Volume 309, number 3, 258–260 FEBS 11511 September 1992
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Heat and cold denaturation of β-lactoglobulin B

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Received 2 July 1992

The thermal denaturation of bovine β-lactoglobulin B was investigated by high-sensitivity differential scanning microcalorimetry between pH 1.5 and 3.0 in 20 mM phosphate buffer. The process was found to be a reversible, two-state transition. Progressive addition of guanidine hydrochloride at pH 3.0 leads to the appearance of a low-temperature calorimetric endotherm, corresponding to the cold renaturation of the protein. Circular dichroism experiments have confirmed the low and high temperature denaturation processes, and have shown some structural differences between both denatured states of β-lactoglobulin B.

β-Lactoglobulin B; Thermal stability; Cold denaturation; Guanidine hydrochloride; Scanning calorimetry; Circular dichroism

1. INTRODUCTION

It is well established that the denaturation of small proteins follows a two-state process with an increase in enthalpy, ΔH, and heat capacity, ΔC_p. This ΔC_p value makes the denaturation enthalpy and entropy, and therefore the Gibbs energy, ΔG, temperature-dependent functions. The ΔG vs. T plot (stability curve) shows a maximum as well as two T values, when ΔG equals zero, which correspond to the heat and cold denaturation of the protein. Thus, cold denaturation of proteins is based on the thermodynamic formalism of protein stability, taking into account the positive ΔC_p value [1]. This apparently paradoxical phenomenon, which should occur with a decrease in enthalpy and entropy, was first predicted almost 30 years ago [2], but was not experimentally proved until very recently [3]. In fact, it has been shown that proteins with the highest ΔC_p and lowest ΔH of denaturation should be suitable for experimental cold denaturation studies [3].

β-Lactoglobulin B (β-LgB) is one of the two main variants of β-lactoglobulin, which is present in abundance in the milk of many mammals. The structure of the bovine protein is known although its function is still uncertain [4]. β-LgB is stable at acid pH values, but as the pH increases it self-associates and undergoes conformational changes, to finally denature under alkaline conditions. The denaturation of β-lactoglobulin has been investigated using different techniques under different conditions and its behaviour has been found to be complicated due to the association of the monomeric protein and the irreversible aggregation of its unfolded state [5–9]. We have carried out a differential-scanning-calorimetry (DSC) study of the protein at low pH and found a rather high denaturation ΔC_p, which led us to look for conditions under which to observe and characterize its cold denaturation. Guanidine hydrochloride (GuHCl) has proved to be very useful to this end and circular dichroism (CD) has confirmed both the heat and cold denaturation of the protein.

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2. EXPERIMENTAL

Thrice crystallized bovine milk β-lactoglobulin B was bought from Sigma. The purity of the protein was checked by SDS-PAGE and electrofocusing. It gave a single band. Protein concentration was determined using an absorption value of 0.96 $ml \cdot mg^{-1} \cdot cm^{-1}$ at 278 nm [8]. GuHCl was from Pierce and its purity was checked as described in [10]. GuHCl concentration was determined by refractive-index measurements [10]. Protein experiments were done in 20 mM phosphate buffer from pH 1.5 to 3.0 with the addition in some cases of different quantities of GuHCl. All other chemicals were of the highest purity available. Distilled, deionized Milli-Q water was used throughout.

DSC experiments were carried out in a DASM-4 instrument with cell volumes of 0.47 ml, under 2.5 atm. Scan rates from 0.5 to 2.0 K/min were employed. Calorimetric traces were analyzed, and protein samples were prepared as described elsewhere [11]. The circular dichroism spectra in the range 200–320 nm were recorded with a Jasco 720 instrument with thermostated cells. Protein concentrations were about 0.7 and 7 mg/ml for the far- and near-UV regions, respectively. Cells of 1 mm path length were used.

3. RESULTS AND DISCUSSION

Fig. 1a shows the original DSC recording of β-LgB in 20 mM phosphate buffer (pH 1.5) including the first

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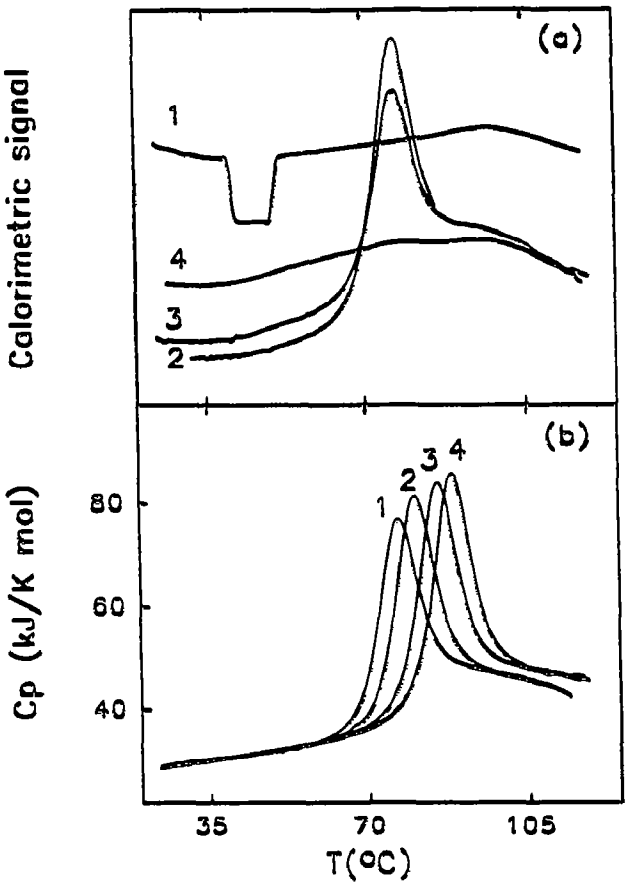

