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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:

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

and

$$K_n(T) = \frac{[N_n]}{[N]^n} \tag{2}$$

$$\Delta H_D(T) = \Delta H_D^\circ + \Delta C_{PD}^\circ (T - T_D) + 1/2 \Delta C_{PD}^{'} (T - T_D)^2 \tag{3}$$

and

$$\Delta H_n(T) = \Delta H_n^\circ + \Delta C_{Pn}^\circ (T - T_n) + 1/2 \Delta C_{Pn}^{'} (T - T_n)^2 \tag{4}$$

where T_D is the temperature at which half of the total protein is in the unfolded state, $T_n$is the reference temperature for the oligomerization process, $\Delta C_{PD}^\circ$and ΔC_{Pn}° are the heat capacity change evaluated at the reference temperature for reactions I and II respectively and $\Delta C_{PD}^{'}$ and $\Delta C_{Pn}^{'}$are their respective temperature dependences.

Once the protein system is defined, its relative partition function is partially derived respect to temperature, keeping pressure and total protein concentration constant, and then, the analytical expression for the enthalpy of the system relative to the reference state, N, is obtained [19]:

$$H - H_N = [\Delta H_D(T)\alpha_D + \Delta H_n(T)\alpha_{Nn}] \left[\frac{1}{1 + (n-1)\alpha_{Nn}} \right] \tag{5}$$

where $\alpha_D = \frac{[D]}{[N]+[D]+n[N_n]}$ and $\alpha_{Nn} = \frac{n[N_n]}{[N]+[D]+n[N_n]}$.

And, finally, the heat capacity of the system relative to the reference state as a function of temperature (T), at constant pressure (P), is derived from the expression of the enthalpy of the system relative to the enthalpy of the reference state:

$$C_P - C_{P,N} = \frac{d(H - H_N)}{dT}. \tag{6}$$

With this set of equations it is possible to simulate the thermograms if the parameters are known or either one can fit the experimental results to obtain these parameters. The parameters in Eqs. (3) and (4) and the equilibrium constant for reaction II, $K_n(T_n)$, evaluated at the reference temperature, were adjusted simultaneously using a global multidimensional annealing procedure which minimizes the root mean square deviation between the experimental and calculated thermograms. *The complete computer codes of both the simulation program and the fitting routines are available on request to the authors.*

2.3. Isothermal titration calorimetry determinations

Isothermal titration calorimetry (ITC) measurements were done using a VP-ITC calorimeter from MicroCal, Llc. (Northampton, MA, USA). β-LG concentration was between 3.1 mg mL^{-1} and 6.6 mg mL^{-1}. The samples were degassed under vacuum prior to titrations. The reference cell was filled with ultra pure water. The protein solution was injected at 5 minute intervals. A 250 μL syringe with a 270 rpm constant rotation was used for all the experiments. The temperature of the titration cell was set at either 30 °C, 40 °C, 45 °C, 50 °C, 55 °C or 60 °C. Raw data were processed and integrated with Origin-ITC 7 software provided by the manufacturer and the parameters for protein association were obtained by fitting the integrated data with Eq. (A1) which is the same as in ref [46] but also includes the heat of dilution as a fitting parameter. The first injection in each experiment was not taken into account for the analysis. Independent experiments at each temperature were analyzed separately and the fitted parameters were averaged.

2.4. Calculation of thermodynamic parameters

Enthalpy and heat capacity changes were calculated using their relationship with changes in the solvent accessible surface area [47,48]. Changes in accessible solvent area (ΔASA) were calculated using the crystal structure of the dimer (PDB code:1BEB), while for the monomers we considered both, rigid body separation of the monomers from the dimer, and rigid body separation plus relaxation of the amino acid side-chains at the dimer interface.

The thermodynamic parameters were calculated using the program STC [49], while the prediction of the side-chain rearrangements upon dissociation were done using the programs SCWRL [50,51] and SCAP [52].

2.5. Fourier-transformed infrared spectroscopy

Fourier-transformed infrared spectroscopy (FT-IR) spectra were performed with a Nicolet Nexus interferometer using a thermostated demountable cell for liquid samples with CaF_2 windows. The spectrometer was flushed with dry nitrogen to reduce water vapor distortions of the spectra. Normally, 50 scans were collected both for the background and the sample at a nominal resolution of 2 cm^{-1}. Sample was prepared dissolving the lyophilized buffer and the protein with 200 μL of D_2O and incubated 24 h at room temperature to allow deuterium exchange of the amide protons.

3. Results

3.1. Calorimetric analysis

The heat capacity profiles of solutions of β-LG at pH = 6.7 are shown in Fig. 1. The thermogram traces evidence two transitions, indicating that two cooperative processes take place as temperature is raised. The first transition, at lower temperature, is evidently smaller and less cooperative than the second one, and presents a clear dependence with total protein concentration. Indeed, as total protein concentration is increased the first transition occurs at higher temperatures (Fig. 1).

