between UpcA which is monoanionic as are the substrates of RNase A and dianionic mononucleotides 2′-CMP and 3′-CMP. The significance of the results obtained in the present work in terms of RNase T_1 catalysis will be the subject of a future study.

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Melting-Profile Analysis of Thermal Stability of Thermolysin. A Formulation of Temperature-Scanning Kinetics†

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ABSTRACT: The melting-profile method consists of a continuous observation of a structural parameter while the temperature of the sample is raised at a constant rate [Fujita, S. C., & Imahori, K. (1974) in *Peptides, Polypeptides and Proteins* (Blout, E. R., Bovey, F. A., Goodman, M., & Lotan, N., Eds.) p 217, Wiley, New York, N.Y.]. An analytical solution to the melting profile was formulated for the two-state irreversible process and called temperature-scanning kinetics. The theory was tested with thermolysin with consistent results, and the thermodynamic parameters of thermal denaturation were calculated: $\Delta H^{\ddagger}$ = 80.3 kcal/mol, $\Delta S^{\ddagger}$ = 153 eu. These values agreed with the corresponding values obtained from the classical constant-temperature relaxation kinetics. The possibilities of temperature-scanning kinetics are discussed.

Melting curves are commonly used to characterize thermal stability of biological macromolecules. They are obtained by plotting some structural parameter such as ultraviolet absorbance or ellipticity of the sample as a function of temperature. The midpoint of transition is generally designated as the melting temperature or T_m to serve as a measure of thermal stability. When in such an experiment the rate of temperature rise is held at a constant value, a plot of a structural parameter against time gives essentially the same curve (as the plot against temperature), which we called the melting profile and proposed as a convenient method to characterize the thermal stability of proteins (Fujita & Imahori, 1974).

In interpreting and evaluating such a type of experiment, reversibility of the transition is an important question. When it is reversible within the time scale of the experiment, the measurements can be made at equilibrium and thermodynamic

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analysis is possible, as has been successfully made for many systems (Tanford, 1968). With an irreversible system, however, the kinetic aspect of the transition can no longer be neglected, and melting curves or melting profiles obtained are dependent on the mode and rate of temperature change. Thus, T_m values and other parameters of the curves were not amenable to thermodynamic analysis in the absence, until now, of a theoretical framework for the irreversible systems.

We have successfully provided a theoretical basis for the dependence of the melting profile and $T_{1/2}$ (which is analogous to T_m) on the rate of temperature rise for the simple case of a two-state irreversible process. The theory, which we propose to call temperature-scanning kinetics, allows calculation of the values of the thermodynamic parameters for the kinetic constants of the process. The present report gives the theory and demonstrates its validity by applying it to the analysis of thermal stability of thermolysin, whose thermal denaturation is known to be first order in enzyme concentration (Voordouw & Roche, 1975).

Theoretical Section

For a two-state endothermic irreversible process N → D assume that the Arrhenius relationship holds for the rate constant k in the temperature range of interest, i.e.

$$k = \exp[A - (E/RT)] \tag{1}$$

where A and E are constants, R is the gas constant, and T is the absolute temperature. (We here treat the endothermic process, but the theory obviously applies to the exothermic process as well.) Starting with a temperature low enough so that k is negligibly small, the temperature of the system is raised at a constant rate v, such that

$$T(t) = T_c + v(t - t_0)$$

where t is the time, t_0 is the time at which $T = T_c$, and T_c is the temperature at which $k = 1$. Rewriting eq 1 with approximation, we have

$$k(T) \simeq \exp\left[\left(A - \frac{E}{RT_c}\right) + \frac{Ev}{RT_c^2}(t - t_0)\right]$$

This approximation is justified, as $v(t - t_0)$ is less than 10% of T_c throughout the temperature range of transitions in the experiments described below. Since the first term in the exponent is 0 by definition of T_c, we obtain

$$k(T) = e^{\alpha v(t - t_0)} \tag{2}$$

with $\alpha \equiv E/RT_c^2$. Letting c stand for the molar fraction of the molecules in the N state, $c = [N]/([N] + [D])$, we have by definition

$$dc/dt = -k[T(t)]c \tag{3}$$

Upon integration with the obvious initial condition $c(t = 0) = 1$, we obtain

$$\ln c(t) = -\int_0^t k[T(t)]\, dt$$

and with eq 2

$$\ln c(t) = \frac{1}{\alpha v}\exp(-\alpha v t_0)[1 - \exp(\alpha v t)] \tag{4}$$

Since $\exp(\alpha v t) \gg 1$ when t is in the neighborhood of t_0, eq 4 gives with approximation

$$\ln c(t) \simeq -\frac{1}{\alpha v}\exp[\alpha v(t - t_0)]$$
$$\simeq -\frac{1}{\alpha v}\exp[\alpha(T - T_c)] \tag{5}$$

This approximation is valid since $\alpha v t$ is much larger than 10 in the temperature range of transitions in the experiments reported below.

