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Role of Bound Calcium Ions in Thermostable, Proteolytic Enzymes. Separation of Intrinsic and Calcium Ion Contributions to the Kinetic Thermal Stability†

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ABSTRACT: The total kinetic thermal stability of a protein molecule, expressed as the total free energy of activation in thermal denaturation reactions, can be separated into an intrinsic contribution of the polypeptide chain and a contribution due to the binding of calcium ions. The theory for this procedure is applied to thermal denaturation data, obtained at the pH of optimum stability, for the serine proteases, thermomycolase and subtilisin types Carlsberg and BPN′, and for the zinc metalloendopeptidases, thermolysin and neutral protease A. The results, obtained from Arrhenius plots at high and low free calcium ion concentrations, reveal a considerable variation in the calcium ion contribution to the total kinetic thermal stability of the various enzymes. In the serine protease group, at 70 °C, the stability is largest for thermomycolase, mainly due to a relatively high intrinsic contribution. For the metalloendopeptidases the total kinetic thermal stability is largest for thermolysin, the difference between thermolysin and neutral protease A being dominated by bound calcium ion contributions. The intrinsic kinetic thermal stability of the polypeptide chain of thermolysin is considerably smaller than that of any of the serine proteases and is probably of the same order of magnitude as that of neutral protease A. Thus, the well known total kinetic thermal stability of thermolysin is due mainly to a *single* calcium ion (Voordouw, G., and Roche, R. S. (1975), *Biochemistry 14,* 4667) that binds with high affinity even at very high temperatures ($K \simeq 6 \times 10^7$ M^{-1} at 80 °C).

The reason for the successful survival of thermophilic microorganisms at the high temperatures preferred or needed by these organisms is intriguing. One may suggest that an increased stability of many intra- and extracellular components is a likely explanation. In the case of the proteins, such an enhanced stability, compared to similar protein molecules isolated from a mesophilic source, has indeed frequently been demonstrated. The data presented are generally limited to the kinetics of the denaturation process: the enhanced stability is thus kinetic and not necessarily thermodynamic in origin. The observation of slow-denaturation kinetics for enzymes isolated from thermophilic microorganisms compared to those from their mesophilic counterparts has led to the description of the former as "thermostable" enzymes. The distinction between "thermostable" and nonthermostable is, however, largely subjective.

Nevertheless, the marked difference in kinetic thermal stability that is observed even between extensively sequence homologous and thus probably conformationally homologous enzymes asks for an explanation. One current strategy aimed at the solution of this problem is to elucidate, in as much detail as is presently possible, the three-dimensional structures of homologous enzymes isolated from a thermophilic and a mesophilic source. By comparing the structures one hopes to obtain pertinent information relevant to the kinetic thermal stability of the "thermostable" enzyme. This approach has led, for instance, to the elucidation of the sequence (Titani et al., 1972) and three-dimensional structure of thermolysin, the thermostable zinc metalloendopeptidase produced by *B. thermoproteolyticus* (Matthews et al., 1972a,b; Colman et al., 1972; Matthews and Weaver, 1974; Matthews et al., 1974). Sequence studies of neutral protease A, the zinc metalloendopeptidase produced by *B. subtilis*, have advanced considerably (Pangburn et al., 1973, 1975, 1976; Pangburn, 1973) and, with 54% of the sequence completed, indicate a high de-

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gree of sequence homology to thermolysin. At the present time, with structural detail on the three-dimensional level available only for thermolysin, the reasons for its kinetic thermal stability are not at all clear. Matthews et al. (1974) suggest, because of the absence of unusual features in the thermolysin structure with the possible exception of the four calcium binding sites, that "the resistance of thermolysin to thermal denaturation might be due, not to a single determinant, but to a combination of factors including calcium binding, hydrophobic interactions, hydrogen-bonding and ionic stabilization". The fact that these authors cannot be more specific about the various contributing factors raises the question whether the problem can be solved at all with the aid of structural data alone. A second approach to the problem is to measure the various contributions by studying the kinetic and, when possible, the equilibrium aspects of denaturation reactions, and comparing the data obtained for the two homologous polypeptide chains.

The two approaches sketched above are not alternatives, but rather have to be seen as complementary to each other. It is with the second approach that we will be concerned in this paper. We will deal only with two contributions to the kinetic thermal stability of the proteolytic enzymes studied: (a) that caused by bound calcium ions and (b) that caused by all other contributing factors (hydrophobic bonding, ionic stabilization, etc.), which we will call the "intrinsic" contribution of the polypeptide chain. The latter approach is based on the fact that, whereas the bound calcium ion and intrinsic contributions can be separated rather easily, the further separation of the intrinsic part is by no means a trivial exercise. A bound calcium ion contribution may be expected for all five proteolytic enzymes, since they all have been shown to bind calcium ions with high affinity. The serine proteases thermomycolase, S. Carlsberg[1] and S. BPN′ (Novo) all bind a single calcium ion with high affinity ($K_{ECa} = 5 \times 10^5$ M^{-1}; Voordouw and Roche, 1975a; $K_{ECa} = 1.1 \times 10^6$ M^{-1}; and $K_{ECa} > 10^{11}$ M^{-1}, respectively, at 25 °C, pH 7.5; Voordouw et al., to be submitted for publication). The apometalloendopeptidases, apothermolysin and aponeutral protease A, bind four ($K_{1,2} = 2.8 \times 10^9$ M^{-2}; $K_3, K_4 > 10^6$ M^{-1}; Voordouw and Roche, 1974) and at least two calcium ions, respectively, at 25 °C, pH 9.0.

Since complications due to subsequent autolysis are likely to cause irreversibility of the denaturation reaction when one studies proteolytic enzymes (Dahlquist et al., 1975), we will only be concerned with the forward kinetics of the reaction and will not attempt to do equilibrium denaturation studies. The theory for the separation of bound calcium ion and intrinsic contributions will be presented first.

Theoretical Section

In order to separate the intrinsic and bound calcium ion contribution to the total kinetic thermal stability, consider a polypeptide chain that, for the sake of simplicity, is in either of the following two macroscopic conformations: the native (N) or the denatured (D) conformation. The pathway for the conversion of N into D is characterized by the presence of *one* activated intermediate (X). N has p calcium binding sites of high affinity. The affinity of a given site i is characterized by the association constants $K_{i,N}$ and $K_{i,X}$ in the conformations N and X, respectively. No changes in the maximal number of calcium ions bound occur in the conversion of N into X; N and X bind, maximally, the same number of calcium ions p ($p = p_N = p_X$). The assumption that $p_N = p_X$ can be verified experimentally, as will be shown below.

