Unusual structural stability and ligand induced alterations in oligomerization of a galectin

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Abstract Abstract L-14, a 14-kDa S-type lectin shows the jelly roll tertiary structural fold akin to legume lectins yet, unlike them, it does not dissociate on thermal unfolding. In the absence of ligand L-14 displays denaturation transitions corresponding to tetrameric and octameric entities. The presence of complementary ligand reduces the association of L-14, which is in stark contrast with legume lectins where no alterations in quaternary structures are brought about by saccharides. From the magnitude of the increase in denaturation temperature induced by disaccharides the binding constants calculated from differential scanning calorimetry are comparable with those extrapolated from titration calorimetry indicating that L-14 interacts with ligands essentially in the folded state.

Key words: S-type lectin; Oligomerization; Differential Scanning Calorimetry

1. Introduction

Lectins, identified at the turn of the century by their ability to agglutinate erythrocytes, are multivalent carbohydrate binding proteins [1,2]. Their carbohydrate specificity makes them useful as probes for monitoring changes that occur in cell surface sugars during development, differentiation, immune response, malignancy, metastasis etc. and as model systems for exploring protein-sugar interactions [3,4]. Lectins are ubiquitously distributed in nature from viruses and bacteria to man. Though originally discovered in plants, biological roles of animal lectins is understood in much greater detail. For example, a number of mammalian lectins are involved in receptor mediated endocytosis of glycoproteins, cellular recognition processes including adhesion, metastasis, apoptosis and immune regulation [5–8].

Animal lectins are classified into two distinct groups that differ in their physicochemical properties and folding patterns [9]. C-type lectins such as mannose binding protein, E-selectin and the hepatic asialoglycoprotein receptor require calcium ion for ligand binding like legume lectins, yet adopt a tertiary structure quite different from the legume lectin fold [9,10]. In contrast, S-type lectins (galectins) display the legume lectin fold and require thiol reducing agents but do not require metal ions for activity [9,11,12].

The most abundant of the S-type lectins is the 14-kDa lectin also designated as L-14, galaptin and galectin [13,14]. The crystal structures of 14-kDa lectin from both a human source and bovine spleen complexed with lactose and *N*-acetyllactosamine, respectively, are available [11,12]. Thermodynamic analyses of L-14-saccharide interactions have shown that like legume lectins the reactions are enthalpically driven with marginal changes in heat capacities and exhibit enthalpy-entropy compensation [15].

DSC studies on a variety of legume lectins have shown that their thermal unfolding transitions are coupled with their dissociation into the constituent monomeric or sub-monomeric units [16,17]. Though ligands enhance their thermal stabilities their overall unfolding transitions are not altered dramatically. Despite the remarkable similarity between the legume lectin fold and that of L-14, DSC data presented here show that they differ strikingly in their mode of thermal unfolding and the influence of ligand on them.

2. Materials and methods

2.1. Preparation and analysis of solutions

L-14 from sheep spleen was prepared as described earlier [15]. L-14 solutions were prepared in the 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M sodium chloride and 10 mM β-mercaptoethanol (PBS-βME), dialyzed overnight against 3000× volume of the same buffer, and centrifuged to remove any insoluble material. The used concentration of L-14 ranged from 0.05 mM to 0.25 mM. The concentration of protein solutions were determined from extinction coefficient of L-14 i.e. $A^{1\%}_{280nm} = 5.5$ [18].

2.2. DSC measurements and analyses

DSC measurements were carried out with a Microcal MC-2 heat conduction Differential Scanning Microcalorimeter which consist of two fixed 1.18-ml cells, a reference cell and a sample cell. Most of the measurements were performed at a scan rate of 20 K h^{-1}. A few scans were also done at 10, 30 and 60 K h^{-1}. The data were analyzed using Origin data analysis software for DSC supplied by the manufacturer [19]. T_m and T_p are the temperature of the half of the transition peak area and the temperature at which the transition peak is at maximum, respectively. The ratio of calorimetric enthalpy (ΔH_c), to the van 't Hoff enthalpy (ΔH_v) i.e. $\Delta H_c/\Delta H_v$ provides the cooperativity of the transition. The binding constant at the denaturation temperature ($K_b(T_{pL})$) is obtained from the increase in the denaturation temperature in the presence of the ligand (T_{pL}), the concentration of the ligand ([L]), and the calorimetric enthalpy of the L-14 dimer as shown by the relationship [16,25] given by:

$$K_b(T_{pL}) = [\exp\{(T_{pL}-T_m)\,\Delta H_c(T_p)/nRT_{pL}T_m\}-1]/[L] \quad (1)$$

where n is the number of ligand binding sites in the L-14 oligomer.

