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Energetic domains analysis of bovine α-lactalbumin upon interaction with copper and dodecyl trimethylammonium bromide

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ABSTRACT

Domain analysis of the dialyzed form of α-lactalbumin (m-α-LA) with varying concentrations of Cu^{+2} and DTAB has been carried out by differential scanning calorimetry (DSC), circular dichroism (CD) and resonance Rayleigh scattering (RLS) to elucidate the effect of the ligands on the thermal and structural properties of m-α-LA. The DSC profile displayed two dissimilar temperature-induced heat-absorption peaks as well as two melting points (T_m = 305 K, T_m = 333 K). The m-α-LA is not a new form of α-LA, but rather contains a mixture of the apo- and holo-forms of α-LA (i.e., a-α-LA and h-α-LA) at low and high temperatures, respectively. The presence of Cu^{+2} as the metal ion and DTAB as the non-metal ion altered the two heat-absorption peaks in such a manner that, with the addition of Cu^{+2} to m-α-LA, the excess molar heat capacity profile showed three sub-peaks, i.e., one sub-peak for a-α-LA at 303.2 K and two other sub-peaks for h-α-LA at 325 K and 334 K. The presence of these peaks was due to the molecular population of the a-α-LA form changing into h-α-LA. Contrarily, when it came to the interaction between DTAB and m-α-LA, the DSC thermogram showed two sub-peaks, i.e., one sub-peak for a-α-LA and another sub-peak for h-α-LA, resulting from the molecular population of the h-α-LA form changing into a-α-LA. The CD experiments on m-α-LA upon interaction with Cu^{+2} and DTAB demonstrated an increment and a decrement, respectively, of the α-helix content relative to that of the protein in the absence of the ligands. However, the α-helix induced by Cu^{+2} as a metal ion inspired one energetics domain in m-α-LA, wherefore it could be deduced that the helicity content caused an increment of the energetics content of α-LA. Hence, Cu^{+2} and DTAB at various concentrations played important roles as good probes for defining the electrostatic moiety for domains of m-α-LA initiated through a dissimilarity with regard to the α-helicity of these domains. The RLS intensity of m-α-LA upon interaction with Cu^{+2} and DTAB determine the DSC and CD results for inducing h-α-LA and a-α-LA respectively.

1. Introduction

In studies of protein folding, α-lactalbumin (α-LA) has received considerable attention over the years. This protein has an amino acid sequence and a conformation comparable to that of *c*-type lysozyme, but also contains a Ca^{+2} binding site giving rise to a stabilization of its native conformation [1,2]. On decalcification, an equilibrium state with a poorly defined tertiary but native-like secondary structure is created [3]. This molten globule conformation is also observed as a transient intermediate step when unfolding and refolding occurs under conditions far from equilibrium [4]. Consequently, a study of the partially unfolded equilibrium states of α-LA would provide information concerning the transient states that are generally encountered in protein folding [5–7].

α-Lactalbumin has also received attention because of its high-affinity Ca^{+2} binding site [1]. This site has been identified as a short loop structure consisting of ten residues, five of which contribute liganding atoms [2,8–11]. The physico-chemical aspects of the metal-binding to the protein have been thoroughly investigated. Ca^{+2} binding stabilizes the protein in its compact native state and current dogma is that partial unfolding is always accompanied by a loss of Ca^{+2} [12]. Vanderheeren and Hanssens have reported that, at neutral moderate Ca^{+2} concentrations (i.e., 2 mM), various metal ions remain associated with bovine α-LA after thermal denaturation of its native structure [10]. At 80 °C, the thermally denaturated Ca^{+2}-loaded α-LA takes on a more secondary structure than that apo-α-lactalbumin (a-α-LA). Moreover, at this temperature, the molten globule character of Ca^{+2}-loaded α-LA is conserved in such a way that the protein retains an accessible hydrophobic core, while the hydrophobic core of the apo-protein is largely lost.

α-LA is a metal-binding protein that competitively binds Ca^{+2} and Na^{+} ions to one specific site, giving rise to a large conformational change of the protein. The removal of bound calcium greatly decreases the thermal stability of α-LA, however the protein retains essentially the same folded conformation [13,14]. One of

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Nomenclature

α-LA	α-lactalbumin
a-α-LA	apo α-lactalbumin
h-α-LA	holo α-lactalbumin
m-α-LA	dialyzed form of α-lactalbumin (mixture of the apo and holo-forms of α-lactalbumin)
DTAB	dodecyl trimethylammonium bromide

the most interesting properties of α-LA is its transition to the molten globule state [15–17]. At temperatures above the thermal denaturation transition (i.e., approximately $T = 323$ K for apo-protein), at intermediate denaturant concentrations (3–4 urea) or when Ca^{+2} dissociates from α-LA at acidic pH (<3), the protein adopts the molten globule conformation. This has been described as a compact state containing a significant degree of the secondary structure present in the native protein, but with a fluctuating tertiary structure [18].