Equation 5 has two implications. (1) Since $T = T_{1/2}$ when $c = {}^1/_2$, we obtain

$$\ln {}^1/_2 = -\frac{1}{\alpha v}\exp[\alpha(T_{1/2} - T_c)]$$

Rearrangement gives

$$T_{1/2} = T_c + \frac{1}{\alpha}\ln(\alpha v \ln 2) \tag{6}$$

Equation 6 implies that if one plots $T_{1/2}$ values obtained from a series of experiments with different v's against $\ln v$, one should obtain a linear relationship, the reciprocal slope of which gives the value of α. When α is known, T_c can be calculated from eq 6. The values of A and E can be calculated through the relations $E = \alpha RT_c^2$ and $A = \alpha T_c$ (v variation analysis).

(2) Equations 5 and 6 give upon elimination of T_c

$$\ln c = -(\ln 2)e^{\alpha(T - T_{1/2})} \tag{7}$$

which implies that c as a function of $T - T_{1/2}$ is independent of the heating rate v. Thus the melting profiles obtained in a series of experiments with varying v should be superimposable over each other. Since eq 7 can be rearranged as

$$\ln\left(-\frac{\ln c}{\ln 2}\right) = \alpha(T - T_{1/2}) \tag{8}$$

one should be able to obtain the value of α from a plot of ln (−ln c/ln 2) vs. $T - T_{1/2}$, data points being derived from any single melting profile. With α known, together with v given in the experiment, one can calculate the values of T_c, A, and E in the same manner as in implication 1 (slope analysis).

Whether these two predictions are borne out in melting-profile experiments and the values of A and E calculated in the above two ways agree with each other is a good test of the validity of underlying assumptions made in the formulation of the temperature-scanning kinetics developed above.

Materials and Methods

Materials. Thermolysin [EC 3.4.24.4] (crystallized three times, lot T31B71) was obtained from Daiwa Kasei, Osaka, and was used as supplied. A 1.3 mg/mL stock solution of thermolysin was prepared after the recommendation of Matsubara (1970) and stored frozen at −20 °C until use. In most of the following experiments the enzyme concentration was 0.16 mg/mL. The concentration of thermolysin was calculated from the absorbance at 280 nm, the absorption coefficient of $\epsilon_{280nm}^{0.1\%} = 1.77$ derived from $\epsilon_M = 66\,300$, and the molecular weight of 37 500 reported by Ohta et al. (1966). Milk casein (according to Hammersten) was a product of Merck, Darmstadt, Germany. All other chemicals were reagent grade and were purchased from Wako Pure Chemicals, Osaka. Glass-distilled water was used throughout the study.

Assay. Caseinolytic activity was measured by the procedure of Matsubara (1970). The reaction mixture (2 mL) was 1% in casein, 5 mM in $CaCl_2$, and 0.05 M in Tris-HCl buffer, pH 8.0. After 10 min of reaction at 35 °C, the increase of absorbance at 280 nm of Cl_3AcOH[1] soluble fraction was read with a Gilford Model 240 spectrophotometer. Enzyme activity

[1] Abbreviations used: CD, circular dichroism; Cl_3AcOH, trichloroacetic acid.

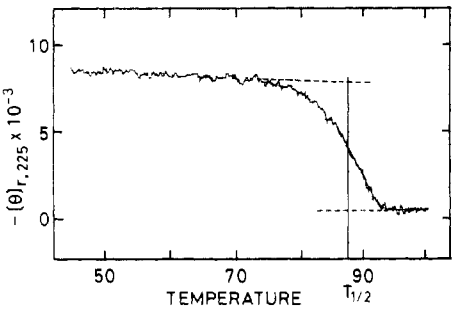

FIGURE 1: The melting profile and the definition of $T_{1/2}$.

was read from the standard curve constructed with known amounts of the enzyme solution.

