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EQUILIBRIUM AND KINETICS OF THE THERMAL UNFOLDING OF α-LACTALBUMIN. THE RELATION TO ITS FOLDING MECHANISM.

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The thermal unfolding of α-lactalbumin has been studied by equilibrium measurements of aromatic difference spectra, and by kinetic measurements of the Joule heating temperature-jump. The unfolding at neutral pH is a reversible two-state transition. The equilibrium transition curves are analyzed by the nonlinear least squares method, which gives correct values of thermodynamic parameters based on the data in a wide range of temperature. The results are discussed in relation to the previous studies on the unfolding by guanidine hydrochloride or by acid. The thermally unfolded state, a partially unfolded species, is shown to be thermodynamically similar to but not identical with the acid state. The folding pathway deduced from the kinetic results is essentially consistent with the folding model of α-lactalbumin proposed previously. Large decreases in entropy and in heat capacity during the reversed activation suggest the packing of the folded segments by hydrophobic interactions, while the forward activation shows a marked temperature dependence, probably caused by the disruption of specific long-range interactions.

1. Introduction

The mechanism whereby proteins fold into their native three-dimensional conformations, under the direction of the amino acid sequence, has been a long sought after goal [1]. A direct way to observe the protein folding is to measure the reversible transition to the native molecule from the structureless polypeptide produced by denaturation [2,3]. As yet, the available data are not sufficient, however, to warrant any conclusion on the mechanism of folding. Most denaturation processes are known to be so highly cooperative [4–6] that the equilibrium measurements, represented by a simple two-state approximation, can give little information about the folding pathway [7]. In such cases, kinetic studies on the protein denaturation by means of rapid reaction techniques may constitute an effective approach to the above problem because much information about reaction pathways is available from the kinetic analysis of unfolding and refolding.

As part of a search for the above problem, we have studied the equilibria and kinetics of the reversible unfolding of bovine and human α-lactalbumins by treatment with acid and with denaturants [8–15]. One of the important features of denaturation has been found in the studies on the unfolding by guanidine hydrochloride, which has demonstrated that the proteins in the denaturant solution undergo a two-stage denaturation involving a stable intermediate [10,14,15]. The quantitative analysis of the denaturation reactions has shown that the above intermediate is identical, in a first approximation, with the denatured state by acid (the A state), which is a quasi-native species, i.e. not completely unfolded, comparable in backbone secondary structures to the native (N) state but rather expanded [8,10]. The transition between the N and the A states was followed kinetically by the stopped-flow pH-jump method by measuring the rapid changes in absorbance [9,11,15] and in circular dichroism [12,15]. On the basis of these experiments, the present authors have proposed a probable model of folding of α-lactalbumin, which can be divided into three steps [14,15]; (i) the incipient formation of backbone helical structures dictated by local interactions, (ii) the packing of the helical segments accompanied with hydrophobic interactions, (iii) the final stabilization by specific long-range

interactions; and this model is also consistent with the theoretical predictions of protein folding presented by other researchers [16,17].

The aim of the present study is to characterize the thermal unfolding of bovine α-lactalbumin by means of equilibrium and kinetic measurements, and to use the results to discuss the protein folding in relation to the previous studies [8–15].

2. Materials and methods

2.1. Materials

Bovine α-lactalbumin was prepared from fresh milk by the method described previously [10]. All chemicals were of analytical reagent grade. The protein concentration was determined spectrophotometrically by the use of a value of $E^{1\%}_{1cm}$ of 20.1 at 280 nm [18]. A molecular weight of 14,200 of α-lactalbumin [19] was used to calculates molar quantities.

2.2. Methods

The temperature-induced difference spectra were measured on a Hitachi Perkin-Elmer 124 spectrophotometer, which is equipped with two separate cell holders for controlling the temperature in sample and reference cells, respectively. The reference cell was kept at 25°C while the sample cell was brought to various temperatures. The temperature of solution in the sample cell, controlled within ±0.1°C, was determined by dipping a thermistor in the cell with a Takara thermistor thermometer, type SPD 01-10A. The optical path used was 10 mm.

