one or more calcium ions with binding constants $K_1^*, K_2^*, \dots K_q^*$, all of the sites being independent, k_{obsd} can be written as:

$$k_{obsd} = k\,\frac{\prod_{i=1}^{q}(1 + K_i^*[Ca^{2+}])}{\prod_{i=1}^{p}(1 + K_i[Ca^{2+}])} \qquad (10)$$

Interacting calcium binding sites can also easily be included in (10) (Voordouw and Roche, 1975).

The dependence of the first-order rate constant of thermal denaturation on $[Ca^{2+}]$ is now easily found (Tanford, 1968, p 265; and 1970, p 74). Assuming that the pathway for the conversion of a native conformation (E_i, E_iCa; $1 \leqslant i \leqslant m$), to a denatured conformation (e.g., E_n) of *lower* free energy than any of the native conformations, goes via an activated intermediate X, that does not bind calcium ions, eq 8 is obtained. Here k_{obsd} is the first-order rate constant for

the thermal denaturation process. When both the native conformation and X bind more than one calcium ion at sites, independent and with different intrinsic affinity, we obtain eq 10. When the native conformation and X bind one calcium ion with binding constants K_1 and K_1*, respectively, we find:

$$k_{obsd} = k\frac{(1 + K_1^*[Ca^{2+}])}{(1 + K_1[Ca^{2+}])} \quad (11)$$

Acknowledgment

The authors are greatly indebted to Miss Carol Milo and Dr. K. Chen for skillful technical assistance.

References

Anfinsen, C. B. (1973), *Science 181,* 223.
Anfinsen, C. B., Schechter, A. N., and Taniuchi, H. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 249.
Bray, G. A. (1960), *Anal. Biochem. 1,* 279.
Cotton, F. A., Bier, C. J., Day, V. W., Hazen, E. E., Jr., and Larsen, S., (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 243.
Delaage, M., and Lazdunski, M. (1967), *Biochem. Biophys. Res. Commun. 28,* 390.
Donovan, J. W. (1969), *J. Biol. Chem. 244,* 1961.
Englander, S. W., and Englander, J. J. (1972), *Methods Enzymol. 266,* 406.
Englander, S. W., and Poulsen, A. (1969), *Biopolymers 7,* 379.
Englander, S. W., and Rolfe, A. (1973), *J. Biol. Chem. 248,* 4852.
Englander, S. W., and Staley, R. (1969), *J. Mol. Biol. 45,* 277.
Epstein, M., Levitzki, A., and Reuben, J. (1974), *Biochemistry 13,* 1777.
Gabel, D., and Kasche, V. (1973), *Acta Chem. Scand. 27,* 1971.
Herskovits, T. T. (1967), *Methods Enzymol. 11,* 748.
Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63,* 530.
Jardetzky, O., Thielman, H., Arata, Y., Markley, J. L., and Williams, M. N. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 257.
Matthews, B. W., Weaver, L. H., and Kester, W. R. (1974), *J. Biol. Chem. 249,* 8030.
Ong, P. S. (1973), Ph.D. Thesis, University of Calgary, Calgary, Alberta, Canada.
Perrin, D. D., and Dempsey, B. (1974), Buffers for pH and Metal Ion Control, Chapman and Hall Laboratory Manuals, London.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Schachman, H. K. (1957), *Methods Enzymol. 4,* 32.
Scheraga, H. A. (1974), in Current Topics in Biochemistry, 1973, Anfinsen, C. B., and Schechter, A. N., Ed., New York, N.Y., Academic Press.
Sillen, L. G., and Martell, A. E. (1964), *Chem. Soc., Spec. Publ. No. 17.*
Tanford, C. (1968), *Adv. Protein Chem. 23,* 121.
Tanford, C. (1970), *Adv. Protein Chem. 24,* 1.
Voordouw, G., Gaucher, G. M., and Roche, R. S. (1974), *Can. J. Biochem. 52,* 981.
Voordouw, G., and Roche, R. S. (1974), *Biochemistry 13,* 5017.
Voordouw, G., and Roche, R. S. (1975), *Biochemistry,* following paper in this issue.
Weber, G. (1972), *Biochemistry 11,* 864.