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# THERMODYNAMICS OF α-LACTALBUMIN UNFOLDING

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Thermodynamic investigations of α-lactalbumin have been performed by isothermal calorimetric guanidine hydrochloride titrations as well as by scanning calorimetric measurements in the presence and absence of guanidine hydrochloride. Compared with lysozyme, α-lactalbumin is less stable, and its changes of enthalpy and heat capacity at unfolding are lower. Thermal unfolding of α-lactalbumin can be described to the first approximation by the two-state transition model even in the presence of guanidine hydrochloride.

## 1. Introduction

Since folding of polypeptide chains to proteins is rather complicated, investigations have always been attractive which elucidate stable intermediates in folding or unfolding reactions. A physically unambiguous criterion for two or multistate behaviour of transitions is equality of van't Hoff and calorimetric enthalpy [1]. This criterion is fulfilled, as a rule, for thermal unfolding of compact proteins having a molecular weight of less than 25 000, i.e., a cooperative folding mechanism seems to be a privilege of small globular proteins [2].

Recently a three-state folding pathway of α-lactalbumin has been deduced from guanidine hydrochloride (GuHCl) denaturation studies [3]. This folding model is based on indirect, chiefly optical investigations [4]. Therefore, it should be desirable to apply additionally the thermodynamic criterion. Unfortunately, thermodynamic data of α-lactalbumin unfolding published so far are already based on assumptions such as the two-state hypothesis or proposed denaturant interaction mechanism [3–9]. Therefore calorimetric investigations of α-lactalbumin unfolding will be presented in this paper, and some parallels with the homologeous lysozyme will be drawn.

## 2. Materials and methods

α-lactalbumin was prepared from bovine fresh milk according to method IIb of Armstrong et al. [10,11]. The electrophoretically homogeneous α-lactalbumin was rechromatographed on Sephadex G 75 in 0.04 molar imidazole buffer pH 6.3 before use. Concentration determinations were performed optically using $E^{1\%}_{1\,cm} = 20.1$ at 280 nm [12].

All reagents used were of analytical grade. GuHCl was purified and checked according to [13].

Scanning calorimetric measurements were carried out on differential scanning microcalorimeter DASM 1M at a heating rate 1 K $min^{-1}$. For the determination of heat capacities the partial specific volume of α-lactalbumin $v = 0.704$ ml $g^{-1}$ [14] was used.

Isothermal calorimetric measurements were performed on a LKB flow calorimeter. For the procedure see refs. [15,16].

## 3. Results

### *3.1. Scanning calorimetry in the absence of denaturants*

α-lactalbumin achieves maximal thermostability in dilute solutions (0.05–0.2%) at neutral pH, i.e.

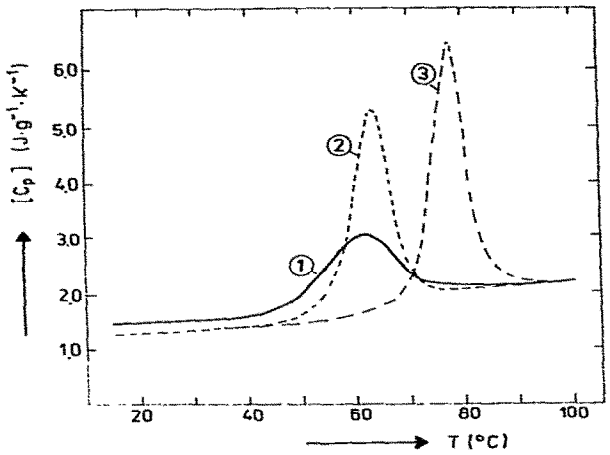


Fig. 1. Temperature dependence of partial heat capacity of α-lactalbumin and lysozyme. (1) α-lactalbumin in 0.04 molar imidazole-HCl buffer pH 6.3 (25°C), (2) lysozyme in 0.04 molar glycine buffer pH 2.4, (3) lysozyme in 0.04 molar acetate buffer pH 4.5.

the transition temperature ($T_{trs}$) remains unchanged within pH range from 5.6 to 7.5. When comparing scanning calorimetric recordings of lysozyme and α-lactalbumin, the following differences are obvious (fig. 1):

(i) the partial heat capacity of α-lactalbumin in diluted solution is higher than those of lysozyme before reaching the transition region. Both proteins exhibit equal heat capacity after thermal denaturation;

(ii) α-lactalbumin is less thermostable than lysozyme;

(iii) α-lactalbumin exhibits lower enthalpy ($\Delta H$) and heat capacity changes ($\Delta C_p$) at unfolding than lysozyme.

