

Physical nature of the phase transition in globular proteins

Calorimetric study of human α -lactalbumin

Wolfgang Pfeil*, Valentina E. Bychkova and Oleg B. Ptitsyn⁺

**Central Institute of Molecular Biology, Academy of Sciences of the GDR, 1115 Berlin, GDR and Institute of Protein Research, Academy of Sciences of the USSR, 142292 Poustchino, Moscow Region, USSR*

Received 26 August 1985

The guanidine hydrochloride-induced unfolding of human α -lactalbumin has been studied by isothermal calorimetry. It has been shown that a cooperative transition takes place only in the concentration interval of the denaturant between 0.3 and 2 mol \cdot l⁻¹. The cooperative transition coincides with the transition detected by circular dichroism in the near-ultraviolet region which reflects the destruction of the specific environment of aromatic side groups. According to scanning calorimetric investigations, the transition disappears in the acid form of the protein where circular dichroism of aromatic side groups is practically absent. At higher concentrations of guanidine hydrochloride, where destruction of the secondary structure and unfolding of the chain are observed, there is no cooperative heat absorption.

Protein unfolding Molten globule state α -Lactalbumin Scanning calorimetry
Isothermal calorimetric titration (Human)

1. INTRODUCTION

The denaturation of proteins [1] or protein domains [2] is a highly cooperative 'all-or-none' process but the physical reasons for this high cooperativity are not completely clear. There are three possible explanations for a highly cooperative protein melting: (i) cooperative expansion of the chain ('globule-coil' transition); (ii) cooperative melting of a secondary structure; (iii) melting of a specific tertiary structure.

It is well known that all types of protein

denaturation lead to the loss of its specific tertiary structure, however the extent of protein unfolding may differ. Denaturation of proteins by strong denaturants (e.g. urea or GuHCl) leads, under some conditions, to the completely unordered and unfolded state, whereas, as a rule, the other types of denaturation can retain some features of protein structure [3]. Recently, in GuHCl denaturation of α -lactalbumin, a stable intermediate has been found which is similar to the acid-denatured protein [4–6]. Various intermediate states of human and bovine α -lactalbumin have been characterized [4–9]. These forms exhibit varying secondary structure content and remain nearly as compact as the native state [7–9] whereas no specific tertiary structure was found. This 'compact state with fluctuating tertiary structure' [7] has been called a 'molten globule' state [10].

It has been suggested that the destruction of specific tertiary structure leads to a cooperative transition [7]. In fact, the acid-denatured form of human α -lactalbumin does not show any all-or-

This paper is dedicated to Professor S.P. Datta, Honorary Chairman on FEBS Letters. Due to technical reasons, this did not appear in the Special Issue (Vol. 194, no. 1)

Abbreviations: H α LA, human α -lactalbumin; B α LA, bovine α -lactalbumin; GuHCl, guanidine hydrochloride; Mops, 4-morpholinepropanesulfonic acid; CD, circular dichroism

none transition upon heating, in contrast to the native protein (see below). This suggests that the main reason for the high cooperativity of α -lactalbumin melting is not the unfolding but the destruction of the specific tertiary structure.

The only direct approach to solving this problem is to measure the effects of heat in the case when both transitions (from the native to intermediate state and from the intermediate to unfolded state) can be followed separately in the same experiment. Isothermal calorimetric titrations of α -lactalbumins open up this possibility as their denaturation by GuHCl is a three-state process going through the intermediate [4–6]. Especially useful for this purpose is human α -lactalbumin as in this protein the two transitions monitored by CD spectra of aromatic and peptide groups are more widely separated than those in bovine α -lactalbumin [6]. Therefore, we use H α LA here to measure the effects of heat upon isothermal calorimetric titration of a protein with GuHCl and to determine which of the two transitions is accompanied by a co-operative heat absorption.

2. MATERIALS AND METHODS

H α LA was prepared according to [11,12] with slight modifications [7]. Protein concentration was determined using an extinction coefficient $E_{1\text{cm},280\text{nm}}^{1\%}$ equal to 18.2 (M_r 14020) [7]. The electrophoretically homogeneous protein was rechromatographed on Sephadex G-75 in 20 mM Tris buffer containing 1 mM CaCl₂ at pH 7.5.

