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Thermodynamic and structural analysis of homodimeric proteins: Model of β-lactoglobulin

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ABSTRACT

The energetics of protein homo-oligomerization was analyzed in detail with the application of a general thermodynamic model. We have studied the thermodynamic aspects of protein–protein interaction employing β-lactoglobulin A from bovine milk at pH = 6.7 where the protein is mainly in its dimeric form. We performed differential calorimetric scans at different total protein concentration and the resulting thermograms were analyzed with the thermodynamic model for oligomeric proteins previously developed. The thermodynamic model employed, allowed the prediction of the sign of the enthalpy of dimerization, the analysis of complex calorimetric profiles without transitions baselines subtraction and the obtainment of the thermodynamic parameters from the unfolding and the association processes and the compared with association parameters obtained with Isothermal Titration Calorimetry performed at different temperatures. The dissociation and unfolding reactions were also monitored by Fourier-transform infrared spectroscopy and the results indicated that the dimer of β-lactoglobulin (N_2) reversibly dissociates into monomeric units (N) which are structurally distinguishable by changes in their infrared absorbance spectra upon heating. Hence, it is proposed that β-lactoglobulin follows the conformational path induced by temperature: $N_2 \rightleftharpoons 2N \rightleftharpoons 2D$. The general model was validated with these results indicating that it can be employed in the study of the thermodynamics of other homo-oligomeric protein systems.

1. Introduction

According to the thermodynamic hypothesis of folding [1], small soluble globular proteins can be thermodynamically present into two well differentiated states: on the one hand, the ***native state*** (N) represents a compact folded conformation, with a high amount of buried water-accessible surface area (ASA) and within a minimum of Gibbs free energy; on the other hand, the ***unfolded or denatured*** state (D), in which most of the amino acid side-chains are exposed to solvent with a high conformational entropy.

Each macroscopic state is an ensemble of microscopic conformational states. The relative low number of degenerated conformers in the N state, represented by a deep valley in the folding energy landscape, can be further stabilized by the interaction with ligands or by protein–protein interactions forming homo or hetero-oligomers. The overall thermodynamic stability of the N state may be deeply affected by the affinity for a ligand, the local concentration of the reactants and the strength of the protein-protein interaction [2–5]. These changes in protein stability can be explained only considering the strength of the binding, determined by the association constant, and the proper intrinsic unfolding properties of the N state. This thermodynamic effect is due to the linkage between binding and unfolding equilibriums and represents a clear example of Le Chatelier's principle. Many studies of ligand binding either to the N state or to the D state of different proteins have been performed as well as the rationalization of these effects through the development of thermodynamic models [2,3,5,6].

Oligomeric proteins as well as protein–protein interactions (PPIs) are extensively present in biology and are considered of crucial importance in regulation of metabolism [7–12]. The thermodynamic parameters obtained from protein thermal unfolding with differential

Abbreviations: N, native state; D, unfolded state; *ASA*, accessible surface area; PPIs, protein-protein interactions; ΔH_{vH}, van't Hoff enthalpy change; ΔH_{cal}, calorimetric enthalpy change; ΔH_D, unfolding enthalpy change; ΔH_n, association enthalpy change; n, oligomerization number; β-LG, β-lactoglobulin; ΔC_{PD}, unfolding heat capacity change evaluated at the reference temperature; ΔC_{Pn}, association heat capacity change evaluated at the reference temperature; $\Delta C'_{PD}$, unfolding heat capacity change temperature dependence; $\Delta C'_{Pn}$, association heat capacity change temperature dependence; rms, root mean square deviation

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scanning calorimetry (DSC) help to understand the physics of protein oligomerization. The model for the analysis of calorimetric protein transitions has been developed by Freire and Biltonen [13] and it states that the enthalpy of the transition is proportional to the population size of unfolded protein. This approach provides the direct calculation of the partition function from the partial heat capacity of the protein solution. The thermodynamics of protein–protein interaction has been developed with this approach for the general homo-oligomeric protein system $N_n \rightleftharpoons nD$[14], then applied to the analysis of the stability of Co-chaperonin GroEs [15] and extended to the study of different lengths of thermolysin C-terminal fragments which follows the three step equilibrium model $N_2 \rightleftharpoons 2N \rightleftharpoons 2D$ [16,17]. However, it has been demonstrated that the proportional model is not accurate for the study of oligomeric proteins and, instead, it is necessary the development of a specific thermodynamic model for each oligomeric protein system [18]. From the statistical thermodynamic study of the equilibrium $N_n \rightleftharpoons nD$ Rösgen and Hinz pointed out that the assumption of proportionality between the temperature induced population and enthalpy changes was not correct [18]. A rigorous analysis of calorimetric data is necessary for the understanding of the energetics of PPIs that in turn controls the behavior of proteins. Burgos et al. [19] have recently developed two general models to describe complex profiles of calorimetric data from unfolding of oligomeric proteins upon heating. These general models were developed following the criterion described by Rösgen and Hinz [18,19] treating the proteins as statistical thermodynamic systems embedded canonically in the buffer solution. Model A assumes a reversible dissociation to native monomers coupled to unfolding ($N_n \rightleftharpoons nN$ and $N \rightleftharpoons D$). Model B considers unfolding linked to the equilibrium between the oligomeric N state and a conformational oligomeric intermediate ($N_n \rightleftharpoons I_n$ and $I_n \rightleftharpoons nD$) [19]. In that previous work we described the thermodynamics of both models and showed their potential prediction capability on the thermal behavior of different oligomeric protein systems. The models are general and could be employed in the study of numerous oligomeric protein systems with different subunit numbers. A great potential advantage is the possibility of the construction of the calorimetric transition baseline after the optimization of the calorimetric trace. In order to validate the models developed we sought a simple dimeric and well known protein system which could be studied with differential scanning calorimetry and separately analyzed its dissociation and unfolding processes.

