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International Journal of Biological Macromolecules 37 (2005) 28–34

INTERNATIONAL JOURNAL OF
Biological
Macromolecules
STRUCTURE, FUNCTION AND INTERACTIONS

www.elsevier.com/locate/ijbiomac

Identification of pitfalls in the analysis of heat capacity changes of β-lactoglobulin A

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Received 27 July 2005; received in revised form 10 August 2005; accepted 10 August 2005
Available online 28 September 2005

Abstract

Information on changes in heat capacity (ΔC_p) of proteins upon unfolding is used frequently in literature to understand possible follow-up reactions of protein denaturation, like their aggregation propensity. This thermodynamic property is intrinsic to the protein's architecture and unfolding and should be independent of the approach used to evaluate it. However, for many proteins, the reported values for ΔC_p vary considerably. To identify whether the origin of these discrepancies lies within the experimental approach chosen and/or in the too simplified unfolding models used in the analysis of the data, we choose β-lactoglobulin A, a relatively small protein, but disputed for its two-state unfolding, and established its ΔC_p from tryptophan fluorescence, near-UV circular dichroism and differential scanning calorimetric measurements. In view of the large variation for the obtained ΔC_p (between 3.2 and 10.1 ± 0.8 kJ/(mol K)), it is evident that: (1) the sensitivity of different approaches to the structural changes; (2) irreversibility of unfolding; (3) non-ideal two-state unfolding behaviour need to be considered prior to interpretation. While the first two points can be addressed by using multiple approaches, the applicability of the selected unfolding behaviour for the analysis is often less easy to establish. In this work, we illustrate that by checking the wavelength-dependence used to detect protein conformational changes a tool is provided that gives a direct insight in the validity of the interpretation in these studies. An experimentally validated determination of ΔC_p allows a more proper use for the mechanistic understanding of protein denaturation and its follow-up reactions, avoiding pitfalls in the interpretation.

Keywords: Protein; Heat capacity; Thermodynamics; Spectroscopy; Calorimetry

1. Introduction

Hyperthermophilic microorganisms have adapted to their ambient temperatures in many ways. An important prerequisite for survival under stress-conditions is that its proteins have to adapt their folding stability properties. On the one hand, the globular protein structure needs to be stable enough to resist proteolytic or chemical degradation, but on the other hand, for proper functioning, dynamic transitions from the native to more unfolded states are essential. Structural stabilization, for example, by intensifying or extending the ionic network on the protein surface, will also result in large activation energies for (partial) unfolding [1]. An alternative route is to widen the temperature-range, where the protein's free energy change for unfolding (ΔG°) is positive, i.e. where the folded state prevails. For a two-state system, the dependence of ΔG° as a function of temperature is typically parabolic, where the sum of all enthalpy contributions to the globular stability determines the magnitude of maximal stability, while the change in heat capacity between the folded and unfolded conformer affects the width of the curve (see e.g. Becktel and Schellman [2]). An increase in heat capacity upon protein unfolding originates from the ordering of polar solvent molecules around the newly exposed non-polar groups in proteins that were originally buried in the core of the native structure. Any residual structure in the unfolded state results, therefore, in a lowering of the change of heat capacity (ΔC_p) upon unfolding. A possible mechanism to achieve this is by introduction of intramolecular disulphides in the protein, since these bonds reduce the conformational space in the unfolded form significantly [3]. On the other hand, internal chemical bonds may hinder a desired local flexibility and complicate the folding pathway considerably.

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doi:10.1016/j.ijbiomac.2005.08.001

The presence of residual structure in denatured proteins has been reported to impart directly any follow-up reactions, like, for example, in amyloid formation. ΔC_p-values are generally directly coupled to changes in accessible surface area (ΔASA) (see e.g. Gill and Wadsö [4]), and provides this information that can be used to develop mechanistic views on protein (un)folding processes. Insight in ΔC_p-values is desirable, not only for a better understanding of life science issues, for example, in understanding the mechanisms behind protein aggregation diseases, but also to optimise the use of proteins in biotechnological applications.

In literature, many different ΔC_p-values for β-lactoglobulin unfolding have been reported, varying between 5 and 12 kJ/(mol K) as the two most extremes [5,6]. In view of the above-described importance for the interpretation of this thermodynamic property, it is the aim of this work to study the origin of these discrepancies in more detail. To this end, a number of tools described in literature to determine ΔC_p is evaluated. Bovine milk whey protein β-lactoglobulin A is a relatively small protein (18.2 kDa), and its structure and thermodynamic properties have been well documented [7,8]. This protein has, however, often been described to unfold via intermediate states [9], while denatured proteins are well-known for their aggregation tendency [10]. Moreover, since at near neutral pH, this protein is dimeric, interference of the dissociation coupled unfolding (DCU) could be expected [11,12]. Together with the presence of a multiplicity of fluorescent chromophores, this protein is an ideal case to study the pitfalls in the interpretation of ΔC_p-values.