Fig. 1. (a) Original DSC recording of (1) base line including a 50 μW calibration; (2) β-LgB sample at pH 1.5, 6.4 mg/ml; (3) reheating of (2); (4) reheating of (3). (b) Partial heat capacity of β-LgB in 20 mM phosphate buffer: (1) pH 1.5; (2) pH 2.0; (3) pH 2.5; (4) pH 3.0. Scanning rate 2.0 K/min.

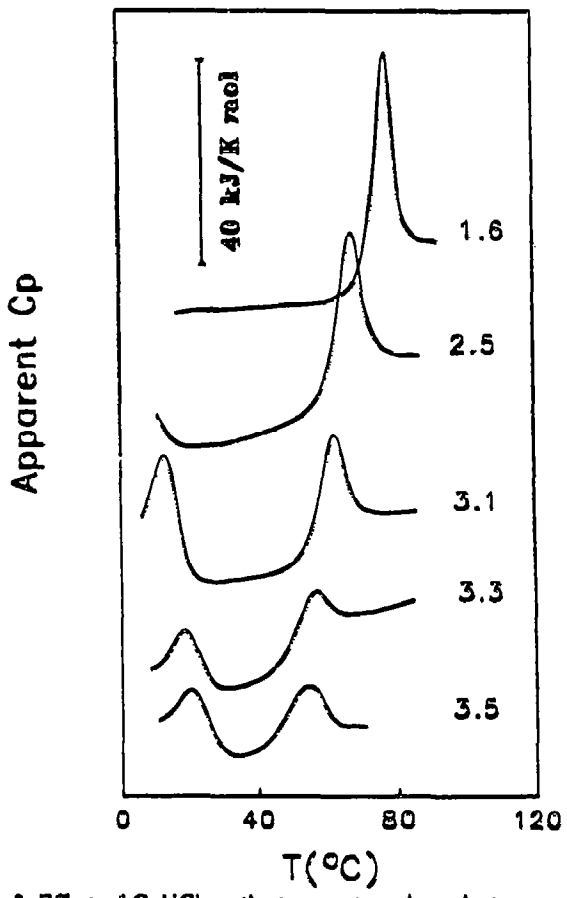

Fig. 2. Effect of GuHCl on the temperature-dependent apparent C_p of β-LgB at pH 3.0. GuHCl molar concentrations are indicated in the curves, which have been shifted along the y axis for display purposes. Scanning rate 0.5 K/min.

heating of the sample, the reheating after completion of the endotherm and the third scan after heating the sample to 120°C. Fig. 1b gives the apparent partial molar heat capacity of the protein in phosphate buffer within the pH range 1.5 to 3.0. The thermal denaturation is reversible under these conditions, although the heating up to 120°C leads to irreversible denaturation as depicted in Fig. 1a. DSC heating at a pH above 3.5 also causes irreversible denaturation with considerable aggregation of the sample at high temperatures. No noticeable protein concentration (3.0 to 10 mg/ml) or scanning-rate (0.5 to 2.0 K/min) effect was detected in the thermograms. The ratio of the calorimetric to the van 't Hoff enthalpies has been found to be very close to unity under all conditions (average value 1.00±0.05). This agrees with the previous assumption using non-calorimetric techniques that the denaturation of β-lactoglobulin follows a two-state mechanism [5,6,8], as well as with a more recent DSC result with the mixture of β-lactoglobulins A and B at pH 2.0 [9]. Nevertheless, DSC experiments at higher ionic strength, 0.5 M NaCl, also show thermal reversibility at these pH values but with a calorimetric to van 't Hoff enthalpy ratio lower than 1.0, indicating probable β-LgB association in the native state (results not shown).

The apparent partial specific heat capacity of the protein at 20°C is 1.5±0.2 J/K·g, whereas its heat capacity predenaturational slope is $(8.2\pm1.0)\times10^{-3}$ J/K^2·g. The heat capacity increase on denaturation, ΔC_p, is on the average 0.65±0.05 J/K·g, although this value slightly changes between pH 3.0 and 1.5. All these values compare well with others reported for globular proteins [12]. The rather high ΔC_p value is expected given the large hydrophobic character of this globular protein [13].