Table 1
Thermodynamic parameters of lysozyme and α-lactalbumin unfolding obtained by scanning microcalorimetry

| | | Lysozyme [a] | α-lactalbumin |
|---|---|---|---|
| $(C_p)^{25°C}$ | J K$^{-1}$ g$^{-1}$ | 1.34 | 1.65 ± 0.08 |
| $T_{trs}$ | °C | 78.5 | 62.0 ± 0.2 |
| $\Delta H$ | kJ mol$^{-1}$ | 590 | 276 ± 9 |
| $\Delta C_p$ | kJ K$^{-1}$ mol$^{-1}$ | 6.6 | 4.0 ± 0.8 |
| $(\Delta H^{cal}/\Delta H^{v.H.})$ | | 1.04 | 1.06 ± 0.03 |

[a] Data from refs. [2,15].

The corresponding thermodynamic data are listed in table 1 for the two proteins under conditions ensuring maximal stability. Based on these data the denaturational Gibbs energy change ($\Delta G$) can be calculated according to eq. (1):

$$\Delta G(T) = \Delta H(T_{trs} - T)/T_{trs} - \Delta C_p(T_{trs} - T) + T\Delta C_p \ln(T_{trs}/T). \quad (1)$$

The results plotted in fig. 2 show the lower stability of α-lactalbumin compared with lysozyme. The Gibbs energy change amounts at 25°C to 21.9 ± 1.8 kJ mol$^{-1}$ for α-lactalbumin, and to 60.7 kJ mol$^{-1}$ for lysozyme [2,15,16]. For comparison, from GuHCl and guanidine thiocyanate denaturation studies of α-lactalbumin $\Delta G$ = (18–28) kJ mol$^{-1}$ has been reported using different extrapolation procedures to zero denaturant concentration [4,17,18].

### 3.2. Isothermal calorimetric GuHCl titration

The isothermal calorimetric GuHCl titration curve of α-lactalbumin (fig. 3) is similar to that of lysozyme [16]. Semiconversion of the GuHCl induced transition

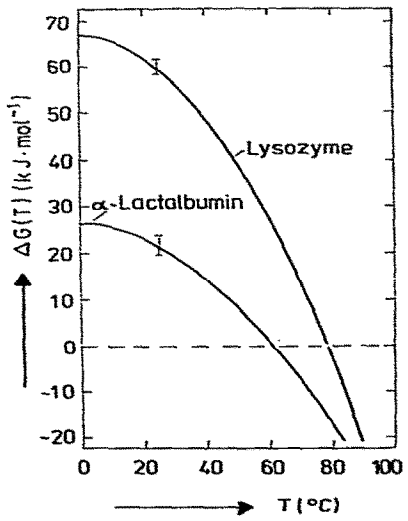


Fig. 2. Denaturational Gibbs energy change of α-lactalbumin and lysozyme versus temperature.