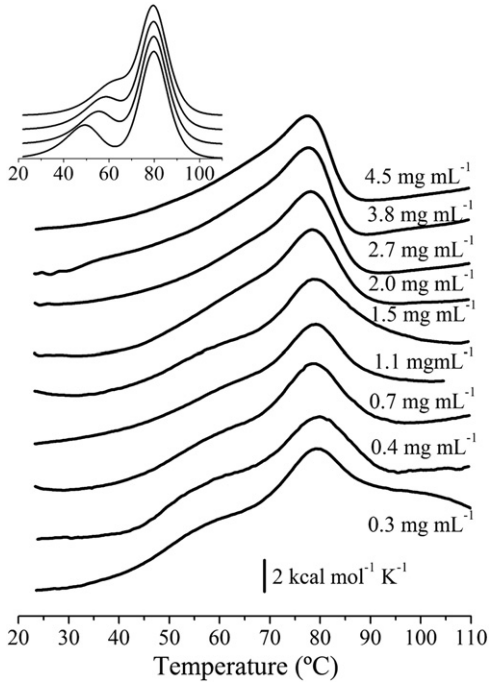

Fig. 1. DSC scans of β-LG at increasing total protein concentration. The crude thermograms were normalized by total protein concentration and the reference scan was subtracted. Inset: Calorimetric profiles simulated with Model A [19] in the same protein concentration range as in the experimental thermograms. Simulation Parameters: $n = 2$, $T_D = 80$ °C, $\Delta H_D^\circ = 60.8$ kcal mol^{-1}, $\Delta C_{PD}^\circ = 0$ kcal mol^{-1} K^{-1}, $K_n = 7.5 \times 10^5$ M^{-1}, $T_n = 35$ °C, $\Delta H_n^\circ = -50$ kcal mol^{-1}, $\Delta C_{Pn}^\circ = 0$ kcal mol^{-1} K^{-1}.

On the contrary, the higher transition is not sensitive to protein concentration up to 2 mg mL^{-1}. At higher protein concentrations (above 2 mg mL^{-1}) the maximum of the second transition ($C_{P\,max}$) slightly shifts to lower temperatures (Fig. 1). Two reasons are plausible to explain this behavior. On the one hand, at higher protein concentrations, the first process becomes close to the second process overlapping in a great extent and the resulting endotherm has its maximum between the two endothermic contributions. On the other hand, the irreversibility discussed above affects the unfolding endotherm shape as the aggregation process is favored as total protein concentration is increased in the sample. Actually, both facts may contribute to the endotherm shape. Therefore, a rigorous thermodynamic analysis should only be applied to calorimetric profiles when β-LG sample has a protein concentration less than 2 mg mL^{-1}.

In the previous work [19], we reported that different calorimetric behaviors could be expected for dimeric protein systems when total protein concentration was changed. One protein system assumed an exothermic protein association reaction and the second one had associated a positive enthalpy of dimerization (Fig. 3 from [19]). From the analysis of the simulated calorimetric profiles and the species concentration profiles it was stated that protein systems with positive self association enthalpy exist as native monomers at low temperature and the dimerization process is favored as temperature is increased. At higher temperatures the dimer dissociates and unfolds in one step. In this situation, there is a change in protein molecularity in both processes. For this reason, both processes, dimerization and unfolding, depend on total protein concentration. On the contrary, a protein with negative dimerization enthalpy exits predominantly as a native dimer at low temperatures. The dimer dissociates as temperature is increased and at high temperature the native monomer unfolds into the denatured monomers.

The inset of Fig. 1 shows the simulated calorimetric profiles of a dimeric protein obtained with the model presented in Section 2.2. The simulations were performed in the same protein concentration range as in the experimental thermograms, with negative enthalpy of dimerization and it is reproduced the same tendency observed in the experimental DSC profiles. β-LG is mainly present as a dimer at near physiological pH values [22–24,29,31,32], hence, it can be proposed that dimerization of β-LG is an exothermic process, and that the first transition is due to dimer dissociation and the second transition is the result of the unfolding of the native monomers according to scheme III:

$$N_2 \rightleftharpoons 2N \rightleftharpoons 2D \quad \text{(III)}.$$

The energetics of β-LG homodimerization at neutral pH (pH = 7) has already been calorimetrically determined by Bello et al. [23]. They performed dissociation ITC experiments between 15 °C and 35 °C and reported that the association process is exothermic with dimerization enthalpy values in the range of −2.4 and −11.3 kcal mol^{-1} and that the association constant is in the order of 10^4 M^{-1}. These results are in accordance with parameters already published from studies of sedimentation equilibrium and light scattering [27,29,32]. They also stated that the association enthalpy is temperature dependent and found a value of -495 ± 9 cal mol^{-1} K^{-1} for ΔC_{Pn}. We employed DSC data to obtain thermodynamic information about association and unfolding of β-LG. Endotherms of β-LG solutions at different concentrations bellow 2 mg mL^{-1} were simultaneously fitted employing the equations for a dimer [19] (Fig. 2, see Section 2.2). The parameters are listed in Table 1 and the deconvolutions of the transitions are shown in Fig. 3, together with the transition baselines obtained from the fitting. The association enthalpy and constant are quite in accordance with the values already reported [23,27,29,32]. The fitting procedure allowed doing the optimization without previous baseline subtraction. This was a great advantage, since the construction of a reliable baseline, before the subtraction, is the most difficult step in the usual processing of DSC scans. Indeed, β-LG has a multiple transition calorimetric profile