The dependence of the denaturation rate constant, k_{obsd}, on the free calcium ion concentration (C) is given by (Tanford, 1968, 1970; Voordouw and Roche, 1975a):

$$k_{obsd} = k_1 \prod_{i=1}^{p} \left(\frac{1 + K_{i,X}C}{1 + K_{i,N}C} \right) \tag{1}$$

Equation 1 clearly shows that a given site i, for proper values of C, can either stabilize, destabilize, or have no influence on the rate constant of interconversion of N into D, depending on the magnitudes of $K_{i,N}$ and $K_{i,X}$ for that site. Two limiting concentration regions are of special interest:

$$k_0 = \lim_{C \to 0} k_{obsd} = k_1 \tag{2}$$

$$k_\infty = \lim_{C \to \infty} k_{obsd} = k_1 \prod_{i=1}^{p} \frac{K_{i,X}}{K_{i,N}} \tag{3}$$

Since neither k_0 nor k_∞ are functions of C it follows that the slope $m = (\delta \log k_{obsd}/\delta \text{ pCa}^{2+})$ of a log k_{obsd} vs. pCa^{2+} plot should approach zero at very low and very high $[Ca^{2+}]$. We have shown previously that $\text{pCa}^{2+} \simeq 2$ is sufficiently high to satisfy the condition $k_{obsd} = k_\infty$: the slope of a log k_{obsd} vs. pCa^{2+} plot approaches zero in this region (Voordouw and Roche, 1975a, Figure 6; Voordouw and Roche, 1975b, Figure 6). This shows that N and X have the same number of high affinity sites ($p_N = p_X = p$); when $p_N \neq p_X$ the slope does not approach zero because C is not eliminated from eq 3. It is worthwhile to recall that at much higher free calcium ion concentrations ($\text{pCa}^{2+} < 0$) calcium ion acts as a protein denaturant (von Hippel and Wong, 1965). The increase in k_{obsd} with increasing $[Ca^{2+}]$ in this range indicates, in terms of the above binding model, that there are low affinity sites for which $p_N < p_X$. We will not be concerned with this concentration range here.

Conditions such that $k_{obsd} \simeq k_0$ ($= k_1$) can be achieved by the use of a calcium chelating agent such as EDTA to reduce the $[Ca^{2+}]$ (see Results).

The free energy change for the stabilization of the conformation N by calcium ions is given by:

$$\Delta(\Delta F_{Ca^{2+}}) = -RT \sum_{i=1}^{p} \ln \frac{K_{i,N}}{K_{i,X}} = -RT \ln \frac{k_0}{k_\infty} \tag{4}$$

where R and T have their usual meaning; $\Delta(\Delta F_{Ca^{2+}})$ is independent of concentration units, since $(K_{i,N}/K_{i,X})$ is a dimensionless quantity. We note that $\Delta(\Delta F_{Ca^{2+}})$ is given by the difference between the free energy change of calcium binding to N ($\Delta F_{Ca^{2+},N}$) and that to X ($\Delta F_{Ca^{2+},X}$):

$$\Delta(\Delta F_{Ca^{2+}}) = \Delta F_{Ca^{2+},N} - \Delta F_{Ca^{2+},X}$$

$$= \left(-RT \sum_{i=1}^{p} \ln K_{i,N}\right) - \left(-RT \sum_{i=1}^{p} \ln K_{i,X}\right) \tag{5}$$

Calcium stabilizes N when $K_{i,N} > K_{i,X}$; $\Delta(\Delta F_{Ca^{2+}})$ is then negative.

The kinetic stability of the polypeptide chain in the absence of calcium ion contributions can be obtained from k_0 (eq 2) as the free energy of activation ($\Delta F^{\ddagger}$) at a given temperature. Absolute reaction rate theory suggests that (Glasstone et al., 1941):

[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; FAGLA, furylacryloylglycyl-L-leucinamide; *N*-Cbz-Gly-NPE, *N*-carbobenzoxylglycine *p*-nitrophenyl ester; S, subtilisin; TEP, tetraethylenepentamine; Tris, tris(hydroxymethyl)aminomethane; $[Ca^{2+}]$ or c and pCa^{2+}, the free calcium ion concentration and its negative logarithm; $[Ca]_T$, the total calcium ion concentration; pH^t, pH at temperature t (°C); OD, optical density.

$$k_0 = \frac{\kappa t}{h} \exp(-\Delta F^{\ddagger}/RT) \quad (6)$$

where κ is Boltzmann's constant and h is Planck's constant. Hence, by determining k_0 and k_∞ at a given absolute temperature T, the intrinsic ($\Delta F^{\ddagger}$) and calcium ion ($\Delta(\Delta F_{Ca^{2+}})$) contributions to the total free energy of stabilization of the conformation N can be determined separately. The total activation free energy is given by:

$$\Delta F^{\ddagger}_T = \Delta F^{\ddagger} - \Delta(\Delta F_{Ca^{2+}}) \quad (7)$$

When $\Delta(\Delta F_{Ca^{2+}})$ is large and negative $k_0 \gg k_\infty$ (eq 4) and it may be difficult to measure k_0 and k_∞ at the same temperature with an experimental technique that makes only a limited range of values for k_{obsd} accessible to measurement. However, by measuring the temperature dependence of k_0 and k_∞ each in their own characteristic temperature range, extrapolations can be made via linear Arrhenius plots to obtain k_0 and k_∞ at the same temperature. This procedure is valid only when: (a) the Arrhenius plots are indeed reasonably linear and (b) the extrapolation is not over too large a temperature range. In view of the high heat capacity of activation, $\Delta C_p^{\ddagger}$, possible for denaturation reactions, this latter possibility leads to a temperature dependence of the enthalpy of activation, $\Delta H^{\ddagger}$, and hence to curved Arrhenius plots.

From a study of the temperature dependence of k_0 and k_∞ other relevant thermodynamic information can be obtained by means of the following equations:

$$\Delta H^{\ddagger} = -R \left(\frac{d \ln k_0}{d\, 1/T}\right) \quad (8)$$

$$\Delta S^{\ddagger} = \frac{\Delta H^{\ddagger} - \Delta F^{\ddagger}}{T} \quad (9)$$

$$\Delta(\Delta H_{Ca^{2+}}) = -R \left(\frac{d \ln (k_0/k_\infty)}{d\, 1/T}\right) \quad (10)$$

$$\Delta(\Delta S_{Ca^{2+}}) = \frac{\Delta(\Delta H_{Ca^{2+}}) - \Delta(\Delta F_{Ca^{2+}})}{T} \quad (11)$$

$$\Delta H^{\ddagger}_T = \Delta H^{\ddagger} - \Delta(\Delta H_{Ca^{2+}}) \quad (12)$$

$$\Delta S^{\ddagger}_T = \Delta S^{\ddagger} - \Delta(\Delta S_{Ca^{2+}}) \quad (13)$$

Alternatively one can obtain $\Delta H^{\ddagger}_T$ by:

$$\Delta H^{\ddagger}_T = -R \left(\frac{d \ln k_\infty}{d\, 1/T}\right) \quad (14)$$

In these equations $\Delta(\Delta H_{Ca^{2+}})$ and $\Delta(\Delta S_{Ca^{2+}})$ are the enthalpy and entropy components of $\Delta(\Delta F_{Ca^{2+}})$, which has been defined by eq 4 and 5. $\Delta F^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ and $\Delta F^{\ddagger}_T$, $\Delta H^{\ddagger}_T$, and $\Delta S^{\ddagger}_T$ are, respectively, the intrinsic free energy, enthalpy, and entropy of activation excluding calcium ion contributions and the total free energy, enthalpy, and entropy of activation including calcium ion contributions (eq 7, 12, 13). It is clear that the magnitudes of all these thermodynamic quantities can be determined by measuring the temperature dependence of k_0 and k_∞ and plotting the data in the Arrhenius plots ($\ln k_0$ and $\ln k_\infty$ vs. $1/T$) the slopes of which are the quantities $\Delta H^{\ddagger}$ and $\Delta H^{\ddagger}_T$, respectively (eq 8 and 14). The other thermodynamic quantities can then be calculated readily.