Calcium Analysis. The concentration of calcium was measured by flame emission at 4227 Å with a Seiko atomic absorption spectrophotometer, Model SAS721.

Circular Dichroism. Circular dichroic (CD) spectra were obtained with a JASCO automatic recording spectropolarimeter, Model J20, which was also employed for the melting-profile analysis.

Refined Melting-Profile Method. The melting-profile analysis consists of a continuous measurement and recording of some structural parameter of the sample while the temperature of the sample is raised at a constant rate (Fujita & Imahori, 1974). In the following study with thermolysin the ellipticity at 225 nm was employed as the structural parameter. The sample (400 μL) was placed in a quartz cell with a 3-mm light path which was embedded in a brass jacket, through which ethylene glycol circulated. The temperature of the ethylene glycol was raised by the use of a Haake bath. The temperature of the sample was directly monitored by a thermistor sensor penetrating the silicone rubber cap to the sample cell. The temperature signal was fed into the X axis of a Rikendenshi XY recorder, Model D-72B, via a Takara thermistor linearizer, Model E311. The CD signal output from the spectropolarimeter was fed into the Y_1 axis of the recorder. Heating rate was recorded by feeding into the Y_2 axis the signal from the time monitor which generated output voltage linearly increasing with time. The whole system was operated in a room maintained at 25 ± 2 °C. The resulting melting profile as a function of temperature is illustrated in Figure 1. As a measure of thermal stability $T_{1/2}$ is defined as the temperature at which the melting profile crosses the midpoint between the two values extrapolated from the native and denatured regions. $T_{1/2}$ is generally dependent on the rate of temperature rise (heating rate). $T_{1/2}$ values obtained were reproducible within 0.1 °C.

Results

Circular Dichroic Spectra. The CD spectrum of the native thermolysin at 25 °C in the 200–250-nm region is given in Figure 2. There is a minimum at 210 nm and a prominent shoulder around 220–225 nm, which is indicative of considerable α-helical structure in accord with the finding of the X-ray analysis (Colman et al., 1972). When the temperature of the sample was brought to and held at 89 °C, the above features of the spectrum were gradually lost until spectrum 2 of Figure 2 was obtained. When the sample was cooled, rapidly or gradually (taking about 2 h), to 25 °C, spectrum 3 was obtained. No turbidity was observed to develop. The sample exhibited spectrum 2 upon reheating the denatured sample to around 90 °C. Since spectrum 3 is reminiscent of that exhibited by poly-L-lysine in random coil conformation (Greenfield & Fasman, 1969), it was concluded that most, if

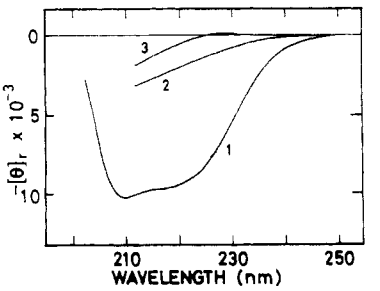

FIGURE 2: Circular dichroic spectra of native and denatured thermolysin in 0.05 M Tris buffer, pH 8.2, and 7.5 mM $CaCl_2$. (1) Native thermolysin at 25 °C; (2) spectrum at 90 °C after thermal denaturation; (3) spectrum at 25 °C after thermal denaturation.

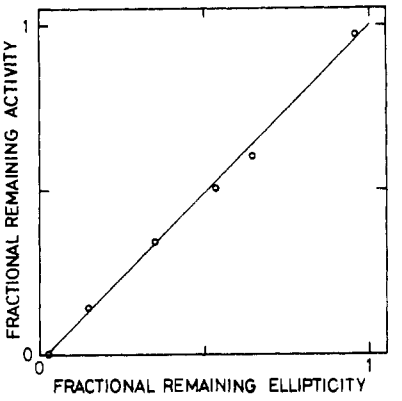

FIGURE 3: Relation between remaining activity and remaining ellipticity at 225 nm of thermolysin after heating interruption. Both quantities are expressed as the fraction of those exhibited by the sample not subjected to melting-profile experiments.

not all, of the ordered secondary structure of thermolysin is lost upon thermal denaturation.