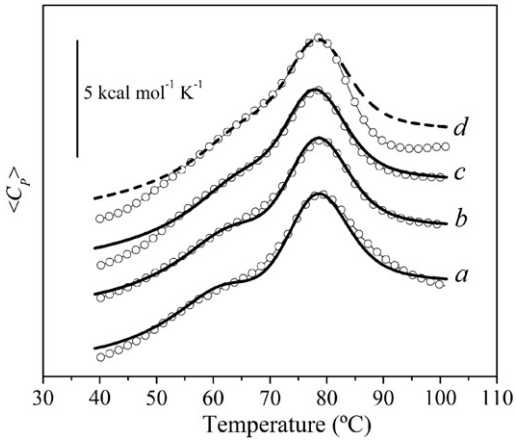

Fig. 2. Optimization of β-lactoglobulin DSC scans at different total protein concentration. Protein concentration (○): (a) 0.7 mg mL^{-1}, (b) 1.1 mg mL^{-1}, (c) 1.6 mg mL^{-1} and (d) 2.0 mg mL^{-1}. The solid line is the optimization obtained with the model developed in Ref. [19] with $n = 2$ and the parameters shown in Table 1. The dashed line is a simulation for 2.0 mg mL^{-1} with the parameters from the optimization.

thus, the construction of a baseline under it, without direct information of the heat capacity changes for each transition and their respective temperature dependences, introduces some probability of error. The results in Fig. 2 show that there was little difference between the experimental endotherms and the optimization results. The specific enthalpy change of unfolding (Table 1) had a lower value (3.3 cal g^{-1} at 75 °C) compared to that found by Privalov for typical compact globular proteins (7–9 cal g^{-1} at 75 °C) but instead, it agreed with the values reported for proteins that would not have a compact conformation according to his analysis [53]. Nevertheless, the crystalline structure of β-LG is the type of a compact and globular conformation [24]. The sum of the areas of the deconvoluted transitions (Fig. 3B) was 99.6 kcal mol^{-1} which was similar to the values reported by other authors for the whole calorimetric transition of bovine β-LG from different sources: 98.4 kcal mol^{-1} [39], 90.8 kcal mol^{-1} [54], and 99.6 kcal mol^{-1} [55]. Another striking result from the DSC fitting was the value of the heat capacity change of association, ΔC_{Pn}, which was among three to five times the value obtained previously with ITC (compare Table 1 with ref. [23]). The value of ΔC_{Pn}, obtained from the DSC fitting, was comparable with the value of the heat capacity change of unfolding per mole of β-LG (Table 1). The increase in heat capacity of a protein solution due to the unfolding process is attributed to the exposure to the solvent of hydrophobic residues in the unfolded state [56,57], while the dissociation of the dimer implies the exposure of the aminoacids located at the dimer interface which, in the case of β-LG, are mainly polar and charged residues [23]. An anomalously large heat capacity change in protein–protein recognition can be accounted by immobilized water molecules at the interface [23,58] although this phenomenon alone would not be sufficient to explain the large magnitude of ΔC_{Pn}. On the other hand, the ITC determinations of ΔC_{Pn} were obtained, as usual, through linear regression of the temperature dependence of ΔH_n in a reduced temperature interval [23]. The DSC traces of β-LG were optimized with no assumptions regarding a linear dependence of $\Delta H_n$with temperature and over a wide temperature range (Table 1). In fact, best rms values were obtained when the temperature dependence of ΔC_{Pn} ($\Delta C'_{Pn}$) had a non zero value. In order to better understand the origin of these discrepancies, we performed ITC dissociation experiments over a broader temperature range and the ΔH_n values at each temperature are shown in Fig. 4 and Table 2. From Fig. 4 it is evident that there are two distinguishable regimes for the ΔH_n temperature dependency. Between 30 °C and 50 °C the slope is soft but from 50 °C to 60 °C the enthalpy change is abrupt. From the slope of the linear regression of these two regimes we determined two values of ΔC_{Pn}(Table 3). The ΔC_{Pn}value of the higher temperature regime is definitely close to the ΔC_{Pn} obtained from the DSC

Table 1
Apparent unfolding and association parameters of β-lactoglobulin at pH = 6.7 optimized employing model A [19] with n = 2[a].

T_D (°C)	ΔH_D[b] (kcal mol^{-1})	ΔC_{PD}[b] (kcal K^{-1} mol^{-1})	$\Delta C'_{PD}$[b] (kcal K^{-2} mol^{-1})	K_n[c] (M^{-1})	ΔH_n[b,c] (kcal mol^{-1})	ΔC_{Pn}[b,c] (kcal K^{-1} mol^{-1})	$\Delta C'_{Pn}$[b,c] (kcal K^{-2} mol^{-1})
75.5	60.8	2.3	−0.016	6.7×10^5	−5	−1.5	0.05

[a] Global parameters estimated from the DSC profiles at different total protein concentration (lines a, b and c from Fig. 2) with rms = 3.997.
[b] Parameter values normalized per mole of monomer.
[c] Values at T_n = 35 °C.