All reagents were of analytical grade. GuHCl was purified and checked according to [13,14], its concentration being determined from the refractive index [14].

Scanning calorimetric measurements were carried out on a Privalov type DASM 1M₃ microcalorimeter [15] at a heating rate of 0.5 K·min⁻¹.

Isothermal calorimetric measurements were performed on an LKB batch calorimeter at 25.0 and 40.0°C. Special attention was paid to blank experiments for complete compensation for the heat of dilution of GuHCl-containing solutions [16]. The calorimetric cells were filled by weighing syringes on an analytical balance taking into account the density of GuHCl solutions as in [17]. The heat of dilution of the protein-containing solution was

found to be negligible for the native protein (N state at pH 7.5, 20 mM Tris-HCl, 1 mM CaCl₂), in contrast to the acidic form (A state at pH 2.0, 50 mM KCl-HCl).

CD spectra were recorded on a modified Roussell-Jouan dichrograph.

3. RESULTS

The results of isothermal calorimetric titrations of H α LA with GuHCl are shown in fig.1. As known from similar titrations of lysozyme [18] and B α LA [19], the function representing the heat of transfer (ΔH) of the protein vs GuHCl concentration can be divided into 4 parts:

- (i) pretransitional region: almost linear increase of $-\Delta H$ starting from zero denaturant concentration (slope s_N);
- (ii) transition region: sigmoidal part exhibiting reduced slope or sigmoidal curvature at appropriate GuHCl concentration;
- (iii) post-denaturational region: almost linear increase of $-\Delta H$, slope s_D ;
- (iv) saturation region: deflection, i.e. no further increase of the $-\Delta H$ function above 6–7 mol·l⁻¹ GuHCl.

In the case of the H α LA titration at 25°C (fig.1a, curve 2), these 4 parts are not clearly distinguished. The pretransitional part (see fig.1c) is a straight line exhibiting the same slope as has been found for lysozyme [18]. At 0.4 mol·l⁻¹ GuHCl, i.e. at the beginning of the two optically observed transitions (see fig.1b), a deflection becomes visible. However, there is no pronounced sigmoidal part until the saturation region becomes visible above 6 mol·l⁻¹ GuHCl.

The optically observed transition in H α LA unfolding could have been invisible in isothermal calorimetry at 25°C due to an apparent enthalpy change close to zero. By the example of lysozyme, however, a marked temperature dependence of transition enthalpy in isothermal calorimetric GuHCl titrations was found [18]. Therefore, H α LA was also titrated at 40°C (fig.1a, curve 3). Here, a sigmoidal transition can be observed exhibiting a midpoint at about 0.9–1.0 mol·l⁻¹ GuHCl and an onset at about 0.25 mol·l⁻¹ GuHCl. From this curve a transition heat can be estimated of about 100 kJ·mol⁻¹ (\approx 24 kcal·mol⁻¹) (including correction for preferential