The kinetic measurements of temperature-jump (*T*-jump) were carried out in a rapid reaction analyzer, Union Giken model RA-1100. The Joule heating temperature pulse [20] was applied to a sample solution of low electrical resistance, containing a suitable amount of electrolyte, by discharging a capacitor in a high-voltage supply (Union Giken, RA-410). A cylindrical quartz cell of 2 mm inner diameter was used. The entire cell was placed in a metal thermostat, which was in thermal contact with the solution and connected with a ground electrode. Rapid changes in transmittance caused by *T*-jump of the solution were memorized and accumulated several times in a micro-computer for processing kinetic data (Union Giken, RA-450). The calibration of the apparatus was carried out using a test solution, 1.4×10^{-5}M phenol red in 1.0 M Tris-HCl (pH 7.9), which exhibits appropriate changes in absorbance at 559 nm and at 423 nm with varying temperature.

All the protein solutions for difference spectra and *T*-jump measurements contained 0.1 M KCl, were buffered with 0.01 to 0.02 M potassium phosphate, and were filtered through a membrance filter (a pore diameter of 0.45 μm) prior to use. The pH values given in the text are indicated values (Hitachi-Horiba model 7 pH meter) at room temperature.

The numerical calculations in the least squares fit for equilibrium transition curves were carried out on a digital computer (FACOM 230-75, Hokkaido University Computing Center).

3. Results

3.1. Equilibrium of thermal unfolding

Difference absorption spectra for the unfolding of α-lactalbumin by heating in water at pH 6.82 in the absence of denaturants, are shown in fig. 1, which are quite similar to the spectra for the acid denaturation [21]. Three major extrema are observed at 284 nm, at 292 nm and at 303 nm, indicating that the aromatic residues buried in the N state are transferred to polar environment during the thermal unfolding. The values of difference extinction coefficients at these extrema are plotted against temperature in fig. 2. The transitions observed were found to be completely reversible, so that thermodynamic treatment of the data is justified.

It is known that the changes in several physical properties of α-lactalbumin are parallel during the thermal unfolding as expected for a two-state transition [22–24]. As given in the next section, kinetic data from *T*-jump experiments also demonstrate a two-state mechanism. Thus, the experimental data in fig. 2 are analyzed by assuming the two-state denaturation, i.e.,

$$N \overset{K_{NH}}{\rightleftharpoons} H, \qquad (1)$$

where H refers to the thermally unfolded state. The equilibrium constant, K_{NH}, of the reaction can be de-

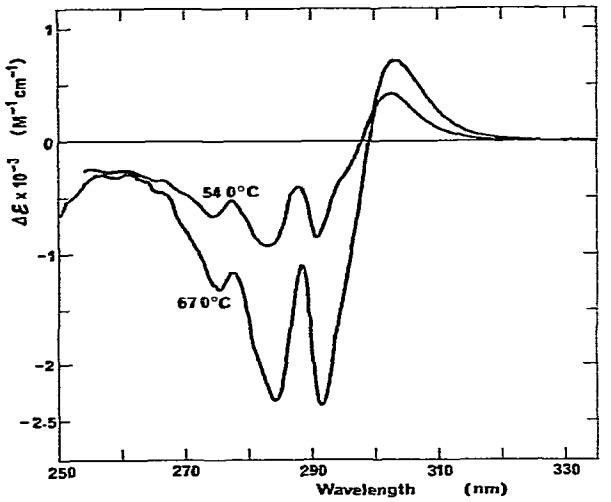

Fig. 1. The temperature-induced difference absorption spectra of α-lactalbumin at pH 6.82 in the absence of denaturants. Reference solution is kept at 25°C, sample solutions are at 54.0°C and at 67.0°C, and these solutions contain 3.22 × 10^{-5}M α-lactalbumin.