fittings (Table 1). We propose that β-LG native dimer would dissociate into native monomers ($N_2 \rightleftharpoons 2N$) in certain temperature interval (up to 50 °C) and above 50 °C the dissociation of β-LG would be between different thermodynamic states, for instance:

$$N_2 \rightleftharpoons 2N^*(\mathrm{IV}).$$

This hypothesis considers a monomeric state that would expose to the solvent not only the aminoacids present at the dimer interface, but also apolar residues from the interior of the protein upon dissociation which could account for the larger value of ΔC_{Pn}. Previous intrinsic fluorescence studies of β-LG proposed that exposure of neutral protein solutions at temperatures below 60 °C resulted in a reversible modification of the hydrophobic regions around the protein tryptophans [37]. The authors suggested a molten globule like state as a product of the heat induced dissociation of the dimer [37]. The unfolding of a heat-swollen monomeric state would be a reasonably explanation to the low value of specific unfolding enthalpy obtained from the fitting of the DSC traces (Table 1). The different values of ΔC_{Pn} found with ITC experiments for two temperature intervals also explain that best DSC fittings were obtained when it was considered that ΔC_{Pn} temperature dependence ($\Delta C'_{Pn}$) had non zero value. Analyses of change in accessible solvent area (ΔASA) were done in order to compare them with the experimental changes observed for ΔC_{Pn}. The analyses of change in accessible surface area between the monomer and the dimer were carried out from de crystal structure data of β-LG dimer at pH = 6.5 considering either a rigid body or taking into account the relaxation of the aminoacid side-chains at the interface, Table 2 and Table 3 (see Section 2.4). The calculated ΔC_{Pn} values were smaller in magnitude than all the experimental estimations supporting the idea that either changes of water molecules at the interface or a conformational change are coupled to the dissociation process.

Fig. 2 also displays (in dashed line) the simulation of the calorimetric profile for 2 mg mL^{-1} total protein concentration employing the parameters obtained in the fitting procedure. Comparing the simulated with the experimental endotherm for 2 mg mL^{-1} it was evident that an exothermic process changed the shape of the unfolding transition, giving additional evidence that above this concentration it is not possible to correctly optimize calorimetric profiles with a model that excludes an irreversible aggregation process of unfolded conformers ($nD \rightarrow D_n$).

The parameters obtained from β-LG endotherms fitting also allowed the simulation of the species profiles against temperature and the different contributions to the DSC traces (Fig. 3). The species profiles for two protein concentrations are shown in Fig. 3 *A*. The model employed in the optimization confirms that at room temperature β-LG is predominately a dimeric protein for these concentration values. The temperature increase induces the dissociation of the dimer into the monomeric state and, finally, the monomeric β-LG unfolds. The areas under the deconvoluted curves were approximately 40 kcal mol^{-1} and 60 kcal mol^{-1} and the widths at the half of the peak were 18.5 °C and 12.8 °C for the first and the second endotherms respectively evidencing that the dissociation of the dimer was a cooperative process. Fig. 3B also shows that the dissociation occurred at higher temperature as total protein concentration was increased while the unfolding process was insensitive to changes in protein

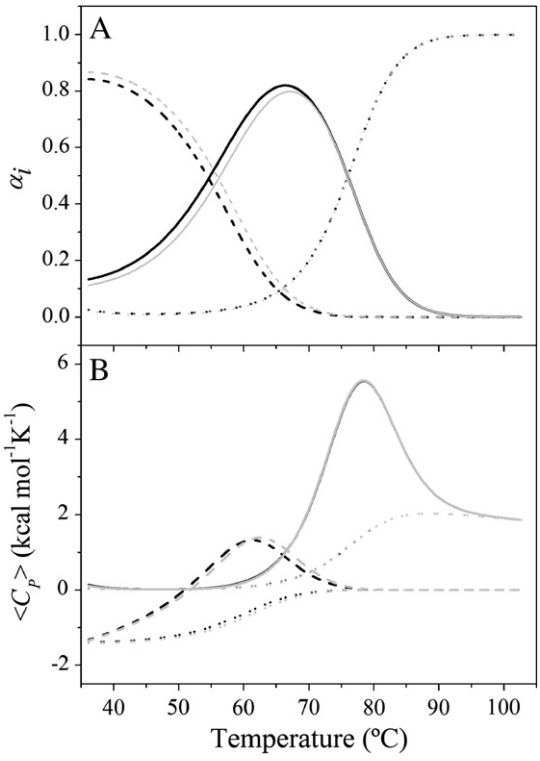

Fig. 3. Temperature dependence of the species profiles (A) and the contributions to the DSC traces (B). (A) Species profiles for 0.7 mg mL^{-1} (*black*) and 1.6 mg mL^{-1} (*gray*) concentrations of β-LG; N (▬), N_2 (▪▪▪) and D (┈┈). (B) Contributions for 0.7 mg mL^{-1} (*black*) and 1.6 mg mL^{-1} (*gray*) concentrations of β-LG: $N_2 \rightleftharpoons 2N$dissociation process (▪▪▪), $N \rightleftharpoons D$ unfolding contribution (▬) and the transition baselines (┈┈).