Interrupted Denaturation. To study the nature of the product of partial denaturation of thermolysin, heating of the sample was interrupted at various points along the melting profile. The sample cell was quickly withdrawn from the spectropolarimeter and chilled in ice–water. The sample was examined at room temperature for the remaining ellipticity at 225 nm. After each experiment a 50-μL aliquot was withdrawn for assay of the remaining caseinolytic activity as described under Materials and Methods. Figure 3 summarizes the relationship obtained between the remaining activity and the remaining ellipticity at 225 nm of partially heat-denatured samples of thermolysin expressed as fractions of those of the native enzyme.

In some of the experiments the interrupted samples were subjected to second melting-profile experiments. These samples exhibited the melting profiles with $T_{1/2}$ values indistinguishable from those in uninterrupted experiments.

Dependence of $T_{1/2}$ on Calcium Concentration. The well-known stabilizing effect of calcium ion was confirmed and found to be saturated at around 7 mM $CaCl_2$ in terms of $T_{1/2}$ (Figure 4). No further change in melting profile was observed with higher levels of $CaCl_2$ up to 66 mM.

Remaining Ellipticity Kinetics. The ellipticity at 225 nm of thermolysin at 25 °C remaining after incubation at various temperatures was followed as a function of the time of heat treatment. Aliquots of thermolysin (500 μL, 0.16 mg/mL) in 50 mM Tris-HCl buffer, pH 8.2, and 7.5 mM $CaCl_2$ were placed in test tubes (1 × 10 cm) and subjected to heat treatment in a water bath. The treatment temperature was controlled to within 0.1 °C. After suitable time intervals the tubes were withdrawn and quickly chilled in ice–water. Remaining ellipticity at 225 nm was measured after standing

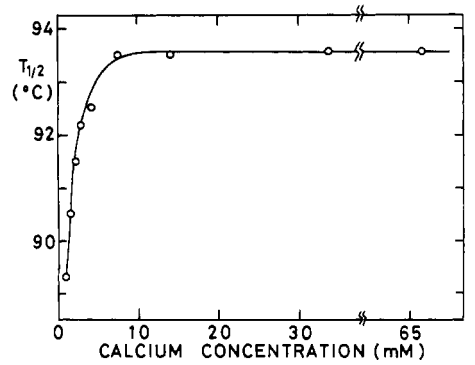

FIGURE 4: Effect of calcium concentration on $T_{1/2}$ of thermolysin in 0.05 M Tris buffer, pH 8.2. The total calcium concentration was determined by the flame emission method. The heating rate was 0.086 deg s^{-1}.

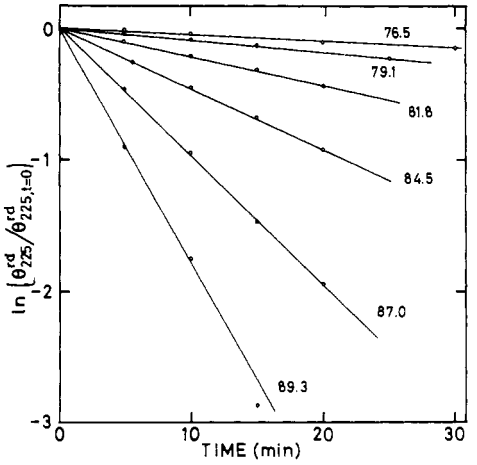

FIGURE 5: Kinetics of irreversible loss of ellipticity at 225 nm of thermolysin incubated at constant temperature in 0.05 M Tris buffer, pH 8.2, and 7.5 mM $CaCl_2$. Θ^{rd} is defined in the text. The numbers in the figure indicate the temperature of incubation in centigrade.

6 h at 25 °C. Remaining destructible ellipticity (Θ^{rd}) is defined as

$$\Theta^{rd} = \Theta^{r} - \Theta^{r}_{\infty}$$

where Θ^{r} refers to the measured ellipticity and Θ^{r}_{∞} to the ellipticity of the completely denatured sample. The latter value is small at 225 nm for thermolysin (see Figure 2). When Θ^{rd} was plotted against time in a semilogarithmic fashion, Figure 5 was obtained. The figure shows that the decay in Θ^{rd} is first order with respect to time. The first-order rate constants obtained from the figure are represented by open circles in Figure 6.

Temperature-Jump Kinetics. The solution of thermolysin (0.16 mg/mL, 0.05 M Tris-HCl buffer, pH 8.2, 7.5 mM $CaCl_2$) was rapidly brought to a constant temperature in a jacketed cuvette placed in the spectropolarimeter. The points were taken from the automatic recordings of ellipticity at 225 nm. They followed an exponential decay kinetics after the temperature reached within 0.1 °C of the final constant value. The rate constants were obtained from the semilogarithmic plots and are represented by filled circles in Figure 6.