It is well known that GuHCl or urea show preferential binding (solvation) to the denatured protein state compared to the native one, thus explaining their denaturing capacity [1]. The effect of increasing concentrations of GuHCl on the reversible DSC behaviour of β-LgB at pH 3.0 is shown in Fig. 2. With increasing GuHCl the heat denaturation temperature and enthalpy decrease and a low temperature transition appears, gradually approaching the high temperature transition. We have obtained a corresponding DSC exothermic

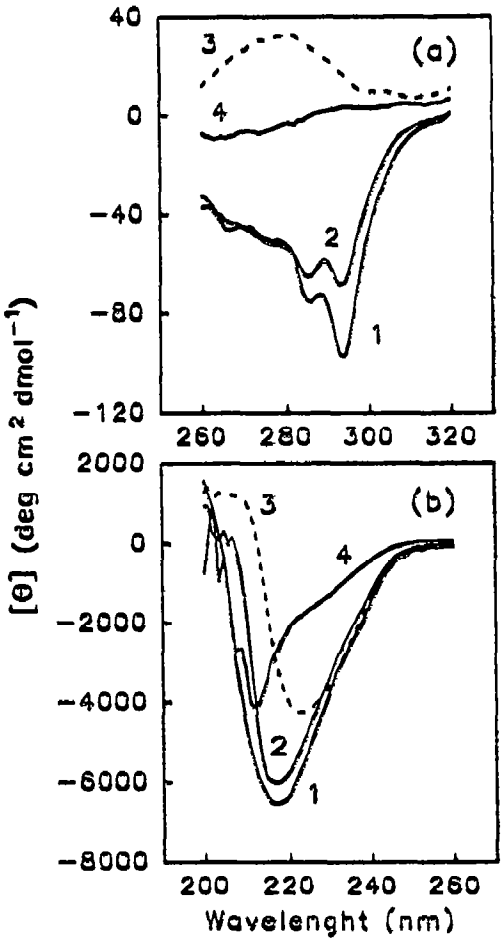

Fig. 3. (a) Near-UV and (b) far-UV CD spectra of β-LgB at pH 3.0 in phosphate buffer. (1) at 33°C; (2), (3) and (4) with 3.5 M GuHCl at 33°C, 88°C and −5°C, respectively.

transition (cold denaturation) by cooling the sample with 3.5 M GuHCl from 45°C to 8°C (results not shown). This behaviour typically resembles one of a cold denaturation process, the experimental accessibility of which depends in this case upon the effect of GuHCl, i.e. the denaturant lowers the protein stability curve, thus making for the cold denaturation temperature to fall into an experimentally suitable range. We have obtained similar DSC results at pH 2.0, and also with β-lactoglobulin A at both pH 2.0 and 3.0 (results not shown). A plot comparable to Fig. 2 was also obtained at pH 2.0 but with urea instead of GuHCl [1]. Despite the fact that the cold denaturation of proteins was predicted by Brandts in 1964 [2], experimental evidence has been reported only recently, and for very few proteins [3,14–16].

In addition to DSC studies, structural techniques, such as CD or NMR, have also been used to confirm protein denaturation at low temperatures under the appropriate conditions [3,14,15]. We have also carried out CD experiments with β-LgB at pH 3.0, with 3.5 M GuHCl, in temperature regions where the protein seems to be native, as well as denatured either at low or high temperatures, and also at pH 3.0 with no denaturant (Fig. 3). In the near-UV region (Fig. 3a) the presence of GuHCl at 33°C has a minor effect on the tertiary structure of the protein, whereas at both −5°C and 88°C this structure is practically absent. Fig. 3b shows a small decrease in the secondary structure at 33°C, due to the effect of GuHCl on the protein. Nevertheless, while at −5°C there is very little secondary structure, a somewhat different state is attained at 88°C with a less disordered structure that at −5°C. As well as confirming the cold denaturation detected by DSC, these results also reveal that both low and high temperature denatured states display CD spectra, i.e. they seem to have different secondary structure. This shows that the final structures derived at extreme temperature appear to be different. This singular situation agrees with the ΔC_p values for both denaturation processes: 0.44 J/K·g for heat denaturation and about 0.6 J/K·g for cold denaturation. Therefore, β-LgB also undergoes heat and cold denaturation, as has been described for a few other proteins [3,13–15], but the conclusion that both processes seem to lead to different states differs from what has been described for some of those proteins. These results, as well as similar studies with β-lactoglobulin A, deserve further investigation which is presently being undertaken in our laboratory.

Acknowledgements: This work was supported by Grant PB90-0876 from the DGICYT, Ministerio de Educación y Ciencia, Spain. We thank our colleague Dr. J. Trout for revising the English text.

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