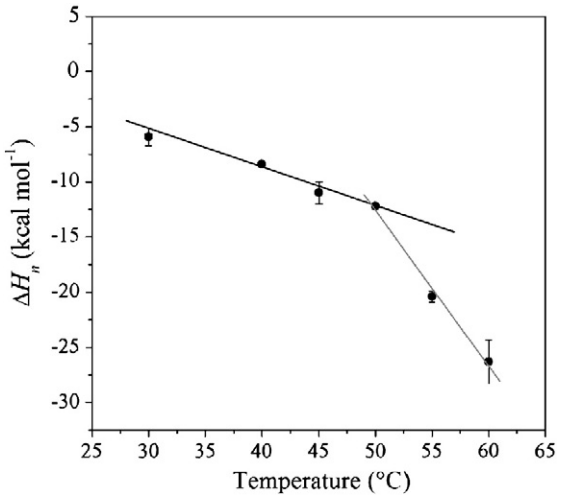

Fig. 4. Association enthalpy of β-LG. Heat of dilution of concentrated solutions of β-LG obtained with ITC at different temperatures.

Table 2
Apparent association parameters of β-lactoglobulin at pH = 6.7 from ITC data and calculated with the ΔASA.

T (°C)	K_n[a] / 1×10^5 M^{-1}	ΔH_n[a] (kcal mol^{-1})	ΔH_{nASA}[b] (kcal mol^{-1})	ΔH_{nASA}[c] (kcal mol^{-1})	ΔH_{nASA}[d] (kcal mol^{-1})
30	1.3 ± 0.5	−5.9 ± 0.8	−8.4	−7.0	−4.9
40	0.7 ± 0.4	−8.4 ± 0.3	−9.5	−8.4	−6.3
45	0.6 ± 0.4	−11 ± 1	−10.0	−9.1	−7.0
50	0.16 ± 0.04	−12.2 ± 0.3			
55	0.10 ± 0.02	−20.4 ± 0.5			
60	0.04 ± 0.01	−26 ± 2			

All the parameter values were normalized per mole of monomer.
[a] Average value from three independent ITC experiments at 30, 40 and 50 °C, from two titrations at 45 and 55 °C and four independent titrations at 60 °C.
[b] From crystal structure.
[c] With side chain relaxation predicted with SCRWL.
[d] With side chain relaxation predicted with JACKAL.

concentration. We would like to point out that the baselines for complex calorimetric transitions are not easily predictable and it would be a more reliable alternative to fit the endotherms without baseline subtraction and considering the parameter $\Delta C'_{Pn}$ in the fit (Fig. 3B).

All in all, DSC analysis performed with our model would provide valuable and accurate information regarding to heat capacity changes and resulted in a strong tool to the study of protein dissociation coupled to unfolding, mainly when complemented with ITC dissociation experiments.

3.2. Fourier transformed infrared spectroscopy analysis

The applicability of the thermodynamic model to the analysis of calorimetric data is subjected to the equilibriums presented explicitly in the model. However, if some conformational change takes place when the protein dissociates the model could still be employed, though both the energetic of the dissociation and of the structural change will be included in the apparent parameters. In order to discern if the change in β-LG oligomerization goes together with modifications in protein conformation we performed FT-IR in D_2O at pD = 6.7 upon heating throughout the range of temperature used in DSC. The direct amide I spectrum at room temperature has the well defined bands expected for this type of protein with a mainly β-barrel structure presenting the main second derivatives peaks at 1623 cm^{-1}, 1633 cm^{-1}, 1647 cm^{-1}, 1663 cm^{-1}, 1677 cm^{-1} and 1691 cm^{-1}, in agreement with those previously reported either for β-LG sub-variant A [59] or B [60]. The bands were analyzed according to the assignments already carried out by other authors [61]. Fig. 5 shows the dependence of the spectra of β-LG with augmented resolution by Fourier deconvolution at increasing temperatures and at different protein concentrations, covering a similar range than those used in DSC experiments. It can be easily visualized that as temperature was increased the absorbance at 1623 cm^{-1} decreased, in a similar way to the shift observed by Casal et al. [60] for β-LG at pH = 7. This band was prominent at 17 °C and completely disappeared above 67 °C (Fig. 5). This rather strong change in the FT-IR profile at 1623 cm^{-1}, particularly in the mentioned interval of temperature, was in keeping with that observed for the first transition in DSC thermograms, assigned to the dimer dissociation (compare with Fig. 2, see also Fig. 6). Interestingly, the change observed in β-LG spectra at 1623 cm^{-1} with T had a clear dependence on total protein concentration since this band became more prominent when the range of β-LG concentration changed from 0.65 mg mL^{-1} to 4.40 mg mL^{-1} at the same temperature (compare the spectra at these concentrations at 47 °C and 57 °C, Fig. 5). Above 75–80 °C the FT-IR profile was distorted and the typical bands associated with the unfolding were evident: 1647 cm^{-1} for disordered conformations and 1615 cm^{-1} assigned to inter-chain aggregation (Fig. 5). In order to assure the assignment of the changes in FT-IR at 1623 cm^{-1} to dimer dissociation, we compared this band intensity normalized with the isosbestic point found at 1627 cm^{-1}. Fig. 6 shows the ratio of the absorbances at 1627 cm^{-1}/1623 cm^{-1} *vs.* temperature at five different increasing concentrations and at each 2 °C after reaching adequate equilibrium time. The change is clearly cooperative and it is coincident with the DSC first transition trace (Fig. 6). To prove reversibility in FT-IR intensity changes at 1623 cm^{-1} we performed different cold-heating cycles up to 55 °C at a protein concentration of 1 mg mL^{-1}. After time equilibration the profile of the original FT-IR spectrum was completely recovered (data no shown), indicating that the changes were reversible in keeping with the DSC data.