At neutral pH and in the presence of calcium, α-LA unfolds cooperatively at high temperatures, giving rise to significant increases in enthalpy and heat capacity [9]. At lower pH, α-LA unfolds in two stages [9,19]. The first stage is highly cooperative and proceeds with a significant and sharp heat absorption. However, the heat capacity at this stage does not reach the value expected for the fully unfolded polypeptide chain [18]. This value is rather reached at the second stage, which proceeds without a significant heat-absorption peak. It is therefore unclear whether this second stage represents a cooperative process with a small enthalpy, or if it corresponds to a gradual process [19]. Analyses of the change in optical properties associated with these two stages and of their dependence on the presence of calcium as well as a comparison with the unfolding of the structurally related equine lysozyme, permit the assignment of the first cooperative stage to the unfolding of the β-domain of α-LA and the second stage to the unfolding of the α-domain [9,19].

The presence of domains that exhibit a temperature-dependent unfolding has also been verified by fluorescence studies [10]. However, it remains unclear as to how independent such domains actually are, i.e., whether one domain is capable of folding and being stable without the other. This point needs to be clarified in order to understand the nature of the intermediate state of α-LA, which is usually regarded as a liquid-like "molten globule" state [20,21], rather than a partly unfolded state of a two-domain protein with one retained domain.

α-LA has often been used as a model protein when investigating the mechanism of protein folding [7,22–24]. This protein typically exhibits a molten globule intermediate during early kinetic folding as well as an equilibrium unfolding intermediate [7,23–25]. As detection and characterization of such folding intermediates are required in protein folding studies, the properties of α-LA have proven to be quite useful for such studies. The molten globule state of the protein has a native-like secondary structure and is compact with regard to its shape. However, it does not present any specific tertiary structure (i.e., specific side-chain packing), and the α-domain is more organized than the β-domain in the α-LA molten globule [23].

α-LA with various concentrations of calcium has been explored by differential scanning calorimetry to elucidate the effect of this ligand on the thermal properties of α-LA [26]. In the presence of excess calcium, the protein unfolds upon heating with a single heat-absorption peak and a significant increase of heat capacity. Analysis of the observed heat effect has shown that this temperature-induced process closely approximates a two-state transition. The transition temperature becomes increased in proportion with the logarithm of the calcium concentration, resulting in a raise in the transition enthalpy as expected from the observed heat capacity increment of denaturation [27–29]. Due to the discovery of the important role of the compact denatured state in many pathological processes associated with protein misfolding and/or the structural rearrangement to non-native conformations, the conformational stability of denatured proteins has been the focus of much interest [30]. In order to understand the role of this state in the development of various kinds of pathological reactions, it is important to know the underlying energetic principles governing the structural stability of this native-like conformation of denatured proteins.

Housaindokht et al. [31] have performed calorimetric studies of the influence of copper and dodecyl trimethyl ammonium bromide on the stability of bovine α-LA. They demonstrated, through the use of sensitive scanning microcalorimetry techniques, that a solution of α-LA at pH 8.0 in dialyzed condition represented a mixture of apo-α-LA (a-α-LA) and holo-α-LA (h-α-LA). These forms do not readily interconvert, and therefore unfold at different temperatures. On the other hand, the interaction between α-LA and ions (e.g., Cu^{+2} and DTAB) showed that the ions altered the partial heat capacity and enthalpy change values of the a-α-LA and h-α-LA forms. Such calorimetric data permitted a reliable determination of the molecular population partition for the interaction of the a-α-LA and h-α-LA forms with Cu^{+2} and DTAB, a quantity difficult to obtain by other methods.

This paper describes an attempt to employ DSC and CD techniques to obtain additional information regarding the energetics domains and the α-helical structure, based on the interaction of Cu^{+2} and DTAB, at various concentrations, with α-LA. The careful characterization of α-LA has in the present study been used to monitor the process of its unfolding and to demonstrate a new aspect of the energetic basis of the structural stability of this protein.

2. Materials and methods

2.1. Materials

Bovine α-LA was obtained from Merck Chemical Co. The purity of the protein was monitored by PAGE under both native and denatured conditions. The concentration of the protein solution was measured spectrophotometrically using an extinction coefficient $E^{\%1}(280\ \text{nm}) = 20.9$ [32] with a correction for light-scattering effects. a-α-LA was prepared according to previously described procedures [33], and the concentrations of a-α-LA and m-α-LA were measured by Bradford assays [34] and Stoschek methods [35]. Dodecyl trimethylammonium bromide (DTAB) and copper sulfate (Cu^{+2}) were obtained from Sigma and Merck, respectively. A visking membrane dialysis tubing {molar mass cut off: 10,000–14,000 g mol^{-1}} was obtained from SIC, Eastleigh, Hampshire, UK. All other materials and reagents were of analytical grade, and solutions were prepared in double-distilled water. A Tris solution of concentration 10 mmol dm^{-3}, pH = 8.0, was used as a buffer.

The visking tubing, employed as the semi-permeable membrane, was boiled three times, each time for 15 min, in ethylene diamine tetra acetic acid (EDTA) and sodium bicarbonate after which it was washed several times with distilled water and stored in (0.2 C_2H_5OH + 0.8 H_2O). The bovine α-LA solution was dialyzed against a buffer (Tris, 10 mM, pH = 8.0), which was exchanged every 8 h. The m-α-LA was ready after 24 h.