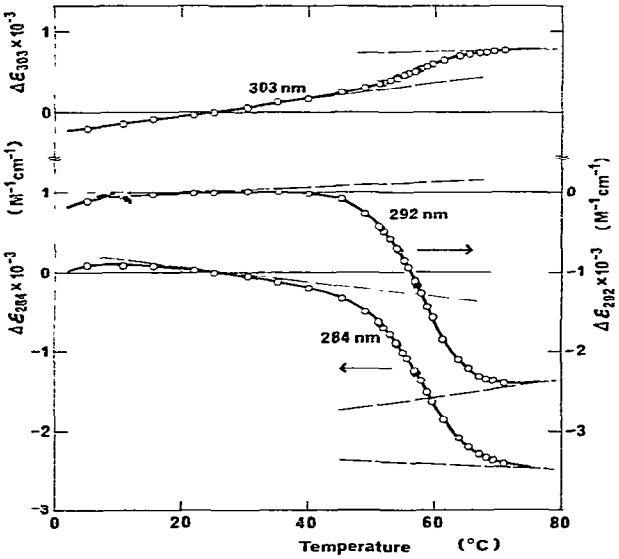

Fig. 2. Transition curves for the thermal unfolding of α-lactalbumin measured at three different wavelengths (284 nm, 292 nm and 303 nm). Reference temperature, pH and protein concentration are the same as in fig. 1. Filled circles show reversals from high temperature. Dashed lines represent the hypothetical values of $\Delta\epsilon_N$ and $\Delta\epsilon_H$.

rived from the spectrophotometric data, as

$$K_{NH} = \frac{\Delta\epsilon - \Delta\epsilon_N}{\Delta\epsilon_H - \Delta\epsilon}. \quad (2)$$

Here, $\Delta\epsilon$ represents the experimental extinction coefficient at a given temperature, and $\Delta\epsilon_N$ and $\Delta\epsilon_H$ are the extinction coefficients of the N and the H states, respectively, which can be usually assumed to vary linearly with temperature, as

$$\Delta\epsilon_N = k_1 T + k_2, \qquad \Delta\epsilon_H = k_3 T + k_4, \quad (3)$$

where k_1, k_2, k_3 and k_4 are temperature-independent extrapolation parameters, and T denotes absolute temperature. As demonstrated by Privalov and Khechinashvili [25], the heat capacity change of thermal unfolding seems to be almost independent of temperature, and then the temperature dependence of the equilibrium constant is expressed by

$$\ln K_{NH} = A \ln(T/T_m) + (A T_m + B)(1/T - 1/T_m), \quad (4)$$

where T_m denotes the transition temperature at which $\ln K_{NH} = 0$, and A and B are temperature-independent parameters. Once these parameters are determined, the thermodynamic quantities, enthalpy, entropy and heat capacity changes (ΔH_{NH}, ΔS_{NH} and ΔC_{pNH}, respectively), of the reaction can be calculated, and at T_m they are as follows

$$\Delta H_{NH}(T_m) = -R\{\partial \ln K_{NH}/\partial(1/T)\}_{T=T_m} = -RB, \quad (5)$$

$$\Delta S_{NH}(T_m) = \Delta H_{NH}(T_m)/T_m = -RB/T_m, \quad (6)$$

$$\Delta C_{pNH} = \partial \Delta H_{NH}(T)/\partial T = RA, \quad (7)$$

where R denotes the gas constant.

In the usual way to estimate these quantities, the logarithmic values of K_{NH}, evaluated from eq. (2) by the aid of the prior extrapolation of $\Delta\epsilon_N$ and $\Delta\epsilon_H$ to

the transition region, are plotted against $1/T$ (van't Hoff plot), and the slope and the curvature of this plot may give the values of ΔH_{NH} and ΔC_{pNH}, respectively. Nevertheless, but in the present case, accurate experimental values of K_{NH} can be obtained over a narrow temperature interval of only about 10° (fig. 2), because of the sharpness of the transition brought about by the high degree of cooperativity. Such a limitation causes nontrivial errors in estimating the thermodynamic quantities especially in estimating ΔC_{pNH} that depends extensively on the predetermination of $k_1 \sim k_4$. Thus, it is necessary to use some alternative method to evaluate the above quantities. Hence, we combine eqs. (2)–(4), and an expression for the extinction coefficient as a function of all the above parameters can be obtained, which will be applicable not only in the narrow range of the transition but also at the extreme temperatures where the almost pure states exist [26], i.e.,

$$\Delta\epsilon = \{(k_1T + k_2) + (k_3T + k_4)\exp[A\ln(T/T_m) + (AT_m + B)(1/T - 1/T_m)]\} \times \{1 + \exp[A\ln(T/T_m) + (AT_m + B)(1/T - 1/T_m)]\}^{-1}. \quad (8a)$$