Table 3
Apparent heat capacity change of β-lactoglobulin association from ITC data and calculated with the ΔASA.

		Experimental[a]	X Ray	SCRWL	JACKAL
ΔC_{Pn} (kcal mol^{-1} $°C^{-1}$)	35 °C–50 °C	−0.33 ± 0.03	−0.11	−0.14	−0.14
	50 °C–60 °C	−1.4 ± 0.1			
ΔASA_{polar}			520	520	444
ΔASA_{Apolar}			540	610	572

[a] Determined from the slope of the ΔH_n at different T. Normalized per mole of monomer.

4. Discussion

In a previous work we reported that different calorimetric traces could be expected for dimeric protein systems with positive and negative enthalpy of association when the total protein concentration was changed [19]. In the case of β-LG, the dissociation is an endothermic reaction (i.e. exothermic association), dimers are stable at relatively low temperature and the dissociation process is favored as temperature is increased. When total protein concentration is augmented the dimer is stabilized and dissociates at higher temperatures, near to the monomer unfolding temperature point, overlapping the DSC trace. Dimer dissociation implies a change in protein molecularity and that is the reason why the overall process depends on total protein concentration, (Figs. 1 and 3 from this work, and see also Fig. 3 in ref [19]). The behavior of the dimerization-dissociation transition is cooperative and reversible in successive heating-cold cycles. The increase in heat capacity of a protein solution due to the unfolding process is mainly attributed to the exposure to the solvent of hydrophobic residues in the unfolded state [56,57]. The dissociation of the dimer also implies the exposure of the aminoacids interacting at the dimer interface which, in the case of β-LG, are mainly polar and charged residues [23]. The apparently anomalous large heat capacity change found for the dissociation of β-LG through the DSC analysis (Table 1) can be accounted by immobilized water molecules at the interface [23,58]. However, ITC dilution experiments performed over a broad interval of temperature evidenced two different kinds of behavior for the association–dissociation of β-LG (Fig. 4). At low temperature the association had a low ΔC_{Pn} value while above 50 °C the enthalpy change of dimerization had a strong dependence with temperature, which resulted in a ΔC_{Pn} value similar to the one obtained from the DSC analysis (Fig. 4 and Table 3). These outcomes may be a consequence of a conformational change associated to protein dissociation when it occurs above 50 °C. The changes observed at 1623 cm^{-1} in the FT-IR curves of β-LG solutions strongly support this idea. As temperature was increased the absorption at that wave number diminished in the range in between 40 and 60 °C in a cooperative manner and the transition shifted towards higher temperatures as protein concentration was raised (Fig. 6). The protein concentration dependence confirms that the spectral change is associated to dimer dissociation. In order to assess even more the correlation between the spectral changes with temperature and the first endothermic process, we compared in Fig. 7 the cooperative changes observed in the FT-IR spectra followed

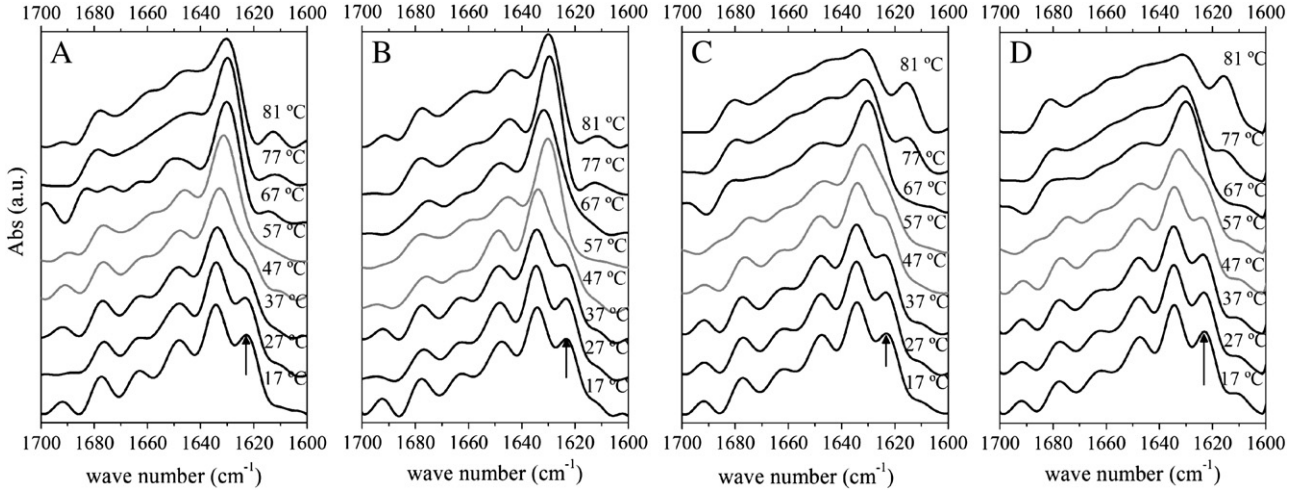

Fig. 5. Temperature dependence of the Fourier self deconvolution of the FT-IR spectra of β-lactoglobulin. Protein concentrations (A): 0.65 mg mL^{-1}; (B): 0.92 mg mL^{-1}; (C): 3.07 mg mL^{-1}; and (D) 4.40 mg mL^{-1}. The arrow indicates the absorbance at 1623 cm^{-1}. The spectra in gray line clearly show dependence of the change in absorbance at 1623 cm^{-1}with total protein concentration.