It may be noted that there is a fundamental difference between the two contributions in the derivation given above: the intrinsic contribution ($\Delta F^{\ddagger}$) to the total kinetic thermal stability ($\Delta F^{\ddagger}_T$) is considered to be an activation free energy change, whereas the bound calcium ion contribution is considered to be an equilibrium free energy change ($-\Delta(\Delta F_{Ca^{2+}})$). This distinction may not be real, however, as is best shown by the following example. For one calcium ion binding to N and X eq 1 reduces to:

$$k_{obsd} = k_1 \frac{(1 + K_{1,X}C)}{(1 + K_{1,N}C)} \quad (15)$$

However, we can also account for a given set of experimental data (k_{obsd},C), which may be adequately described by eq 15, in an alternative fashion using:

$$k_{obsd} = \frac{k_1}{1 + K_{1,N}C} + k_2 \quad (16)$$

where $K_{1,N}$ has the same numerical value in both equations; k_2 is the denaturation rate constant for the native conformation with a bound calcium ion, and $k_1 + k_2$ the rate constant for the native conformation without bound calcium ion. Rearranging eq 16 one obtains:

$$k_{obsd} = (k_1 + k_2) \frac{\left\{1 + \left(\frac{k_2}{k_1 + k_2}\right) K_{1,N}C\right\}}{(1 + K_{1,N}C)} \quad (17)$$

Noting that $k_1 = (k_1 + k_2)$ and defining $(k_2/(k_1 + k_2))K_{1,N} \equiv K_{1,X}$, it is seen that the differences between eq 15 and 16 are only conceptual, *not* mathematical. Hence, in eq 15 the change in k_{obsd} with C is assigned to a parallel change in the net free energy of calcium ion binding, $\Delta(\Delta F_{Ca^{2+}})$, whereas in eq 16 it is assigned to a parallel change in the activation free energy, $\Delta(\Delta F^{\ddagger}_{Ca^{2+}})$, due to calcium ion binding. The equivalence of eq 15 and 16 shows that is impossible to tell, from an experimental recording of k_{obsd} as a function of C, whether the calcium ion stabilization resides in $\Delta(\Delta F_{Ca^{2+}})$ or in $\Delta(\Delta F^{\ddagger}_{Ca^{2+}})$.

When the rate of association-dissociation of calcium ions to their binding site(s) is fast relative to the rate of unfolding the stabilization is an equilibrium phenomenon and is characterized by eq 15; when it is slow, eq 16 is more realistic. Noting that denaturation rates are on the minute time scale (see Results), whereas association-dissociation reactions of proteins with small ligands generally take place on the millito microsecond time scale, it follows that eq 16 is probably the more realistic one in the present case.

It is for the latter reason that we have treated the calcium contribution to $\Delta F^{\ddagger}_T$ as an equilibrium free energy change and the intrinsic contribution as a free energy of activation in the derivation given above.

Experimental Section

Materials. Thermolysin (lot no. 54C-0211), S. Carlsberg (lots no. 33C-3270 and 94C-0245), and S. BPN′ (lot no. 13C-2360) were from Sigma Chemical Co. and were used without further purification. Neutral protease A from *B. subtilis* purified by affinity chromatography (Pangburn et al., 1973) was obtained as a frozen solution (~3 mg/ml) in 0.1 M NaCl, 0.01 M $CaCl_2$, and 0.05 M 2-(*N*-morpholino)ethanesulfonic acid, pH 5.0, through the courtesy of Dr. K. A. Walsh, University of Seattle, Seattle, Wash. Thermomycolase, the extracellular serine protease from the fungus *Malbranchea pulchella*, was purified as described previously (Ong, 1973; Voordouw et al., 1974; Ong and Gaucher, 1976). *N*-Cbz-Gly-NPE and FAGLA were from Sigma Chemical Co. and Cyclo Chemical Co., respectively. Na_2 EDTA-$2H_2O$ and TEP were obtained from Fisher and J. T. Baker Chemical Co., respectively. All other chemicals used were reagent grade.

Methods

Determination of Enzyme Concentration. Enzyme concentrations of dialyzed stock solutions were routinely deter-

mined from uv absorbance measurements at 280 nm, using a Beckman DB-G grating spectrophotometer. We used the following molar extinction coefficients (ϵ in M^{-1} cm^{-1}) at 280 nm for the calculation of the enzyme concentration. Thermomycolase: ϵ = 43 900 (Voordouw et al., 1974), S. Carlsberg: ϵ = 23 500 (Markland and Smith, 1971), S. BPN′: ϵ = 32 300 (Markland and Smith, 1971), thermolysin: ϵ = 52 400 (Voordouw and Roche, 1974), and neutral protease A: ϵ = 55 000 (Pangburn, 1973; Pangburn et al., 1973).

Enzyme Assays. The serine proteases thermomycolase, S. Carlsberg, and S. BPN′ were assayed with the use of *N*-Cbz-Gly-NPE as substrate, according to the procedure described previously (Voordouw et al., 1974). Tris-HCl (0.01 M, pH 8.00), containing 0.01 M $CaCl_2$ and 0.1 M NaCl, was used as the assay buffer. The assay reaction was initiated by adding to 2.6 ml of this buffer 100 μl of enzyme solution and 200 μl of 1.5×10^{-3} M *N*-Cbz-Gly-NPE in acetonitrile. The rate of increase in optical density at 400 nm (ΔOD_{400} min^{-1}) and 30 °C, corrected for the nonenzymatic substrate hydrolysis, was taken as a measure of the enzyme activity. The zinc metalloendopeptidases thermolysin and neutral protease A were assayed according to Feder (1968) at 30 °C, using 0.05 M Tris (pH 7.50), 0.01 M $CaCl_2$, 0.03 M NaCl, and 10^{-3} M FAGLA as the assay buffer. The rate of decrease in optical density at 345 nm (ΔOD_{345} min^{-1}) was taken as a measure of the enzyme activity. The assay procedure is described below.