Direct measurement of ΔC_p is complicated. Generally, two indirect approaches are employed to establish ΔC_p-values for protein unfolding. The first is based on the determination of ΔG, by determining the fraction unfolded (f_u) and folded (f_f) protein using a spectroscopic tool as a function of a denaturant concentration, like that of urea or guanidinium, using the following equations [13]:

$$\Delta G^\circ = -RT\ \ln K = -RT \ln\left(\frac{f_u}{f_f}\right)$$

$$\text{and} \quad \Delta G = \Delta G_{(H_2O)} - m\,[\text{denaturant}] \tag{1}$$

where R is the gas constant and m an experimentally determined parameter. Performing these studies at various temperatures allows one to construct the free energy envelope as described by Pace and Shaw [14]:

$$\Delta G(T) = \Delta H\left(1 - \frac{T}{T_m}\right) + \Delta C_p\left(T - T_m - T\ \ln\frac{T}{T_m}\right) \tag{2}$$

where ΔH is the enthalpy change of the denaturation process with a midpoint temperature T_m. Determination of ΔH and T_m, using, for example, differential scanning calorimetry, provides then a data-set that can be analysed using Eq. (2) with ΔC_p as single fitting parameter.

An alternative approach can be followed based on the definition (e.g. Pace and Laurents [15]):

$$\Delta C_p = \frac{\delta(\Delta H)}{\delta(T_m)} \tag{3}$$

In this case, a system-parameter is varied, like pH, ionic strength, or other components that affect the solvent quality. This provides a series of ΔH's with corresponding T_m's, from which ΔC_p can be established from the slope of the relation.

Often, however, in literature, a single approach is selected for the determination of ΔC_p without validation of its applicability. This work aims to re-establish the reported differences in ΔC_p for a single batch of β-lactoglobulin A and to illustrate how pitfalls in the interpretation can be identified.

2. Materials and methods

2.1. Materials

β-Lactoglobulin (b-Lg) was isolated and purified (>98% purity) from fresh cow milk (A:B ratio 60:40) using the protocol as described by de Jongh et al. [16]. Separation of the two genetic variants was performed on a Source 15Q column (Pharmacia) eluting the proteins in 20 mM bis-Tris (pH 6.0) buffer and using a 0–1 M NaCl gradient in 10 column volumes, and detection of the proteins at 214 nm. The two variants displayed a separation at baseline level. The pooled fractions were dialysed extensively against demineralized water and stored at −40 °C. β-Lactoglobulin A (b-LgA) had a purity >99% as demonstrated by SDS-PAGE analysis and capillary electrophoresis.

2.2. Fluorescence spectroscopy

2.2.1. Isothermal unfolding experiments

A 10 M urea stock solution was prepared by dissolving 1.1 g of urea/ml of phosphate buffer (composed of equal amounts of a 20 mM Na_2HPO_4 and 20 mM NaH_2PO_4; (pH 7.0)). A 100 μl of a protein stock solution (0.75 mg/ml in 10 mM phosphate buffer pH 7.0) was diluted in 1.5 ml of urea-solution in phosphate buffer, yielding final urea concentrations between 0 and 8 M. First, the samples were incubated at 20 °C for at least 15 h, a condition shown to be sufficient to reach an apparent equilibrium in fluorescence intensity (unpublished results). Prior to measurement, the samples were incubated for at least 1 h in the water bath connected to the fluorescence cell at a selected experiment temperature (between 10 and 45 °C). After incubation, the sample was transferred to a thermostatted 1 ml quartz cell. The fluorescence emission was recorded between 300 and 400 nm using a Cary Med Eclipse (Varian) fluorimeter upon excitation at 295 nm using a scan speed of 100 nm/min and slit widths of 5 nm.

2.2.2. Thermal unfolding experiments

A 0.05 mg/ml protein, containing 0–1.2 M urea, was prepared as described above. The sample was heated from 15 to 100 °C with a heating rate of 1 K/min. The fluorescence emission was recorded at 360 nm upon excitation at 295 nm using slit widths of 5 nm.