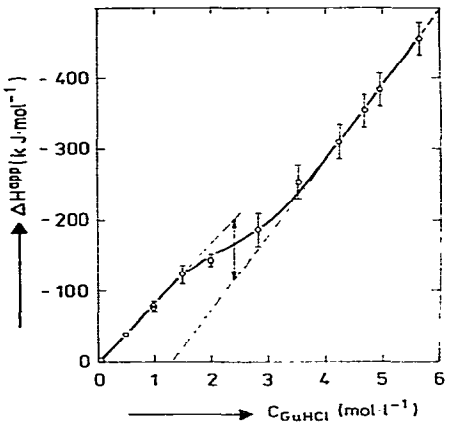


Fig. 3. Isothermal calorimetric titration curve of α-lactalbumin with GuHCl at 25°C in 0.04 molar imidazole at pH 6.3. It follows $\Delta H^{app} = 84 \pm 17$ kJ mol$^{-1}$ and after correction of "preferential binding heat" $\Delta H = 122 \pm 19$ kJ mol$^{-1}$. The correction was performed according to the approach described elsewhere [16] using the preferential binding parameter $\Delta n = 13.5$ [17], and total number of binding sites $a_D = 84$ [14].

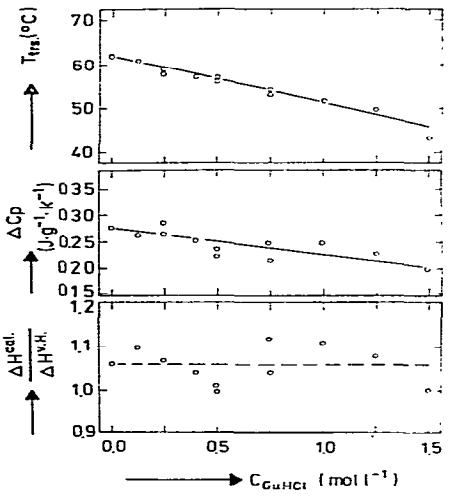


Fig. 4. Transition temperature ($T_{trs}$), heat capacity change ($\Delta C_p$), and ratio ($\Delta H^{cal}/\Delta H^{v.H.}$) of α-lactalbumin unfolding versus GuHCl concentration.

occurs at about 2.4 mol$^{-1}$ GuHCl consistently with the reduction of molar ellipticity of α-lactalbumin at 222 nm [4]. The enthalpy change obtained by GuHCl titration $\Delta H^{25°C} = 122 \pm 19$ kJ mol$^{-1}$ is in fair agreement with $\Delta H^{25°C} = 130 \pm 17$ kJ mol$^{-1}$ from thermal denaturation as well as with the results of [7]. When comparing the calorimetric GuHCl titration curves of α-lactalbumin and lysozyme [16] we find the same slope in the postdenaturational region. This corresponds to the nearly identical number of sites available for denaturant interaction of both proteins [14]. A nontrivial difference exists in the predenaturational region. α-lactalbumin should have more sites available for denaturant interaction in the predenaturational region since its "preferential binding" parameter $\Delta n$ is considerably lower than that of lysozyme (see legend to fig. 3). In contrast, the initial slope of the titration curve $s_N = (\partial \Delta H_i'/\partial c)_T$ of α-lactalbumin is reduced by about 25% compared with that of lysozyme.

### 3.3. Scanning calorimetry in the presence of GuHCl

As reported above, α-lactalbumin unfolding follows different mechanisms: three-state at GuHCl induced denaturation [3,4], and two-state at thermal denaturation [9]. Therefore, a transition from one mechanism into the other is to be expected if thermal denaturation of α-lactalbumin is performed in the presence of GuHCl. The results of scanning calorimetric measurements of α-lactalbumin unfolding in GuHCl solution, however, do not show any deviation from the two-state mechanism (fig. 4). With increasing GuHCl concentration the ratio ($\kappa$) of calorimetric ($\Delta H^{cal}$) and van't Hoff ($\Delta H^{v.H.}$) enthalpy ($\kappa = \Delta H^{cal}/\Delta H^{v.H.}$) remains constant within the experimental error, whereas the transition temperature ($T_{trs}$) and heat capacity ($\Delta C_p$) of α-lactalbumin decrease.

## 4. Discussion

α-lactalbumin unfolding seems to be rather complicated as compared with other small globular proteins. Various denatured states and different unfolding mechanisms of α-lactalbumin from bovine milk have been proposed on the basis of optical studies and viscosity measurements involving inorganic protein denaturants and temperature [3–9,21,22].