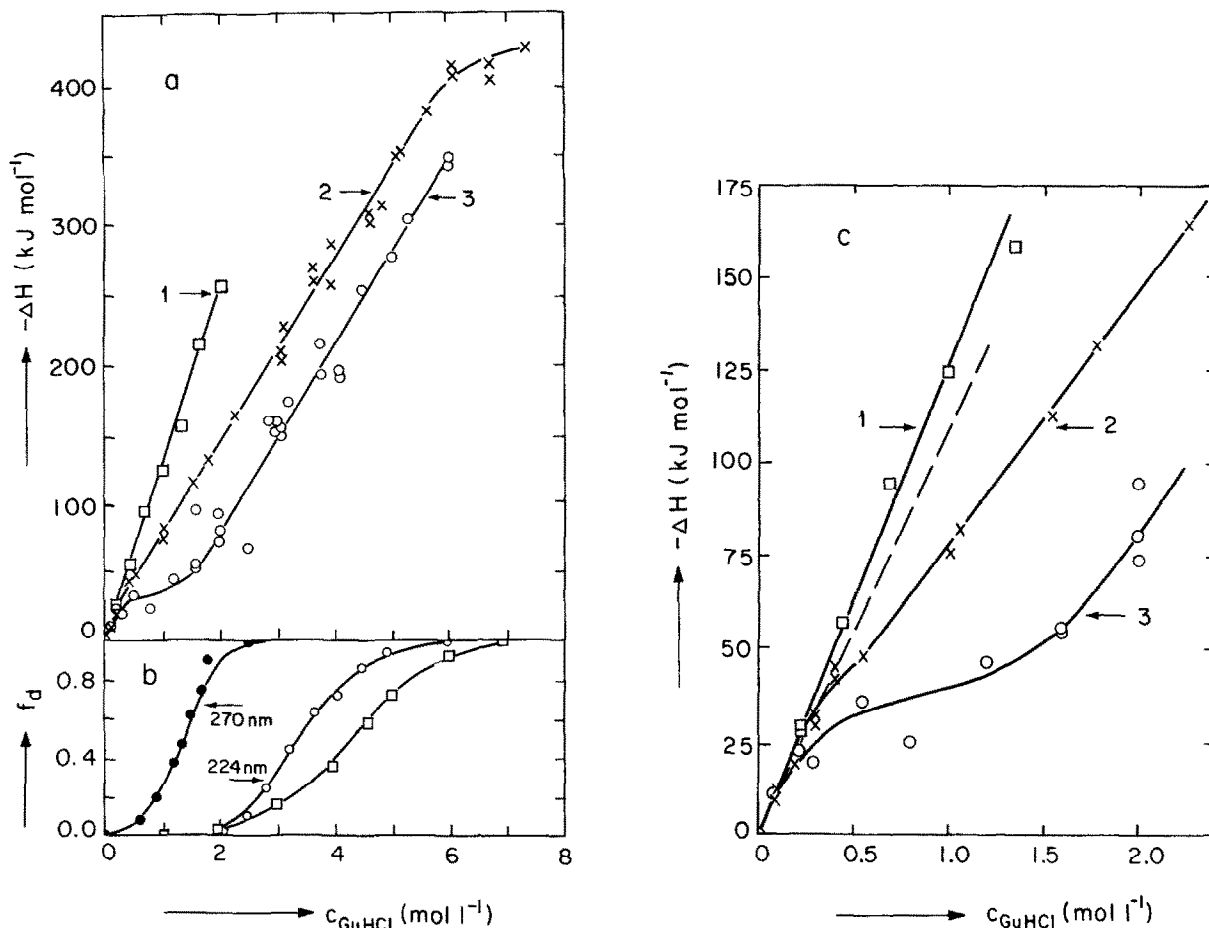


Fig.1. (a) Heat of the transfer ΔH of $H\alpha$ LA in GuHCl: curve 1: acid form in 50 mM KCl-HCl (pH 2.0) at 25°C, curve 2: neutral form in 20 mM Tris-HCl + 1 mM CaCl₂ at 25°C, curve 3: neutral form at 40°C. (b) Relative changes of molar ellipticities of $H\alpha$ LA vs GuHCl concentration at 25°C: pH 7.5 at 270 nm (●) and 224 nm (○); pH 2.0 at 222 nm (□). (c) ΔH at low GuHCl concentrations. Symbols as in panel a; (---) initial slope of the ΔH curve for the neutral form at 25 and 40°C.

binding according to [18,19]). From calorimetric titrations of the homologous lysozyme [18] it is known that the slope, s_D , in the post-denaturational region is increased compared with that of the pretransitional region, s_N . This observation is in agreement with the idea that a denatured protein exhibits more denaturant-binding sites than a native one. For $H\alpha$ LA, however, such an increase in the slope of the titration curve cannot be observed. Furthermore, there is no indication of sigmoidal curvature parallel to the changes of the CD spectrum in the far-ultraviolet region as displayed in fig.1b.