The points from the above two types of constant-temperature relaxation experiments, represented by open and filled circles in Figure 6, are in close agreement.

v Variation Analysis. The melting profiles were obtained from experiments with various values of heating rate (v) ranging from 0.0049 to 0.094 deg s^{-1}. The melting profiles were superimposable over each other. When $T_{1/2}$ values were plotted against ln v, Figure 7 was obtained. The points fell

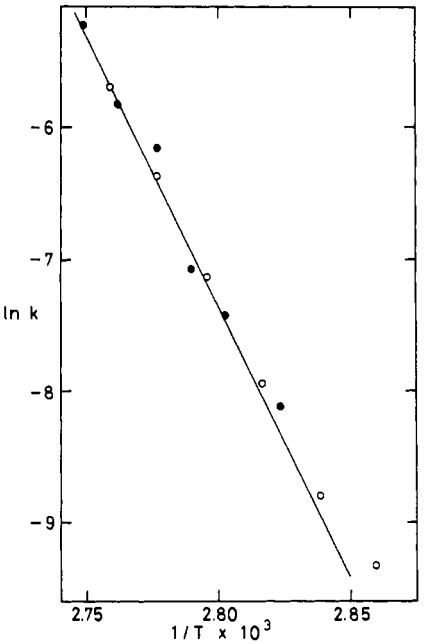

FIGURE 6: Comparison of the first-order rate constants obtained by the CD melting-profile, temperature-scanning kinetic analysis and those by conventional constant-temperature relaxation kinetic analyses. The solid line represents the result of v variation analysis, while open and filled circles show the results of the remaining ellipticity kinetics and the temperature-jump kinetics, respectively.

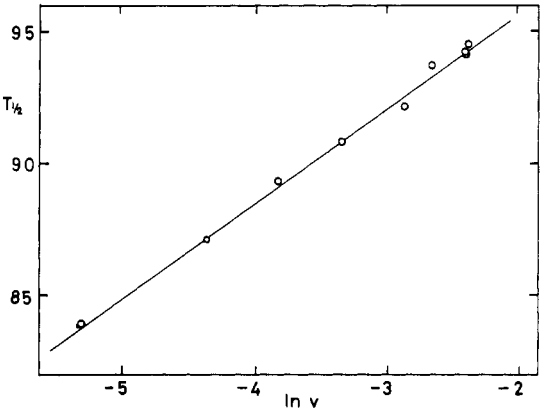

FIGURE 7: The v variation analysis. The $T_{1/2}$ values of the melting profiles obtained with various values of v ranging from 0.0049 to 0.094 deg s^{-1} are plotted against ln v.

on a straight line, giving α = 0.280 deg^{-1}, T_c = 381.7 K, E = 81.0 kcal/mol, and A = 106.8, corresponding to $\Delta H^{\ddagger}$ = 80.3 kcal/mol and $\Delta S^{\ddagger}$ = 153.4 eu. A line corresponding to these values is drawn in Figure 6 to show the close agreement with the results of the conventional constant-temperature relaxation kinetics.

Slope Analysis. The data points taken at 1° intervals from the denaturing region of the melting profile with v = 0.0049 deg s^{-1} were replotted according to eq 8 to yield Figure 8. The points fell on a straight line as predicted, giving α = 0.281 deg^{-1}, T_c = 382.0 K, E = 81.3 kcal/mol, and A = 107.1, corresponding to $\Delta H^{\ddagger}$ = 80.6 kcal/mol and $\Delta S^{\ddagger}$ = 154.0 eu. These values are in good agreement with those obtained by v variation analysis.

Discussion

The theory of temperature-scanning kinetics developed above offers a sound basis, heretofore unavailable, for the analysis of melting profiles of irreversible processes. As the relation between the heating rate and the observed $T_{1/2}$ of the

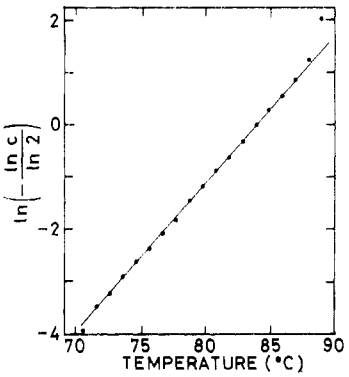

FIGURE 8: The slope analysis of a melting profile of thermolysin. The data points from the denaturing region of the melting profile with v = 0.0049 deg s^{-1} are replotted according to eq 8.

melting profile is given by eq 6, the values of thermodynamic parameters of the process can be obtained from melting profiles at different heating rates. Equation 8 further shows that a single melting experiment can give the same values. The latter fact demonstrates that the new method can extract more information from a limited amount of material than the conventional constant-temperature relaxation kinetics.