The fitting of this model function to all the experimental points in fig. 2 makes it possible to obtain values of parameters, A, B and T_m, and simultaneously the values of $k_1 \sim k_4$ by using the nonlinear least squares method (the Gauss-Newton method) based on iterative adjustment [27,28]. In practice, the following alternative function, equivalent to eq. (8a), has been used for the sake of convenience in the initial guess of the extrapolation parameters

$$\Delta\epsilon = \{[k_1(T - 298) + k_2'] + [k_3(T - 343) + k_4']\exp[A\ln(T/T_m) + (AT_m + B)(1/T - 1/T_m)]\} \times \{1 + \exp[A\ln(T/T_m) + (AT_m + B)(1/T - 1/T_m)]\}^{-1}. \quad (8b)$$

The iterative procedure has been applied until the residual sum of squares between the experimental and the analytical values of $\Delta\epsilon$ has been minimized. The full adjustment of the parameters, however, not infrequently resulted in an increase in the residual sum of squares, and in such cases, sequential halving of the adjustment could give rise to satisfactory convergence [27,28].

The solid curves in fig. 2 show the analytical values of $\Delta\epsilon$, calculated independently at three different wavelengths, according to eq. (8b). The best fit values of thermodynamic parameters are shown in table 1 together with the unbiased estimates of probable errors for the parameters, which could be calculated from the standard deviation of $\Delta\epsilon$ and the variance-covariance matrix elements. The values of the parameters at different wavelengths agree reasonably well with each other, and this shows the applicability of the present procedure.

3.2. Kinetics of thermal unfolding

Kinetic experiments were performed by the T-jump method by following the absorbance change at 292 nm. At sufficiently high temperatures (temperature after T-jump > 60°C), all the relaxation curves associated with the unfolding were observed in a very short time interval as a single exponential decay term (fig. 3a), as

$$\Delta A(t) = A_1 + A_2\exp(-k_{ap}t) \quad (9)$$

where $\Delta A(t)$ is an observed change in absorbance at time t, and k_{ap} is the apparent first-order rate constant. At rather lower temperatures, on the other hand, the process of temperature returning slightly overlapped the kinetic process of the thermal unfolding, and in appearance, it was seen as if two kinetic phases were observed (fig. 3b). Because temperature returning can be approximated to obey an exponential decay process (Newton's law of cooling), the overall change in absorbance is expressed in terms of two exponentail decay terms, given by

$$\Delta A(t) = A_1\exp(-k't) + A_2\exp[-(k_{ap} + k')t] \quad (10)$$

where $\exp(-k't)$ corresponds to the term for the temperature decay process. In the present case, the applied temperature pulse is less than 10°, and k_{ap} is larger than 8.9 s^{-1} at all the temperatures employed,

Table 1
Thermodynamic parameters for unfolding of bovine α-lactalbumin at neutral pH

	The parameters for the thermal unfolding (N ⇌ H) calculated from the data at three different wavelengths			The parameters for the transitions between the three states caused by guanidine hydrochloride and acid [a]		
	284 nm	292 nm	303 nm	N ⇌ A	A ⇌ D	N ⇌ D
T_m (K)	330.59 ± 0.16	330.72 ± 0.12	330.78 ± 0.80	–	–	–
ΔG (kJ • M^{-1})	–	–	–	4	3	7
ΔH (kJ • M^{-1})	222.3 ± 3.0	226.3 ± 2.3	227 ± 10.3	210	100	310
ΔS (J • M^{-1}K^{-1})	673 ± 9.2	684 ± 7.0	686 ± 32.6	620	300	920
ΔC_p (kJ • M^{-1}K^{-1})	6.48 ± 0.18	6.24 ± 0.17	4.40 ± 3.72	4.1	1.8	5.9