Intrinsic Denaturation Rate Constants as a Function of pH. The pH dependence of the intrinsic denaturation rate constants was studied at low [Ca^{2+}] by using a high concentration of EDTA in order to eliminate calcium contributions to the thermal stability. An exception was made for neutral protease A, which was found to be instantaneously inactivated upon addition of excess EDTA, even at room temperature (25 °C). Stock solutions of thermomycolase, S. Carlsberg and S. Novo (all 10^{-5} M) were prepared in 0.1 M NaCl, 10^{-4} M $CaCl_2$ with the pH adjusted to 7.8 by addition of a few drops of 1 M Tris. The reaction was initiated by addition of a 0.1-ml aliquot of these solutions to 4.0 ml of 0.01 M Tris–acetate, 10^{-4} M EDTA, NaCl to ionic strength 0.1, and $3 < pH < 11$ at 60 °C. The decrease in active enzyme concentration [E] was followed by withdrawing periodically 0.5-ml samples of which 0.1 ml was assayed after cooling in ice. First-order rate constants for thermal denaturation (k_{obsd}) were obtained from linear plots of ln ($[E]^{-1}$) vs. time. Values for the pH at 60 °C, pH^{60}, were obtained from the known pH^{23} via an experimentally established plot of pH^{23} vs. pH^{60} for 0.01 M Tris–acetate, 10^{-4} M EDTA, NaCl to ionic strength 0.1. The corrections found experimentally agreed well with those calculated from the known temperature dependence of the proton binding constants of the buffer salts (Sillen and Martell, 1964, 1971). Tests were made to establish whether indeed the intrinsic rate constant was measured by varying the ratio of $[EDTA]_T$ to $[Ca]_T$ keeping $[EDTA]_T$ in excess. No variation of the measured k_{obsd} was found beyond experimental error, except for thermolysin at pH^{45} 5.0 (see Results). The procedure for thermolysin was similar to the one described above. A stock solution of 10^{-4} M thermolysin in 1 M NaCl, 10^{-4} M $CaCl_2$ with the pH adjusted to 9.0 by addition of a few drops of 1 M Tris was used; 0.2 ml of this solution was diluted into 4.0 ml of 0.01 M Tris–acetate, 10^{-4} M EDTA, and 0.045 M NaCl at 45 °C. Since EDTA causes at least partial inhibition of thermolysin at the concentration levels present in the assay mixture, the remaining enzyme, which has not been irreversibly denatured in the thermal reaction, was reactivated before assay by addition of 0.1 ml of 1 M Tris, pH 7.50, 3×10^{-4} M $Zn(OAc)_2$, and 0.01 M $CaCl_2$ to the 0.5-ml sample withdrawn from the incubation mixture. A 0.2-ml aliquot of the resulting 0.6 ml reactivated enzyme solution was then assayed with 2.0 ml of FAGLA. Values for pH^{45} were obtained from the known pH^{23} from an experimentally determined plot of pH^{23} vs. pH^{45}, as above.

The dependence of the intrinsic thermal denaturation rates of holoneutral protease A on pH was measured in the *absence* of EDTA because of the reactivation problems mentioned above. A stock solution of 8.3×10^{-5} M enzyme in 0.1 M NaCl, 10^{-3} M $CaCl_2$, pH 5.8, was used; 0.05 ml of this solution was diluted into 1.0 ml of 0.01 M Tris–acetate, 0.09 M NaCl at 60 °C. Samples of 0.250 ml were withdrawn periodically and 0.2 ml of these assayed with FAGLA after cooling in ice.

Temperature Dependence of Denaturation Rate Constants. The temperature dependence of the intrinsic denaturation rate constant, k_I, was determined at the pH of maximum stability for the various enzymes, using exactly the same procedures as in the previous section. Because of the high temperature coefficient of the proton association constant of Tris, the pH^{23} of the solutions was adjusted for every experimental temperature (t) such as to have always the same pH^t. The temperature dependence of the denaturation rate constants was also determined at high calcium ion concentration (k_∞) by addition of 0.015 M $CaCl_2$ to the reaction mixture in the absence of EDTA, adjusting the NaCl concentration to keep a constant ionic strength of 0.1, and leaving the rest of the procedure as above.

Miscellaneous. Enthalpy of activation values were calculated from Arrhenius plots of the experimental data (ln k_0 or ln k_∞ vs. $1/T$) by means of linear-regression analysis, using a Wang Model 462 calculator. All pH adjustments were performed using a Fisher Accumet Model 320 pH meter, equipped with an expanded scale. All incubations were done in a Colora Ultrathermostat circulating water bath with a temperature control of ±0.1 at 70 °C.

Results

Intrinsic Thermal Denaturation Rate Constants: Dependence on pH. Since the first-order rate constant for the thermal denaturation of a protein is generally strongly dependent on pH (Laidler and Bunting, 1973), a comparative study of different proteins at a single pH value is not very meaningful. In order to evaluate differences in the *intrinsic* kinetic stability of the serine proteases thermomycolase, S. Carlsberg and S. BPN′, the dependence of the intrinsic denaturation rate constant k_I was measured as a function of pH. Under the experimental conditions given in the legend to Figure 1, the [Ca^{2+}] is sufficiently low that calcium contributions to k_{obsd} are eliminated, i.e., $k_{obsd} = k_I$. Even for S. BPN′, which has a very high calcium binding constant at 25 °C (see introduction), no change in the experimental rate constant k_{obsd} is observed as the ratio $[EDTA]_T/[Ca]_T$ is decreased (Table I). The data presented in Figure 1 represent, therefore, truly intrinsic rate constants for *all* three proteins, given as a function of pH at 60 °C. The lines in Figure 1 are not calculated on the basis of any theoretical model (e.g., Laidler and Bunting, 1973; Tanford, 1970) but are just smooth curves drawn through the experimental data. A few well known features are apparent in Figure 1: the enzymes have a region of optimal kinetic stability around neutral pH and the rate constant k_I increases at both high and low pH (Laidler and Bunting, 1973). The intrinsic kinetic stability of thermomycolase is clearly larger than that of the two subtilisins in the neutral pH region *at this temperature.* S. Carlsberg is more stable than S. BPN′ at $6.0 < pH < 9.0$. The three enzymes have comparable kinetic stabilities

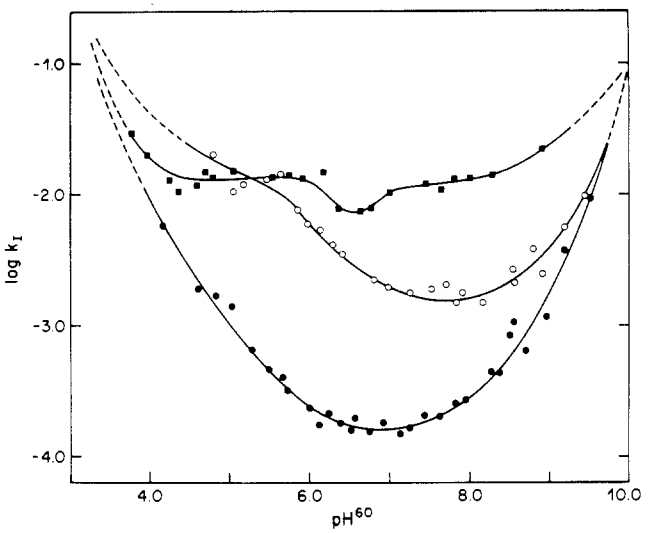

FIGURE 1: Dependence of the intrinsic denaturation constant (k_I) on pH^{60}. The logarithm of k_I (s^{-1}) has been plotted for thermomycolase (●), S. Carlsberg (○), and S. BPN′ (■). Experiments were done at 60 °C in 0.01 M Tris-acetate, 10^{-4} M EDTA, NaCl to ionic strength 0.1, and $[Ca]_T = 3.7 \times 10^{-6}$ M at an enzyme concentration of 2.4×10^{-7} M. The abscissa represents the pH of the solution *at* 60 °C. The solutions at pH^{60} ≥ 9.0 contained an additional 5×10^{-3} M glycine.