as the ratio at 1627 cm^{-1}/1623 cm^{-1} with the fraction of progress of monomeric β-LG state with temperature, calculated with the model proposed ($N_2 \rightleftharpoons 2N$, $N \rightleftharpoons D$), using the thermodynamic parameters shown in Table 1 and employing the same protein concentration. The dependence on the total protein concentration observed in the cooperative and reversible changes of the FTIR results allowed us to relate them to the degree of interaction between monomers. Absorbance at 1623 cm^{-1} has been assigned to anti parallel β-sheet structure as well as the 1634 cm^{-1} band [62]. The difference between them is that the former establishes stronger hydrogen bonds and it does not show isotopic effect [62]. The β-hairpin peptide conformation was characterized by a strong absorbance around 1620 cm^{-1}. It was proposed that β-strands connected by turns instead of long loops, as it is in the case of β-hairpin conformation, consist in a more rigid structure that would explain the differences observed between the amide I spectra of anti parallel β-sheet and β-hairpin conformations [63]. The β-barrel of β-lactoglobulin is formed by β-strands connected either by turns and loops, that is why the amide I spectra of this protein presented a strong absorbance at 1623 cm^{-1} and at 1634 cm^{-1}. Our results showed that the temperature induced dissociation of β-LG would produce a less rigid structure indicated by the lost of the 1623 cm^{-1} band and by the change in the center of gravity of the spectra towards higher wave numbers (Fig. S2, in Supplemental data). All this information indicates that the dissociation takes place with a discernible conformational change when it is induced by heat, then the unbound monomer would be

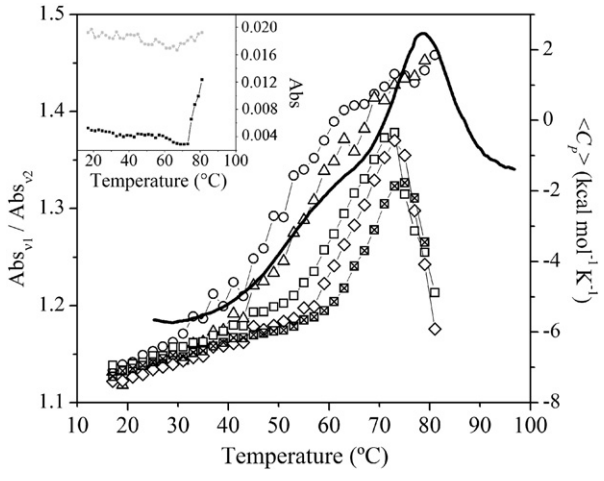

Fig. 6. Conformational changes upon β-lactoglobulin dissociation. Absorbance ratio between ν1 (1626.7 cm^{-1} wave number) and ν2 (1622.8 cm^{-1} wave number) as a function of temperature at different total β-lactoglobulin concentration: (○) 0.65 mg mL^{-1}; (△) 0.92 mg mL^{-1}; (□) 3.07 mg mL^{-1}; ( ) 4.40 mg mL^{-1}; ( ) 9.20 mg mL^{-1}. The solid line is the experimental calorimetric profile of β-lactoglobulin for 0.7 mg mL^{-1} of total protein concentration. *Inset:* Absorbances assigned to irreversible intermolecular aggregation (1615 cm^{-1} (■)) and to disordered structure (1647 cm^{-1} (■)) as a function of temperature.