[a] The values at 330.7 K, calculated from the data in ref. [14].

while the apparent value of k' is 0.4 ~ 0.8 s^{-1}. We could apply eq. (10) in a good approximation to the data at lower temperatures (temperature after T-jump between 52° and 60°C). In these calculations, the first term in eq. (10) was evaluated from the absorbance measurement in a long time interval. This term was subtracted from the total absorbance changes, and the value of k_{ap} was estimated from the initial portion of the resultant relaxation curve after subtraction in a sufficiently short time interval (~200 ms), where k_{ap} can be regarded nearly as a constant. Typical examples of relaxation curves are shown in fig. 3, and all of the results are summarized in table 2. These results show that the time course associated with the thermal unfolding process per se can be expressed as a single exponential decay term, and that the kinetic amplitude of absorbance change for the unfolding is in a good agreement with that expected from the equilibrium transition curve. These are the strong evidence that the thermal unfolding of α-lactalbumin is a two-state transition..

On the basis of the two-state mechanism, the microscopic rate constants for the forward and the reversed processes, k_f and k_r, can be evaluated from the equilibrium constant K_{NH} ($=k_f/k_r$) and the apparent rate constant k_{ap} ($=k_f + k_r$), and they are plotted against $1/T$ in fig. 4. On the assumption that the heat capacity changes in the activation processes are independent of T, the temperature dependence of k_f and k_r are given by

$$\ln(h/kT)k_f = A_f \ln(T/T_0) + B_f(1/T - 1/T_0) + C_f, \quad (11)$$

$$\ln(h/kT)k_r = A_r \ln(T/T_0) + B_f(1/T - 1/T_0) + C_r, \quad (12)$$

where h and k denote the Planck and the Boltzman constant, respectively, A_f, B_f, C_f, A_r, B_r and C_r are temperature-independent parameters, and T_0 is an arbitrary constant for excluding round off errors (in this case, a value of 335 K is used). The best fit of the experimental data to each of the above equations was obtained by the simple linear lest squares method, and is shown as a solid curve in fig. 4. The free energy, enthalpy, entropy and heat capacity changes for each of the activation processes can be derived from the best fit values of $A_f \sim C_r$, and they are shown in table 3.

4. Discussion

The values of thermodynamic parameters derived from the present analysis are compared with those values for the transitions of α-lactalbumin between the three states, the N, the A and the fully unfolded (D) states, shown in the previous studies (table 1) [11,14]. The similarity in the parameters is found between the thermal unfolding and the N ⇌ A transition, and suggests that the H state is a partially unfolded species as well as the A state [10]. Takesada et al. [23] have shown that the H state of α-lactalbumin has an appreciable amount of folded structures on the basis of the backbone circular dichroism spectra. The aromatic difference spectra also support the similarity between the H and the A states (fig. 1). However, nontrivial differences also exist in the spectral details of far ultraviolet circular dichroism [10,23]; e.g., the spectral intensity around 222 nm is about 20% smaller in H than in A.