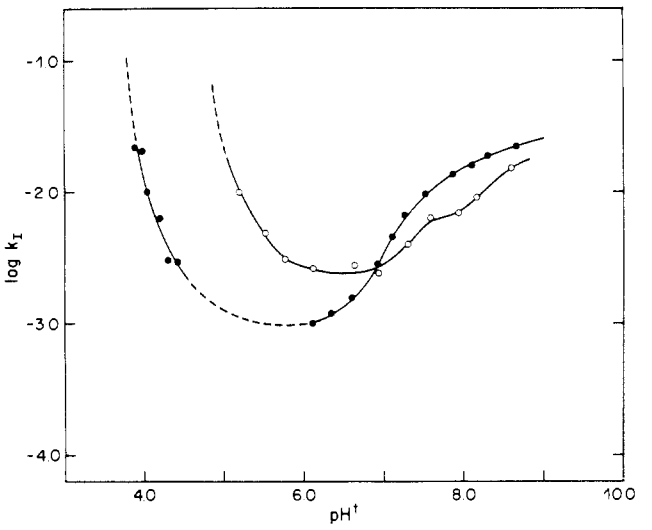

FIGURE 2: Dependence of the intrinsic denaturation rate constant (k_I) on pH′. The logarithm of k_I (s^{-1}) has been plotted (a) for thermolysin (●) at 45 °C in 0.01 M Tris-acetate, 10^{-4} M EDTA, NaCl to ionic strength 0.1 and $[Ca]_T = 2.4 \times 10^{-5}$ M, and (b) for neutral protease A (○) at 60 °C in 0.01 M Tris-acetate, NaCl to ionic strength 0.1, and $[Ca]_T = 5.6 \times 10^{-5}$ M; enzyme concentrations were 4.8×10^{-6} M and 4.0×10^{-6} M, respectively. The abscissa represents the pH of the solution *at* 45 or 60 °C.

in the acid and basic regions of the pH profiles. The relatively small change of k_I for S. BPN′ in the region $4.0 < pH < 9.0$ is noteworthy.

In contrast to the serine proteases studied, the pH dependence of k_I for the homologous zinc metalloendopeptidases, thermolysin and neutral protease A, cannot be studied conveniently at the same temperature with the techniques used in this study. Thus, although it has a high total kinetic thermal stability, its k_I is such that thermolysin is most conveniently studied at 45 °C. Neutral protease A, on the other hand, although it has the lowest total thermal kinetic stability of the five enzymes studied, has to be studied at 60 °C in order to get conveniently measurable rates at $[Ca^{2+}]_T = (5.6 \times 10^{-5}$ M). The data obtained for thermolysin *at 45 °C* and neutral protease A *at 60 °C* are presented in Figure 2. Since the pH profile for neutral protease A has been obtained in the absence of EDTA, for the reasons given above (see Methods), the data shown in Figure 2 for this enzyme are *not* truly intrinsic rates but still contain a calcium contribution. The discrepancy between the k observed at $[Ca^{2+}]_T = 5.6 \times 10^{-5}$ M and the true k_I is probably not too large, however, since only a small degree of calcium stabilization of neutral protease A was observed (Figure 3), when $[Ca^{2+}]_T$ was varied 300-fold. For the latter reason and also taking into account the significant difference in temperature at which the two curves have been obtained, one is tempted to conclude that neutral protease A may actually have a higher intrinsic stability than thermolysin. This conclusion cannot be pressed too far, however, for the reasons indicated above.

TABLE I: Thermal Denaturation Rate Constants for S. BPN′ at pH^{60} = 7.25 and 60 °C at Various Ratios of Total EDTA to Total Calcium Ion Concentration ($[EDTA]_T/[Ca]_T$).[a]

$[Ca]_T$ (M)	$[EDTA]_T/[Ca]_T$	log k_{obsd}
2.5×10^{-6}	40.0	−2.13
7.5×10^{-6}	13.3	−2.12
1.25×10^{-5}	8.0	−2.12
2.25×10^{-5}	4.4	−2.14
5.25×10^{-5}	1.9	−2.17

[a] Conditions were otherwise similar to those described in the legend to Figure 1. The $[EDTA]_T$ was kept constant at 10^{-4} M. Since k_{obsd} does not vary with the ratio: $k_{obsd} = k_I$.

With thermolysin, truly intrinsic rates can be measured, which are independent of the ratio of total EDTA to total calcium concentration, when EDTA is in excess (Table II). The measured rate constant (k_{obsd}) shows no dependence on the latter ratio except in the region of pH 5 (Table II). Apparently, the "chelating power" of EDTA relative to that of the calcium binding site(s) is insufficient to create complete site unsaturation at this pH. Consequently, no data for k_I are presented in this pH range (Figure 2). It is worthwhile to note here that the intrinsic kinetic stability of thermolysin is smaller than any other enzyme studied (Figures 1 and 2), with the possible exception of neutral protease A. The well known thermostability of thermolysin is therefore dominated by calcium ion binding (see below).

Under the conditions of our experiments, holothermolysin is likely to be converted, at least in part, to zinc-free apothermolysin due to the presence of EDTA in the reaction mixture. Comparisons with denaturation rates obtained for zinc-free apothermolysin samples, prepared by dialysis against 0.01 M TEP, pH 9.00, 10^{-4} M $CaCl_2$, and NaCl to ionic strength 0.1, showed no difference within experimental error. These results are in agreement with those of Holmquist and Vallee (1974), who showed that holo- and apothermolysin denature at the same rate, and indicate in addition that there are *no* autolysis contributions to the rate of the inactivation process. This conclusion can also be drawn from the observation that the thermal denaturation rates for all five enzymes were always first order in enzyme concentration with respect to time. Autolytic degradation reactions, on the other hand, are always second order in enzyme concentration with respect to time (Voordouw and Roche, 1975a,b). The formation of small peptides, noted in the thermal denaturation of thermolysin (Dahlquist et al., 1975), thus follows the rate-determining first-order denaturation step, in agreement with the scheme

TABLE II: Thermal Denaturation Rate Constants for Thermolysin at 45 °C and at Various Ratios of Total EDTA to Total Calcium Ion Concentration ($[EDTA]_T/[Ca]_T$) for Various Values of pH^{45} [a]

	$([EDTA]_T/[Ca]_T)$		
pH^{45}	4.2	3.2	2.1
3.94	1.74	1.74	1.71
5.02	3.10	3.21	3.37
5.98	3.12	3.10	3.12
6.63	2.80	2.92	2.87
7.43	2.16	2.12	2.13
8.40	1.65	1.65	1.71

[a] Conditions were otherwise similar to those described in the legend to Figure 2. The $[EDTA]_T$ was kept constant at 10^{-4} M. The tabulated data are ($-\log k_{obsd}$), k_{obsd} in s^{-1}.

proposed by these authors.

It is clear from an examination of Figures 1 and 2 that the pH dependence of k_I varies considerably for the five proteolytic enzymes investigated. It is, thus, not logical to examine the temperature dependence of k_I at the same pH value for all enzymes. We, therefore, chose to determine the latter dependence at the characteristic pH of optimal intrinsic kinetic stability for each enzyme. Since the value of this optimal pH shows little temperature dependence (Laidler and Bunting, 1973; Glasstone et al., 1941) this choice has the added experimental advantage that, at any temperature, $\partial \ln k_I/\partial$ pH $\simeq$ 0. Hence, errors in the measured k_I due to errors in pH adjustment for the different temperatures (see Methods) are judged to be minimal. The following values for the pH of optimal stability have been used: thermomycolase, 6.8 (60 °C), S. Carlsberg, 7.2 (60 °C), S. BPN′, 6.6 (60 °C), thermolysin, 6.0 (45 °C), neutral protease A, 6.5 (60 °C). The temperature dependence of k_∞, at saturating $[Ca^{2+}]$, was determined at this same pH value for each enzyme, since k_∞ is directly proportional to k_I (eq 2 and 3).