The three-state mechanism of α-lactalbumin unfolding is well documented [3,4]. In some contradiction, thermal unfolding of α-lactalbumin proceeds in a two-state process. This has been proposed on the basis of aromatic difference spectra [9] and is now confirmed by the calorimetric results presented here. The thermodynamic criterion, i.e. the ratio between calorimetric and van't Hoff heat, gives analogous unfolding of other small globular proteins ($\kappa$ = 1.05 ± 0.03 [2]) under conditions of maximal protein stability $\kappa = \Delta H^{cal}/\Delta H^{v.H.} = 1.06 \pm 0.03$. The characterization of the denatured states of α-lactalbumin is somewhat contradictory. The completely unfolded state of α-lactalbumin in GuHCl (D-state) and the acid denatured one (A-state), which contains ordered structure to a considerable extent, are well characterized [3,4,7]. The thermal denatured protein (H-state) has been described as being similar to the A-state [9]. The far ultraviolet circular dichroism spectra, however, are different for the two forms [5], indicating unlike the A-state a significantly lower content of ordered structure of α-lactalbumin at 71°C where the transition is almost complete (see fig. 1). Furthermore, the heat capacity of α-lactalbumin after thermal denaturation is equal to that of the completely unfolded lysozyme [16]. Finally, $\Delta H^{25°C}$ has the same value for GuHCl and for heat denaturation, which would be impossible if either $\Delta H$ or $\Delta C_p$ of a transition H → D were unequal to zero. Therefore, thermally denatured α-lactalbumin must be close to the completely unfolded form, the D-state. In other words, the residual structure observed at thermal unfolding cannot measurably contribute to the enthalpy change.

A similar tertiary structure of lysozyme and α-lactalbumin has been proposed on the basis of homologeous amino acid sequences and identical position of disulfide linkages [23,24]. Nevertheless thermodynamic parameters of unfolding are strikingly different for the two proteins (table 1). As shown in fig. 2, the stability of α-lactalbumin is considerably lower than that of lysozyme. Furthermore, the heat capacity change ($\Delta C_p$) which results mainly from exposure of apolar residues at unfolding [25], amounts for α-lactalbumin to only 2/3 of the lysozyme value. Since partial heat capacities of the two proteins are equal above the transition region, the lower $\Delta C_p$ of α-lactalbumin must be due to its higher partial heat capacity below transition. Therefore a high amount of apolar residues can be assumed to be in contact with water already in the "native" α-lactalbumin structure.

The same behaviour is reflected by the statistical mechanical treatment as given in the appendix, which does not differentiate between hydrogen bonds and nonpolar contacts, but it shows that less secondary bonds must be broken at α-lactalbumin unfolding compared with lysozyme. The conclusion that a fraction of apolar groups of native α-lactalbumin is exposed to the solvent is consistent with some experimental observations.

Compared with lysozyme α-lactalbumin exhibits:

(a) higher heat capacity, at standard temperature, and a relatively low stabilization energy (fig. 2);

(b) a lower content of slowly exchanging peptide bonds in H-D exchange studies [5];

(c) higher reducibility of the disulfide bonds [26];

(d) higher fluctuation around the average structural state (see appendix);

(e) a lower preferential interaction parameter $\Delta n$ at GuHCl denaturation [17].

Therefore it can be concluded that not only is the structure of α-lactalbumin in solution more flexible but is maintained by less noncovalent contacts than the lysozyme structure. The presence of moderate GuHCl concentration at thermal denaturation causes lowering of thermostability of lysozyme without change of $\Delta C_p$ [16] whereas both transition temperature and $\Delta C_p$ of α-lactalbumin decrease (fig. 4). This indicates loosening of α-lactalbumin structure under the influence of increasing denaturant concentration already below unfolding conditions. However, the effects brought about by adding GuHCl to α-lactalbumin (below 1.5 molar) reflect only gradual changes: $\Delta C_p$ and $T_{trs}$ in fig. 4, and $\Delta H^{app}$ in fig. 3 change monotonously without any transition-like behaviour. Furthermore, the thermodynamic criterion does not indicate any significant deviation from the two-state mechanism of α-lactalbumin unfolding even in the presence of GuHCl (fig. 4).