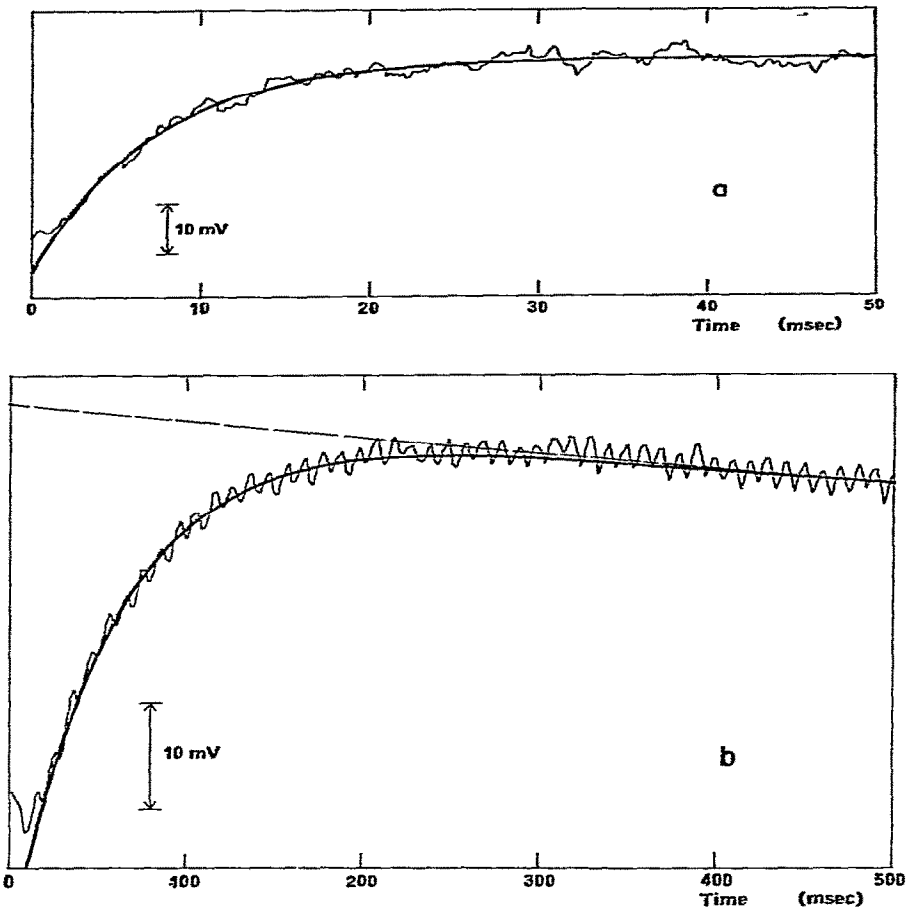

Fig. 3. Typical relaxation curves (transmittance versus time) of the thermal unfolding of α-lactalbumin at pH 6.82 in the absence of denaturants. Protein concentration is 3.13×10^{-5}M. A 10 mV of output voltage corresponds to a transmittance change of 0.19% of the total transmittance of the solution. (a) A 6 times averaged curve and the *T*-jump from 59.0° to 68.1°C, and (b) a 3 times averaged curve and from 50.5° to 58.2°C. Heavy solid curves are theoretically drawn with the best fit to the observed curves, according to eq. (9) (a) and to eq. (10) (b). A dashed line in (b) represents the temperature decay term.

Thus as a conclusion, the H state, a partially unfolded species involving a considerable amount of backbone ordered structures, is thermodynamically similar to rather than identical with the A state.

On the other hand, in our previous study on the unfolding of human α-lactalbumin [15], we have detected another partially unfolded species, having more folded backbone structures than the A state, at ph lower (pH < 3) than required for the N ⇌ A transition. Thus, there are at least three partially unfolded species, of which aromatic difference or aromatic circular dichroism spectra are very similar to each other, but different in the degree of folding of backbone structures. Such variation in the partially unfolded species may arise from the lack of extensive cooperation of long-range specific interactions to stabilize the intermediate structures. The folding process from the D to the partially folded structure has been shown to be dominated

Table 2
Temperature-jump kinetics for the thermal unfolding of bovine α-lactalbumin at pH 6.82.