Temperature Dependence of the Denaturation Rate Constants: Determination of the Calcium Ion Contribution to the Thermal Stability. The temperature dependence of the denaturation rate constants for all five enzymes was studied at low levels of calcium ion (k_0 or k_I) and at saturating levels (k_∞) in order to determine for each enzyme: (a) the intrinsic enthalpy, $\Delta H^{\ddagger}$, and entropy, $\Delta S^{\ddagger}$, of activation, and (b) the calcium ion contributions to the total kinetic thermal stability $\Delta(\Delta H_{Ca^{2+}})$ and $\Delta(\Delta S_{Ca^{2+}})$, as outlined in the Theoretical Section. Measurements were done under the conditions outlined in the legend to Figure 3. The data are presented in Figure 3 as Arrhenius plots. The slope of these plots equals $(-\Delta H^{\ddagger}/R)$ when $\ln k_0$ and $(-\Delta H^{\ddagger}{}_T/R)$ when $\ln k_\infty$ is plotted. It is clearly seen from Figure 3 that the binding of calcium ions stabilizes all the enzymes against thermal denaturation. The free-energy change associated with this stabilization at a given temperature is proportional to the vertical distance between the two Arrhenius plots. The magnitude of this free-energy change and its temperature coefficient vary considerably among the various proteins of the group examined in this study.

Data obtained for the intrinsic activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the five enzymes are given in Table III, together with values for $\Delta H^{\ddagger}{}_T$ and $\Delta S^{\ddagger}{}_T$. The values tabulated for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for neutral protease A may be systematically

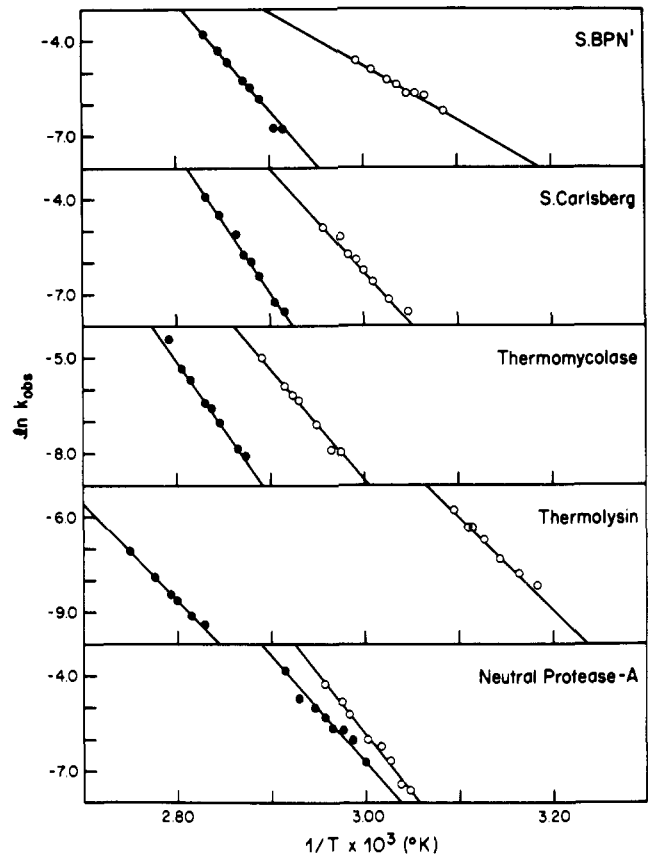

FIGURE 3: Arrhenius plots for the thermal denaturation of the various proteolytic enzymes. The temperature dependence of the intrinsic denaturation rate constant, k_I, was studied in 0.01 M Tris-acetate, 10^{-4} M EDTA, and NaCl to ionic strength 0.1 (○), except for neutral protease A, where the EDTA was omitted. The temperature dependence of the denaturation rate constant at calcium ion saturation, k_∞, was studied in 0.01 M Tris-acetate, 0.015 M $CaCl_2$, and NaCl to ionic strength 0.1 (●). Measurements were done at the pH of optimum stability for each enzyme (see text). The natural logarithm of the observed rate constant (k_{obsd} in s^{-1}) is plotted against the reciprocal of the absolute temperature.

in error due to a residual calcium contribution as explained above. From the data in Table III, the enthalpy, $\Delta(\Delta H_{Ca^{2+}})$, and entropy, $\Delta(\Delta S_{Ca^{2+}})$, contributions to the calcium ion stabilization of the five proteolytic enzymes can be readily calculated (see Theoretical Section). The results are presented in Table IV. Apart from those for neutral protease A, the data presented for thermolysin are also likely to be in error, since they have been obtained via an extrapolation of the Arrhenius plots over a large temperature interval. It is not likely that the temperature dependence of $\Delta H^{\ddagger}$ can be neglected over a temperature span of 40 °C. Consequently, a much higher value for $\Delta(\Delta S_{Ca^{2+}})$ is to be expected and also a higher, probably positive value for $\Delta(\Delta H_{Ca^{2+}})$. Errors of this type are likely to be smaller for the other enzymes, since the Arrhenius plots are linear within experimental error and the temperature extrapolation covers a much smaller span (5–19 °C). The apparent linearity (the average correlation coefficient $\bar{r} = 0.995$ for the ten lines presented in Figure 3) is a consequence of the fact that only a limited range of temperatures has been investigated. However, this does not mean that $\Delta C_P{}^{\ddagger}$ is negligible for the denaturation reactions investigated.

Discussion

The five proteolytic enzymes studied, which were all shown to bind at least one calcium ion at 25 °C (Voordouw and

TABLE III: Intrinsic Enthalpies and Entropies ($\Delta H^{\ddagger}$,$\Delta S^{\ddagger}$) and Total Enthalpies and Entropies ($\Delta H^{\ddagger}_{T}$, $\Delta S^{\ddagger}_{T}$) of Activation for the Proteolytic Enzymes, Obtained from the Arrhenius Plots in Figure 3.

Enzyme	pH	T (°K)[a]	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)	T (°K)[a]	$\Delta H_{T}^{\ddagger}$ (kcal/mol)	$\Delta S_{T}^{\ddagger}$ (eu)
S. Carlsberg	7.20	333	62.6 ± 2.8	117 ± 8	348	90.0 ± 3.4	188 ± 13
S. BPN′	6.60	329	33.4 ± 2.1	32 ± 7	348	72.0 ± 1.2	138 ± 4
Thermomycolase	6.80	341	72.8 ± 2.9	142 ± 10	353	85.4 ± 2.3	171 ± 7
Thermolysin	6.00	318	57.2 ± 2.2	107 ± 7	358	66.0 ± 1.7	109 ± 6
Neutral protease A	6.50	333	71.1 ± 3.4	143 ± 11	338	60.6 ± 4.0	110 ± 3

[a] Average temperature for which data have been obtained.