What may be the reason for the different behaviour of α-lactalbumin and lysozyme in GuHCl solution? The most likely explanation is that GuHCl penetrates into the more flexible structure of α-lactalbumin, disrupts a few noncovalent bonds, thus diminishing $\Delta C_p$ as observed by scanning calorime-

try (fig. 4) and flattening the initial slope of calorimetric titration curve (fig. 3). This, however, is important for the understanding of the noncoincidence of optical changes of α-lactalbumin at GuHCl titration monitored by circular dichroism at different wavelengths [3,4]. Gradual loosening of protein structure accompanied by solvation phenomena can disturb the spectral properties of aromatic residues already below unfolding conditions. Therefore we have to ask whether the intermediate observed in GuHCl denaturation of α-lactalbumin [3,4] can be considered as a "natural" state on the protein folding pathway.

## Appendix

### *Statistical mechanical treatment*

A better understanding of why thermodynamic of unfolding of the two homologous proteins is so different can be reached applying Ikegami's statistical mechanical treatment of structural changes of proteins [19,20]. According to this approach the structure of protein can be defined by the fraction of secondary bonds (number of possible bonds $N_0$) being in bonded state. Thus Gibbs energy of the structural state ($x$) of a protein can be expressed by average bond energy ($\epsilon$), cooperative energy ($ZJ$), chain entropy ($\alpha$) and a parameter ($\gamma$) specifying the hydrophobic nature. These parameters, which reflect average properties of a protein, can be obtained by a least square fitting of scanning calorimetric recordings [20].

From the statistical mechanical model it follows that the parameters characterizing the noncovalent contacts of both proteins are identical (table 2). The only difference is in $N_0$, the number of secondary bonds, which amounts for α-lactalbumin to only 2/3 of the lysozyme value.

The statistical mechanical model according to Ikegami [19] distinguishes between "structural transitions" and "gradual structural changes". The Gibbs energy plotted against the structural state parameter ($x$) shown in fig. 5 exhibits two minima for both proteins, corresponding to the "native" and "denatured" states, i.e. the typical profile of proteins undergoing

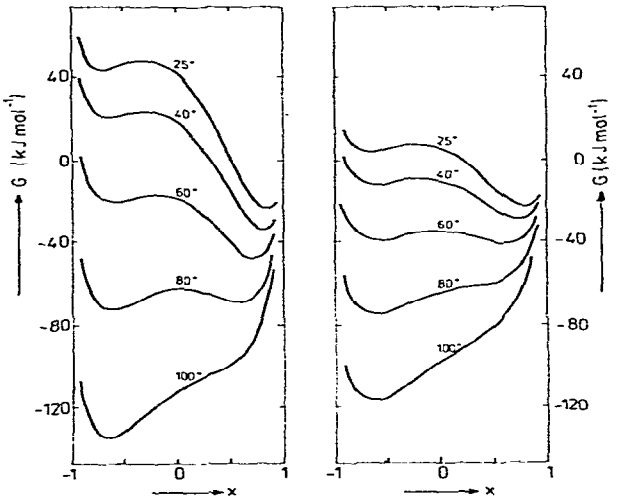


Fig. 5. Gibbs energy versus structural state parameter $x$. Left: lysozyme; right: α-lactalbumin.