Our circular dichroic studies have confirmed that thermolysin undergoes an irreversible thermal denaturation. Furthermore the two-state nature of the process of the thermal denaturation of thermolysin is suggested by the facts that (1) a linear relationship was found between activity and ellipticity of partially denatured thermolysin (Figure 3), (2) the second melting experiment gave the normal $T_{1/2}$ value, (3) the ellipticity was lost according to first-order kinetics at constant temperature (Figures 5 and 6), and (4) the melting profiles recorded at three different wavelengths, 215, 225, and 235 nm, gave the essentially identical $T_{1/2}$ value (unpublished observation). This agrees with earlier findings by Voordouw & Roche (1975). The thermal stability of thermolysin as a function of calcium ion concentration (Figure 4) is consistent with the result of inactivation kinetics reported (Voordouw & Roche, 1975), the stabilizing effect saturating somewhat below 10 mM Ca^{2+}.

The temperature-scanning kinetics as formulated for the two-state irreversible process was tested with thermolysin with 7.5 mM $CaCl_2$. When $T_{1/2}$ was plotted against ln v, a straight line was obtained in accordance with the theoretical prediction (v variation analysis). When the melting profile was replotted according to eq 8, a straight line was obtained as the theory requires (slope analysis). $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values calculated from the two independent analyses are tabulated in Table I together with the corresponding values obtained from classical constant-temperature relaxation kinetics (Figures 5 and 6). Although the values from constant-temperature kinetics are somewhat smaller than those from temperature-scanning kinetics, it is shown in Figure 6 that the data from both types of experiments agree with each other. This agreement supports the validity of the assumptions made in the formulation and in its application to thermolysin, showing the usefulness of temperature-scanning kinetics and confirming the essentially two-state nature of thermal denaturation of thermolysin.

A recent work of Voordouw et al. (1976) on the stability of thermolysin found that the thermal denaturation at pH(45 °C) 6.00 is accompanied by $\Delta H^{\ddagger}$ = 66.0 kcal/mol and $\Delta S^{\ddagger}$ = 109 eu, which are smaller than our values of $\Delta H^{\ddagger}$ = 80.3 kcal/mol and $\Delta S^{\ddagger}$ = 153 eu from v variation analysis at pH(25 °C) 8.2. Whether the difference is related to the different pH used remains to be seen.

Table I: Enthalpies and Entropies of Activation ($\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$) for Thermal Denaturation of Thermolysin[a]

method	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)
temperature scanning kinetics		
v variation analysis	80.3	153
slope analysis	80.6	154
constant temperature relaxation kinetics	76.4	143

[a] In 0.05 M Tris-HCl, pH(25 °C) 8.2, and 7.5 mM $CaCl_2$.

Among many possible applications of the melting-profile analysis will be the study of forces that stabilize the higher structure of proteins and protein–ligand interaction (Fujita, 1978). Enthalpic and entropic contributions would be conveniently estimated by the slope analysis (eq 8). In the present state of limited experience, we are not fully aware in what aspect temperature-scanning kinetics is more informative than or complementary to the conventional constant-temperature kinetics.

Although we have shown the usefulness of the temperature-scanning kinetics only for a simple case of a two-state irreversible process, it will be possible to extend the theory to more complex kinetic processes. Computer simulation may be of help in the last resort. The melting-profile method as described above is also open to further technical refinements. For example, the reproducibility of the melting profile with the present setup is most dependent upon the reproducibility of the heating rate. If a computer-controlled heating system is available, the quality of the data should be improved. The melting-profile method is applicable with any structural parameter of the sample that can be continuously monitored, be it ultraviolet absorption, fluorescence emission, etc.

Although we illustrated the temperature-scanning kinetics with a slow process, the theory applies to rapid processes as well. An apparatus that can give a linear temperature elevation in milliseconds will be useful. The melting-profile method together with temperature-scanning kinetics will find a variety of applications.

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