TABLE IV: Standard Enthalpy, $\Delta(\Delta H_{Ca^{2+}})$, and Entropy, $\Delta(\Delta S_{Ca^{2+}})$, Changes Associated with the Calcium Ion Stabilization of the Proteolytic Enzymes.[a]

Enzyme	pH	$\Delta(\Delta H_{Ca^{2+}})$ (kcal/mol)	$\Delta(\Delta S_{Ca^{2+}})$ EU
S. Carlsberg	7.20	−(27.4 ± 4.4)	−(71 ± 15)
S. BPN′	6.60	−(38.6 ± 2.4)	−(106 ± 8)
Thermomycolase	6.80	−(12.6 ± 3.7)	−(29 ± 12)
Thermolysin	6.00	−(8.8 ± 2.8)	−(2 ± 9)
Neutral protease A	6.50	+(10.5 ± 5.2)	+(33 ± 17)

[a] The data for neutral protease A and thermolysin are tentative for reasons outlined in the text.

TABLE V: Intrinsic ($\Delta F^{\ddagger}$) and Total ($\Delta F^{\ddagger}_{T}$) Free Energies of Activation for the Five Enzymes Studied.[a]

Enzyme	$\Delta F^{\ddagger}$	$\Delta F^{\ddagger}_{T}$	$-\Delta(\Delta F_{Ca^{2+}})$
S. Carlsberg	22.5 ± 5.5	25.5 ± 1.0	3.0
S. BPN′	22.4 ± 0.3	24.6 ± 0.2	2.2
Thermomycolase	24.1 ± 0.5	26.7 ± 0.1	2.7
Thermolysin	20.5 ± 0.2	28.6 ± 0.4	8.1
Neutral protease A	22.0 ± 0.4	22.9 ± 3.0	0.8

[a] Calculated from Table III for 70 °C. The calcium ion contribution, $\Delta(\Delta F_{Ca^{2+}})$, to the thermal stability is obtained using eq 7. Units are kcal/mol.

Roche, 1974, 1975a; the calcium binding data for the subtilisins and neutral protease A will be submitted for publication), are all stabilized by calcium against thermal denaturation (Figure 3, Tables III and IV). The data obtained allow us to separate the total free energy of activation for the denaturation process into an intrinsic and a bound calcium ion contribution at a given temperature. As an example, we show in Table V the magnitudes of these contributions to the thermal stability of the various enzymes at 70 °C, calculated from the data in Table III. The large calcium ion contribution to the thermal stability of thermolysin, apparent also from the large vertical distance between the two Arrhenius plots for this enzyme (Figure 3), is undoubtedly the most striking feature of the results.

Thermomycolase is the most "thermostable" enzyme of the serine protease group, mostly due to its relatively high intrinsic stability (Figure 1 and Table V) under the given experimental conditions. The calcium ion contributions are very similar for these three enzymes in this temperature range. The differences in "thermostability" are not very large for the three serine proteases. Because the distribution of kinetic thermal stabilities is likely to be continuous, the classifications "thermostable" and "nonthermostable" are completely arbitrary. Since this point has been ignored by earlier workers and a dividing line is necessary in order to make the terms somewhat more meaningful the following definition is proposed: thermostable enzymes are those that have total kinetic thermal stabilities $\Delta F^{\ddagger}_{T} \geq 25.0$ kcal mol^{-1} at 70 °C under conditions (pH, pCa^{2+}, ionic strength, etc.) that maximize their total kinetic thermal stability. Under this definition we see that S. Carlsberg just qualifies as a thermostable enzyme, S. BPN′ is 0.5 kcal mol^{-1} short, while thermomycolase and thermolysin are well above, and neutral protease A is well below the 25.0 kcal mol^{-1} dividing line.

As was pointed out in the Results section, the separation of $\Delta F^{\ddagger}_{T}$ into $\Delta F^{\ddagger}$ and $\Delta(\Delta F_{Ca^{2+}})$ for both thermolysin and neutral protease A is likely to be systematically in error. In the case of neutral protease A the $[Ca^{2+}]$ may not have been low enough for the measurement of truly intrinsic rates, since EDTA was omitted in the k_i determination for the reasons outlined in the Methods and Results sections. The calcium ion contribution may thus be somewhat larger, and the intrinsic contribution similarly smaller than shown in Table V. The absolute value of $\Delta F^{\ddagger}_{T}$ remains unaffected, however.

The possible systematic error in the values of $\Delta F^{\ddagger}$ and $\Delta(\Delta F_{Ca^{2+}})$ for thermolysin has quite a different origin. The neglect of the temperature dependence of both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ may not be justified, since protein denaturation reactions are frequently characterized by high heat capacities of activation ($\Delta C_p^{\ddagger}$). This poses problems in the extrapolation (40 K) of the Arrhenius plot for thermolysin in the absence of calcium from its average temperature range (318 K, see Table III) into the temperature range of the Arrhenius plot obtained at saturating calcium levels (358 K). Values for $\Delta C_p^{\ddagger}$ are known to vary widely for proteins, depending on how closely the structure of the activated intermediate state (X), which is the state of highest free energy along the unfolding pathway, resembles either N or D. The upper limit is probably in the range 2000 cal mol^{-1} deg^{-1}, which is the order of magnitude of the increase in heat capacity upon complete denaturation (Tanford, 1968, 1970); X is then close to D. The lower limit may be smaller than 100 cal mol^{-1} deg^{-1} (Lumry and Biltonen, 1969), justifying neglect of $\Delta C_p^{\ddagger}$ corrections even over fairly large temperature ranges; X is then close to N. Although we cannot estimate $\Delta C_p^{\ddagger}$ from the data presented in Figure 3, it is important to see how a $\Delta C_p^{\ddagger}$ of say 300 cal mol^{-1} deg^{-1} affects the calcium ion and intrinsic contributions to $\Delta F^{\ddagger}_{T}$. For the intrinsic enthalpy and entropy of activation at 358 K we then obtain, by extrapolation from 318 K: $\Delta H^{\ddagger}$ (358) = 69.2

kcal/mol, $\Delta S^{\ddagger}$ (358) = 145 eu. Combined with $\Delta H^{\ddagger}_T$ (358) = 66.0 kcal/mol and $\Delta S^{\ddagger}_T$ (358) = 109 eu this gives $\Delta(\Delta H_{Ca^{2+}}) = +3.2$ kcal/mol and $\Delta(\Delta S_{Ca^{2+}}) = +36$ eu. These corrected enthalpy and entropy contributions to the calcium ion stabilization of thermolysin differ from the data in Table IV both in sign and magnitude. Since the changes in $\Delta(\Delta H_{Ca^{2+}})$ and $\Delta(\Delta S_{Ca^{2+}})$ compensate at least in part, the calcium ion contribution, $\Delta(\Delta F_{Ca^{2+}})$, to the total kinetic thermal stability is not so sensitive. At 70 °C we calculate from the data for thermolysin $\Delta(\Delta F_{Ca^{2+}}) = -8.1$ kcal/mol (Table V), whereas we obtain by using the corrected data $\Delta(\Delta F_{Ca^{2+}}) = -9.2$ kcal/mol. The main conclusion to be drawn from this analysis is, thus, that by neglecting $\Delta C_p^{\ddagger}$ corrections (Table V), we may *underestimate* the $\Delta(\Delta F°_{Ca^{2+}})$. For thermolysin the latter contribution may be a few kcal/mol larger than that shown in Table V, depending on the value of $\Delta C_p^{\ddagger}$ chosen to correct the data. Consequently, we may conclude that the well known kinetic thermal stability of thermolysin is due *only* to an unusually large calcium ion contribution to $\Delta F^{\ddagger}_T$: the intrinsic contribution ($\Delta F^{\ddagger}$) of thermolysin to $\Delta F^{\ddagger}_T$ is lower than that of any of the serine enzymes and is similar in magnitude to that of neutral protease A. The kinetic thermal stability of thermolysin is, therefore, dominated by the $\Delta(\Delta F_{Ca^{2+}})$ contribution of its bound calcium.