Table 2
Results of least squares fit of scanning calorimetric recordings according to Ikegami [19,20] a)

| Parameter | Dimension | α-Lactalbumin pH 6.3 | Lysozyme pH 2.4 | Lysozyme pH 4.5 |
|---|---|---|---|---|
| $N_0$ | | 118 | 185 | 178 |
| $ZJ$ | kJ mol$^{-1}$ | 6.45 | 6.53 | 6.82 |
| $\alpha$ | J K$^{-1}$ mol$^{-1}$ | 18.0 | 18.1 | 17.5 |
| $\epsilon$ | kJ mol$^{-1}$ | 4.27 | 4.35 | 4.35 |
| $\gamma$ b) | | 2.82 | 2.85 | 2.74 |

a) For more details the reader is referred to the original papers [19,20]. Additionally included into the fitting procedure were the contributions from the internal degrees of freedom, which enables further decrease of variance.
b) Number of water molecules affected by the change in state per secondary bond.

a "structural transition". However, compared with lysozyme the two minima of α-lactalbumin are less well separated from each other indicating lower stability of the native protein in accordance with the Gibbs energy calculation shown in fig. 2. It should be added that the measure of fluctuation ($\overline{\Delta x^2}$) of a protein molecule around the average structural state ($\bar{x}$) gives a ratio between α-lactalbumin (pH 6.3) and lysozyme (pH 4.5) of about 2.5 : 1 below and above the transition region indicating higher flexibility of α-lactalbumin in both, the native and denatured states.

## References

[1] R. Lumry, R. Biltonen and J.F. Brandts, Biopolymers 4 (1966) 917.
[2] P.L. Privalov and N.N. Khechinashivili, J. Mol. Biol. 86 (1974) 665.
[3] K. Kuwajima, J. Mol. Biol. 114 (1977) 241.
[4] K. Kuwajima, K. Nitta, M. Yoneyama and S. Sugai, J. Mol. Biol. 106 (1976) 359.
[5] H. Takesada, M. Nakanishi and M. Tsuboi, J. Mol. Biol. 77 (1973) 605.
[6] K. Takase, K. Niki and S. Arima, Agr. Biol. Chem. 40 (1976) 1273.
[7] T. Okuda and S. Sugai, J. Biochem. (Tokyo) 81 (1977) 1051.
[8] A. Jankowski and I.Z. Siemion, Studia biophysica 68 (1978) 61.
[9] K. Kuwajima and S. Sugai, Biophys. Chemistry 8 (1978) 247.
[10] J. McD. Armstrong, H.A. McKenzie and W.H. Sawayer, Biochim. Biophys. Acta 147 (1967) 60.
[11] J.McD. Armstrong, K.E. Hopper, H.A. McKenzie and W.H. Murphy, Biochim. Biophys. Acta 214 (1970) 419.
[12] M.J. Kronman and R.E. Andreotti, Biochemistry 3 (1964) 1145.
[13] Y. Nozaki and C. Tanford, J. Biol. Chem. 245 (1970) 1648.
[14] J.C. Lee and S.N. Timasheff, Biochemistry 13 (1974) 257.
[15] W. Pfeil and P.L. Privalov, Biophys. Chemistry 4 (1976) 23.
[16] W. Pfeil and P.L. Privalov, Biophys. Chemistry 4 (1976) 33.
[17] S. Sugai, H. Yashiro and K. Nitta, Biochim. Biophys. Acta 328 (1973) 35.
[18] K. Takase, K. Nitta and S. Sugai, Biochim. Biophys. Acta 371 (1974) 352.
[19] A. Ikegami, Biophys. Chemistry 6 (1977) 117.
[20] M.I. Kaneshisa and A. Ikegami, Biophys. Chemistry 6 (1977) 131.
[21] R.N. Sharma and C.C. Bigelow, J. Mol. Biol. 88 (1974) 247.
[22] S. Maruyama, K. Kuwajima, K. Nitta and S. Sugai, Biochim. Biophys. Acta 494 (1977) 343.
[23] W.J. Browne, A.C.T. North, D.C. Phillips, K. Brew, T.C. Vanaman and R.L. Hill, J. Mol. Biol. 42 (1969) 65.
[24] P.K. Warme, F.A. Momany, S.V. Rumball, R.W. Tuttle and H.A. Scheraga, Biochemistry 13 (1974) 768.
[25] W. Kauzmann, Advan. Protein Chem. 14 (1959) 1.
[26] K.S. Iyer and W.A. Klee, J. Biol. Chem. 248 (1973) 707.