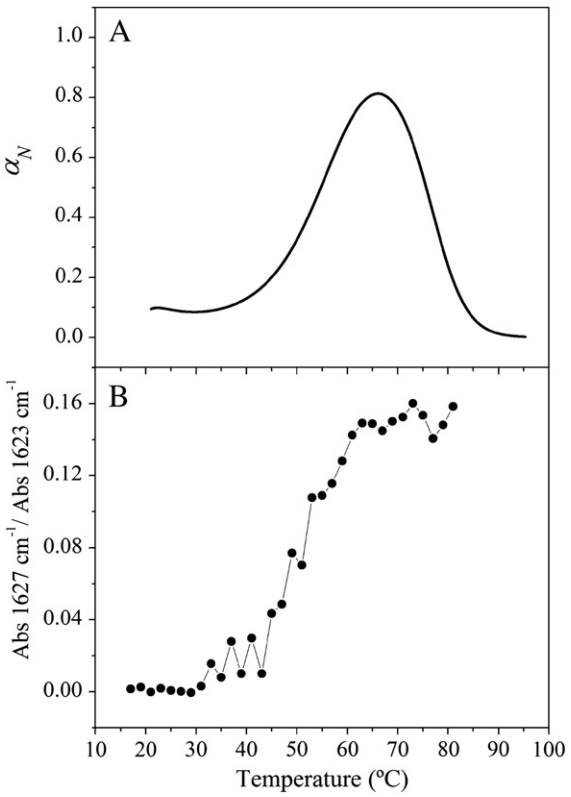

Fig. 7. Correlation between FT-IR analysis with DSC data for β-Lactoglobulin dissociation previous to monomer unfolding. (A) Fraction of native monomer (α_N) as function of temperature simulated according to the model for a total protein concentration of 0.65 mg ml^{-1} using the parameters obtained from thermogram fitting in Fig. 2 and Table 1. (B) Absorbance ratio between ν1 (1626.7 cm^{-1} wave number) and ν2 (1622.8 cm^{-1} wave number) as a function of temperature at the same protein concentration of (A).

distinguishable from the monomer in the dimeric form as a less compact conformer and the reaction should be written as: $N_2 \rightleftharpoons 2N^*$. This means that the unfolding reaction would be: $N^* \rightleftharpoons D$, and it is a plausible explanation to the relative low value of unfolding enthalpy obtained from the DSC fittings comparing with the values obtained for typical globular and compact proteins [53]. This last consideration does not affect the thermodynamic analysis provided that the information obtained is treated as global macroscopic parameters which include the parameters of the microscopic reactions. In this sense was the proposal of Cairoli et al. [37] who found that dissociation of dimeric β-LG upon heating produced changes in the tryptophans fluorescence that indicated the presence of a molten-globule like state. It is interesting to note that β-LG dissociation and unfolding was studied at increasing concentrations of urea at pH 7.0 and 37°C and the results were in agreement with this model induced by temperature [64]. The intrinsic fluorescence intensity of β-LG increased in a cooperative manner with a midpoint transition centered at a urea concentration value that was dependent on protein concentration revealing the dissociation process. The transition shifted to much higher urea concentration when the position of the fluorescence maximum was monitored and it was insensible to changes in protein concentration evidencing that it corresponded to the unfolding of the monomeric species [64]. The authors proposed that the unfolding of β-LG induced by urea would follow the following steps: $N_2 \rightleftharpoons 2N \rightleftharpoons 2D$, and they discard the probability of a partially unfolded state as they observed that the changes of ANS dye intensity bound to the native β-LG were coincident with the transition of the position of the fluorescence maximum of tryptophan [64]. However, neither of the two tryptophans of each protein unit is located at the dimer interface (see PDB:1BEB), suggesting that some kind of conformational change would have produced the increase in the tryptophans intensity when the dimer dissociated.

Fig. 3 *B* shows that the baselines for complex calorimetric transitions are not easily predictable and it would be a more reliable alternative to fit the endotherms without baseline subtraction by considering the parameter $\Delta C'_{Pn}$ in the fit with an adequate model of unfolding. The model proposed in this report fits quite well with the experimental DSC traces of β-LG at relatively low protein concentration. Assuming a correct model of dissociation for β-LG coupled to unfolding the changes in heat capacity and enthalpy can be better deconvoluted for both cooperative processes (see Fig. 3). Protein concentration higher than 1.6 mg mL^{-1} (see Fig. 2) induced an irreversible aggregation after unfolding which distorted the thermogram at upper temperatures.

The interactions and forces that keep the monomer–monomer inter-chain stability are similar in nature to those involved in the intra-chain folding of the protein. We found an endothermic enthalpy and a positive value in heat capacity change upon β-LG dissociation that was coincident to parameters already found at neutral [23,27,29,32] and acid pH [35]. The association process has a considerable effect to the global stability of the dimer. Protein–protein association affects the thermal stability of β-LG due to the association constant coupled to unfolding and also because the dissociation above 50 °C has a conformational change associated that would be the cause that changes in heat capacity in both the dissociation and the unfolding temperature dependent processes have similar values. As the heat capacity changes become higher the overall stability of the protein decreases [65,66].

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Appendix A. Equation for the analysis of ITC results

Curves were analyzed with the following equation [46]:

$$q_i = \frac{V\Delta H_{dis}}{[M]_{syr}}\left[\frac{K_{dis}}{4}\left(\left(1+\frac{8[M]_{Ti}}{K_{dis}}\right)^{1/2}-1\right)\right]-\frac{K_{dis}}{4}\left(\left(1+\frac{8[M]_{T(i-1)}}{K_{dis}}\right)^{1/2}-1\right)\left(1-\frac{v}{V}\right) - \frac{K_{dis}v}{4V}\left(\left(1+\frac{8[M]_{syr}}{K_{dis}}\right)^{1/2}-1\right)+q_{dil}. \quad \text{A1}$$

Where

q_i	Heat of dissociation normalized per mol of total protein injected in injection *i*.
V	Cell effective volume.
ΔH_{dis}	Enthalpy of dimer dissociation normalized per mol of total protein as monomer.
K_{dis}	Dissociation constant.
v	Volume of injection *i*.
$[M]_{syr}$	Total protein concentration in the syringe.
$[M]_{Ti}$	Total protein concentration in the cell after injection *i*.
$[M]_{T(i-1)}$	Total protein concentration in the cell before injection *i*.
q_{dil}	Heat of the titrant dilution.

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