We have shown already that of the four calcium binding sites in thermolysin only *one* contributes to the kinetic thermal stability (Voordouw and Roche, 1975b). This site is apparently of high affinity even at high temperatures. From the data in Table IV, after calculation of $\Delta(\Delta F_{Ca^{2+}})$ and using eq 4 with $p = 1$, we obtain $K_N/K_X \simeq 10^5$ for this site in the range 70–80 °C. Since we have found that $K_X = 600$ M^{-1} at 80 °C (Voordouw and Roche, 1975b), we conclude that this site has a binding constant $K_N \simeq 6 \times 10^7$ M^{-1} even at these high temperatures. Thus, the binding of calcium ion to and the stabilizing effect of this site are characterized by only small enthalpy changes, causing little temperature dependence (see also Table IV). In contrast, the binding and the stabilizing effect of the single calcium ion, so tightly bound by S. BPN′ ($K_{ECa} > 10^{11}$ M^{-1} at 25 °C), are completely enthalpy driven (Table IV: $\Delta(\Delta H°_{Ca^{2+}})$ large and negative) leading to a strong temperature dependence and therefore little stabilization in the high-temperature region. The difference is most clearly seen from Figure 3: whereas the two Arrhenius plots for S. BPN′ converge rapidly with increasing temperature the plots for thermolysin are nearly parallel.

We do not want to speculate on the origins of these differences *on a molecular level,* nor do we want to expand further on which calcium binding site in thermolysin may be involved beyond what has been said in a previous paper (Voordouw and Roche, 1975b). The most important conclusion that can be drawn from the work presented here is that by *measuring* intrinsic and calcium ion contributions and by interpreting the data in the way described above definitive statements can be made with regard to the reasons for the kinetic thermal stability of thermolysin. It is doubtful whether such conclusive results could be obtained on the basis of the presently available structural data alone. The latter are, however, a necessary prerequisite for possible interpretations on a molecular level. Thus, it will be interesting to see whether the conclusion drawn in this paper that the difference in kinetic thermal stability between thermolysin and neutral protease A is mainly due to different calcium ion contributions, can be interpreted on a molecular level by, for example, the absence of the surface calcium binding sites Ca(3) and/or Ca(4) in neutral protease A after solving the latter structure by x-ray crystallographic methods. Evidence for the absence of Ca(4) in neutral protease A is accumulating from sequence (Pangburn et al., 1975, 1976) and calcium binding studies of this protein (Voordouw and Roche, to be submitted for publication).

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Binding of Adenosine 3′,5′-Monophosphate Dependent Protein Kinase Regulatory Subunit to Immobilized Cyclic Nucleotide Derivatives†

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ABSTRACT: Several cyclic nucleotide derivatives with aminoalkyl side chains attached to the purine ring were synthesized and their interactions with adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase were studied before and after immobilization to CNBr-activated Sepharose 4B. The soluble N^6-substituted derivatives were as effective as cAMP itself in activating protein kinase and were more effective than 8-substituted cAMP derivatives, whereas the 2-substituted cAMP derivatives and the cGMP derivatives were the least effective. All of the synthetic derivatives tested were poor substrates for beef heart phosphodiesterase being hydrolyzed at rates less than 2% for that of cAMP itself. Utilizing methodology developed to evaluate the affinity of protein kinase for immobilized cyclic nucleotides it was found that all of the immobilized cyclic nucleotides interacted with protein kinase in a biospecific manner as judged by the following criteria: (1) the immobilized cyclic nucleotides competed with cAMP for the binding sites on protein kinase; (2) the analogous spacer-arm did not compete; and (3) the effects of enzyme concentration, MgATP, and cleavage of the cyclic phosphate ring on the interactions of protein kinase with the immobilized cyclic nucleotides were the same as previously shown for free cAMP. In addition, the immobilized ligands were bound with the same order of effectiveness as the analogous soluble ligand. The observed K_a for the activation of 0.005 μM protein kinase by N^6-$H_2N(CH_2)_2$-cAMP was increased from 0.23 to 3 μM by the process of immobilization. This increase was unaffected by the coupling density and spacer-arm length. The observed K_b for 0.10 μM protein kinase binding to immobilized N^6-$H_2N(CH_2)_2$-cAMP was increased as the molecular sieving exclusion limit of the matrix used was decreased indicating that at least part of this decrease in apparent affinity upon immobilization is due to exclusion of the enzyme from a portion of the matrix and therefore from a fraction of the immobilized ligand molecules.

The enzyme, cAMP[1]-dependent protein kinase (EC 2.7.1.37), has been shown to exist as a tetramer having two different types of subunits, a catalytic subunit (C) which catalyzes the phosphorylation reaction, and a cAMP binding regulatory subunit (R) which inhibits the activity of the catalytic subunit in the absence of cAMP (Erlichman et al., 1973; Beavo et al., 1975). The mechanism for the activation of the enzyme involves its dissociation by cAMP as shown below:

$$R_2C_2 \text{ (inactive)} + 2\text{cAMP} \rightleftharpoons 2C \text{ (active)} + R_2(\text{cAMP})_2 \quad (1)$$

Several studies on the purification of the regulatory subunit by affinity chromatography using a variety of immobilized cAMP derivatives have been carried out (Wilchek et al., 1971; Cuatrecasas, 1972; Tesser et al., 1974; Jergil et al., 1974; Ramseyer et al., 1974; Dills et al., 1975a,b; Rieke et al., 1975). In these studies it was observed that the protein kinase was dissociated by the immobilized cyclic nucleotides but in only a few cases were successful elutions of the bound regulatory subunit described (Ramseyer et al., 1974; Dills et al., 1975a,b; Rieke et al., 1975). In order to develop better methodology for the purification of this protein, as well as to gain a better understanding of certain quantitative aspects of affinity chromatography in general, the binding of protein kinase to a variety of immobilized cyclic nucleotides was studied.

Experimental Section

Chemicals. Polyox was obtained from Union Carbide; 6-Cl-cPRMP, 8-Br-cAMP, and 8-Br-cGMP from Boehringer-Mannheim, [8-^{3}H]cAMP from Schwarz/Mann, 1,9-diaminononane from Chemical Procurement, Inc., and the other alkyl diamines and 2-aminoethanethiol hydrochloride from Aldrich. 2-Cl-cAMP was a gift from Dr. M. Helen Maguire of the University of Kansas Medical Center. CNBr-activated Sepharose 4B was obtained from Pharmacia and was swollen in 5 volumes of 1 mM HCl for 15 min before use. Sephadex G-10 and Sephadex G-150 from Pharmacia and Bio-Gel A-150 from Bio-Rad were activated with CNBr by the method

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[1] The abbreviations used are: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; cIMP, inosine 3′,5′-monophosphate; cPRMP, purine riboside 3′,5′-monophosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetracetic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.