The intermediate in $H\alpha$ LA unfolding in $\sim 2 \text{ mol} \cdot \text{l}^{-1}$ GuHCl has been found to be similar to the acid (A) form of the protein in the absence of GuHCl at pH 2 [6,7]. In contrast to the native protein, the A form does not show any cooperative transition upon heating in scanning calorimetry (fig.2). In isothermal calorimetric titration, the A form markedly differs from the native protein. The initial slope of the calorimetric titration curve is increased, and no transition (or deflection) is visible below $2 \text{ mol} \cdot \text{l}^{-1}$ GuHCl (fig.1a, curve 1). Similar behaviour has been found for thermally denatured lysozyme [18].

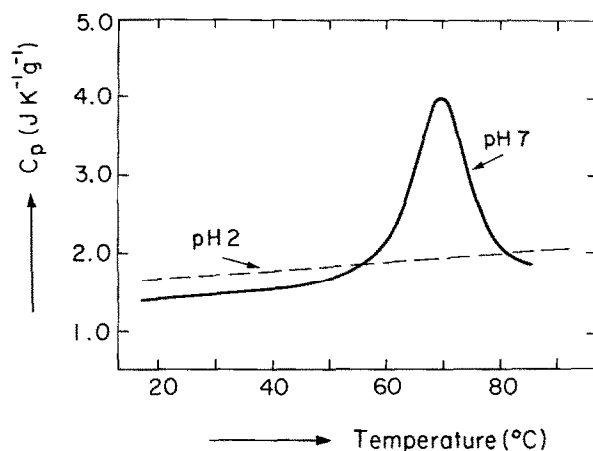


Fig.2. Heat capacity vs temperature: (—) neutral form (10 mM Mops, 0.1 mM CaCl_2 , pH 7.0), (---) acid form (50 mM KCl-HCl, pH 2.0) of H α LA.

4. DISCUSSION

GuHCl-induced H α LA unfolding is characterized by two consecutive transitions originally found from CD spectra in the near- and far-ultra-violet regions [6]. The calorimetrically observed transition corresponds to the first one, i.e. to the destruction of the specific environment of aromatic side chains. The transition is accompanied by an enthalpy change of $\Delta H = 100 \text{ kJ} \cdot \text{mol}^{-1}$ at 40°C. Comparing curves 2 and 3 in fig.1, a substantial heat capacity change responsible for the temperature dependence of the transition enthalpy can be suggested. The transition enthalpy, however, is considerably lower than that of the thermal transition of the calcium-free form of H α LA: $\Delta H = 254 \pm 15 \text{ kJ} \cdot \text{mol}^{-1}$ ($\approx 60 \text{ kcal} \cdot \text{mol}^{-1}$) at 40°C (Sadowski, M. and Pfeil, W., unpublished). The acid form of H α LA, which is characterized by the absence of the specific environment of aromatic side chains, does not show a cooperative transition below $2.0 \text{ mol} \cdot \text{l}^{-1}$ GuHCl (fig.1a, curve 1) or upon heating (fig.2).

A heat effect of the second transition at $2\text{--}5 \text{ mol} \cdot \text{l}^{-1}$ GuHCl which corresponds to the destruction of the secondary structure (fig.1b) and to the unfolding of the chain [7] cannot be found by means of the calorimetric titration. This means that the effect is either absent or rather small and therefore compensated by the increase in enthalpy

of the additional GuHCl binding upon this transition.

In conclusion, the first of the two consecutive structural transitions in H α LA unfolding by GuHCl, which is characterized by the destruction of the specific tertiary structure of the protein [6,7], is a highly cooperative one with remarkable enthalpy and entropy changes. The second transition, which corresponds to destruction of the secondary structure and unfolding of the chain [7], is not identified by calorimetric titrations. It appears as a gradual change with marginal enthalpy of the transition. This result is consistent with the recent theoretical consideration put forward on phase transitions of proteins [20], according to which the main reason for the phase transition is destruction of the protein specific tertiary structure rather than the secondary structure or unfolding of chain.

ACKNOWLEDGEMENTS

The authors are indebted to Dr V.V. Filimonov for valuable discussions and to N.V. Kotova for excellent technical assistance.

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