Temperature after T-jump (°C)	Temperature pulse (degree)	k_{ap} (s^{-1})	$\Delta A_{kinetic}$ [a] / $\Delta A_{equl.}$
52.4	8.0	8.93	0.97
53.2	5.8	9.06	1.00
53.3	9.1	8.35	1.07
55.1	4.8	9.51	0.93
55.3	8.0	9.46	0.92
56.1	5.8	12.8	1.08
57.0	9.8	14.2	1.09
58.2	8.0	15.8	1.04
58.2	8.0	17.4	1.23
58.4	5.8	13.3	0.97
62.8	3.8	31.8	0.98
63.6	9.1	46.1	1.00
63.6	9.1	40.6	1.01
63.6	9.1	46.5	1.08
63.6	9.1	39.3	0.94
63.6	9.1	47.4	0.96
64.0	6.9	48.6	0.93
64.8	5.8	58.8	1.01
65.5	4.8	72.0	0.89
66.1	9.1	74.1	0.98
66.2	9.1	77.0	1.01
67.0	8.0	78.9	1.00
67.6	6.9	101	0.75
68.1	9.1	143	0.95
69.8	9.1	179	0.91
69.8	9.1	208	0.75

[a] The ratio of the observed kinetic amplitude to that expected from the equilibrium transition curve in fig. 2.

by local interactions and may be approximated as a one-dimensional transition system such as the coil-to-helix transition, while the transition between the N and the partially folded states can be interpreted in terms of a cooperative two-state mechanism [14,15]. In such a system, the partially unfolded state observed is a macroscopic species distributed over a large number of microscopic states, and the lack of the cooperation of the long-range interactions makes the distribution so broad and motile that the average distributional properties of the species are expected to

Table 3
Kinetic parameters for the thermal unfolding of bovine α-lactalbumin [a]

	Forward activation	Reversed activation
$\Delta G^{\ddagger}$ (kJ • M^{-1})	75.9 ± 0.05	75.9 ± 0.05
$\Delta H^{\ddagger}$ (kJ • M^{-1})	235 ± 4	10 ± 4
$\Delta S^{\ddagger}$ (J • $M^{-1}K^{-1}$)	480 ± 12	−200 ± 12
$\Delta C_p^{\ddagger}$ (kJ • $M^{-1}K^{-1}$)	2 ± 1.0	−4 ± 1.0

[a] At pH 6.82 and 330.7 K.

be significantly affected by temperature or by pH.

The kinetic results from the T-jump experiments are essentially consistent with the folding model of α-lactalbumin proposed previously [14]. The large entropy loss and the large decrease in heat capacity are associated with the reversed activation process (table 3), and suggest that the partially folded segments such as α-helical segments existing in the H state may pack together by the aid of hydrophobic interactions. This packing process is analogous to the reversed activation

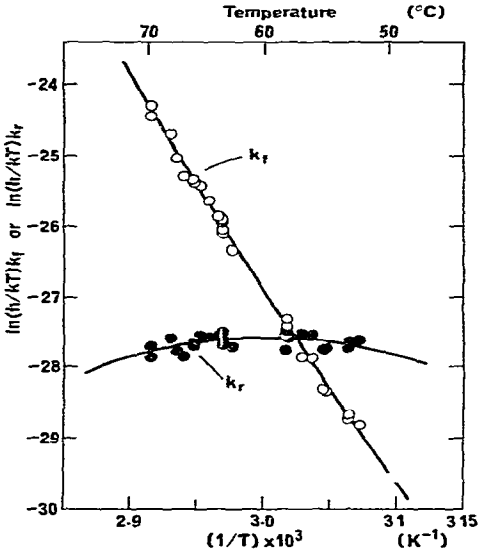

Fig. 4. The dependence of $\ln(h/kT)k_f$ and $\ln(h/kT)k_r$ on $1/T$. The best fit curves are shown by solid curves.

process of the N ⇌ A transition [11] and brings about the entropic feature of the activation. On the other hand, the forward activation process of the unfolding shows a marked temperature dependence (fig. 4) and involves large enthalpic factors (table 3) as well as the forward activation of the N ⇌ A transition [11]. These schemes for the folding and unfolding are also in accordance with the experimental findings from the kinetics of unfolding of other globular proteins, chymotrypsinogen A [29] and lysozyme [30]. The precedence of hydrophobic interactions in the folding process from the H state may arise from the non-specific features of the interactions, and then the final stabilization of molecular structures may be brought about by the specific long-range interactions such as the formation of charge pairs and hydrogen bonds between residues, which cause the marked enthalpic barrier for the forward activation [14,15].

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