By perusal of existing literature on thermal unfolding of oligomeric proteins we found that the β-lactoglobulin (β-LG) is quite suitable to experimentally test the Model A reported in ref. [19]. β-LG is the major whey protein of ruminant species [20] and the naturally occurring level in cow's milk is on average about 3 mg mL^{-1} [21]. β-LG has 162 amino acid residues with a molecular weight of 18400 and it is well known that it exists in the dimeric form at neutral pH at protein concentrations over 1 mg mL^{-1}. Moreover, several works performed by different techniques have reported that no other oligomerization state is present at this pH [22–32]. β-LG belongs to the lipocalin family characterized to be small secreted proteins with a highly conserved antiparallel β-barrel with nine β-strands and one α-helix, which encloses a hydrophobic pocket as internal binding site interacting with small fatty acids, retinol, vitamin D and also cholesterol [20,33]. The crystal structure of the dimeric form of β-LG has been reported previously [24,34], and the association constant was determined by several techniques and in different experimental conditions and the values informed are in the range 10^3–10^6 M^{-1} [23,27,35,36]. Given the enormous biotechnological value of β-LG and the importance of heating of cow's milk for commercial purpose there is great amount of literature referred to the effect of heat [37–41]. In the present work we performed calorimetric experiments of neutral β-LG solutions. The DSC data were analyzed with model A [19] in order to obtain the thermodynamic parameters that govern the dissociation and unfolding of β-LG. The comparison with the ITC results clearly demonstrated the complementarity between both calorimetric techniques for a rigorous analysis of the dissociation and unfolding processes of an oligomeric protein. Furthermore, the analysis was completed with infrared spectroscopy experiments to correlate the structural and stability changes. From our work it arose that bellow 50 °C β-LG dissociates into monomeric native units ($N_2 \rightleftharpoons 2N$) and above 50 °C the dissociation is coupled to a conformational change ($N_2 \rightleftharpoons 2N^*$) previous to the protein unfolding ($N^* \rightleftharpoons D$). The results show the potential of the general thermodynamic model in the analysis of the energetics of protein homo-oligomerization which can be used, in turn, for the analysis of a given particular protein oligomeric system.

2. Material and methods

2.1. Materials

β-lactoglobulin A (β-LG) from bovine milk was from SIGMA (cat. no. L7880) and was used without further purification. Only one band was observed when protein purity was checked by SDS-PAGE 12% in absence of beta-mercaptoethanol. The buffer employed for all the experiments was 100 mM sodium phosphate (pH 6.7) prepared in ultra pure water and the reagents were from Merck and Cicarelli. For all the experiments, protein concentration was determined by absorbance spectrophotometry with a $\varepsilon^{1\%} = 9.6$ at 278 nm.

2.2. Differential scanning calorimetry measurements

Thermograms were obtained using a MicroCal VP-DSC calorimeter from MicroCal Llc. β-LG concentration ranged between 18 and 243 μM. All the solutions were filtered (0.45 μm, Millipore membranes) and degassed before loading in the calorimetric cell. The reference cell was filled with buffer and a pressure of 26 p.s.i. was applied to both cells. A scan rate of 60 °C/h was used in all the experiments. Buffer–buffer scan was subtracted to the crude sample scan and subsequently normalized for total protein concentration. The resulting thermogram was analyzed with an optimization routine. A reversible three states model for oligomeric proteins was utilized to obtain the thermodynamic parameters of the dissociation and unfolding processes [19]. When reheating a complete unfolded β-LG solution no endotherm was observed in the DSC traces (data not shown). A possible justification for the applicability of reversible thermodynamics to apparently irreversible processes has been discussed previously [42–45] for the case where reversible unfolding is followed by a rate-limited irreversible step. Both reactions, the dissociation and the unfolding, could be considered reversible as it was observed in an experiment in which a β-LG solution (1 mg mL^{-1}) was heated 15 °C from the starting temperature (10 °C) and cooled down to the starting temperature, then heated 20 °C and cooled down again, and so on, and it was observed that the signals were superposed (Fig. S1 in the Supplementary data). The signal was not fully recovered only when the solution was heated over the temperature of the maximum of the second transition. This means that an irreversible aggregation process occurs immediately after the protein unfolding takes place and, therefore, it is plausible to apply a thermodynamic model to the calorimetric data.

Briefly, the model assumes a two state unfolding transition coupled to the native protein oligomerization equilibrium.

$$N \rightleftharpoons D \quad \text{(I)}$$
$$nN \rightleftharpoons N_n \quad \text{(II)}$$

The equilibrium constants and enthalpy changes for reactions I and II are:

$$K_D(T) = \frac{[D]}{[N]} \tag{1}$$