3. Results and discussion

3.1. Thermal unfolding of L-14

A representative DSC scan for L-14 is shown in Fig. 1 along with the fit of the transition peak data to the A↔B two-state transition model. Deconvolution of the transition peak shows that it consists of two entities unfolding at different temperatures. The results of the fit of the transition peak

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Abbreviations: DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; L-14, 14-kDa S-type lectin

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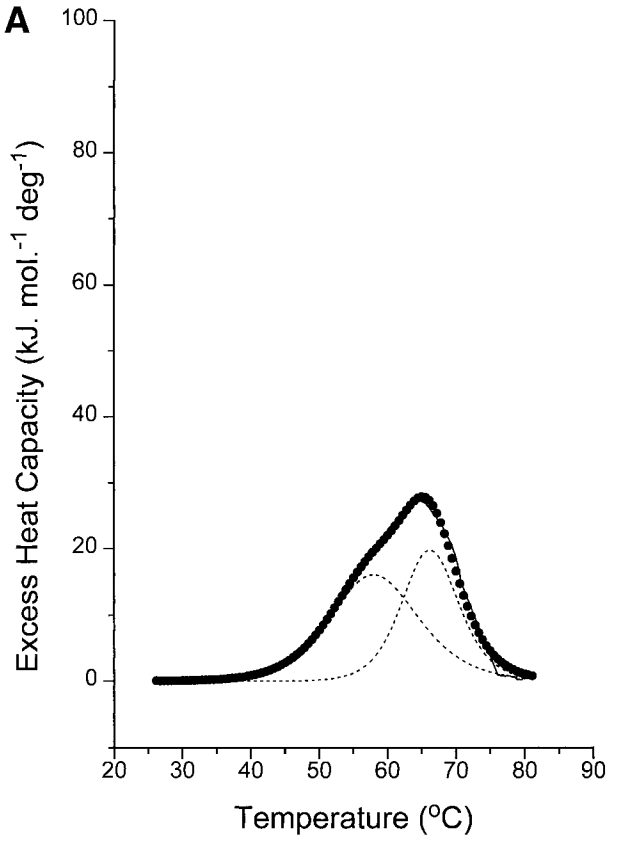

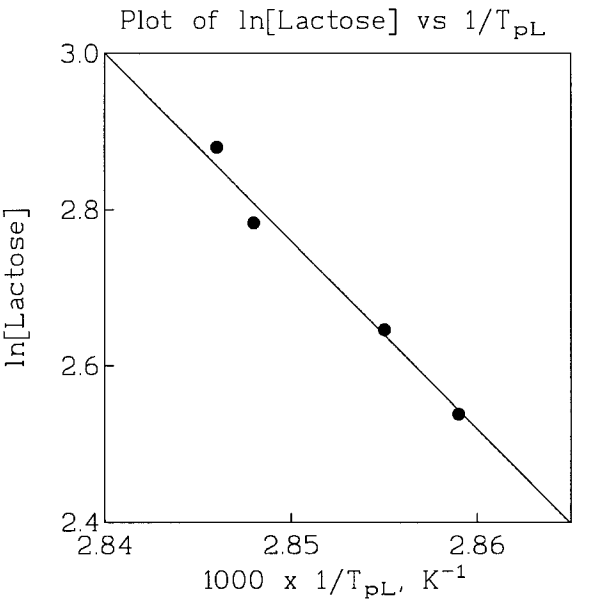

Fig. 2. A plot of ln[Lactose] vs. $1/T_{pL}$ for the L-14 transitions in the presence of ligand. The straight line is the best linear least squares fit of the data.

data are given in Table 1. Fits of the transition for a single unfolding species or greater than 2 unfolding entities were statistically inconsistent with the data. No endotherm was observed on subsequent re-scanning of the sample suggesting that both the transitions in the absence of ligand are irreversible. To rule out any kinetic effects on the thermal unfolding the data were also collected at scan rates of 10–60 K h^{-1} (Table 1). The results at higher scan rates exhibit only a slight increase (1.2 K) in T_m with increased scan rates but the thermodynamic parameters, ΔH_v and ΔH_c are similar. The equilibrium two-state transition model was, therefore, applied to these data instead of the irreversible model of Sanchez-Ruiz et al. [20]. The basis for this assumption is that the transition may be treated as a sequence of two processes where the first process is the reversible unfolding of the protein described by thermodynamic parameters T_m, ΔH_v and ΔH_c which is followed by a slower irreversible process e.g. aggregation. This treatment first demonstrated by Sturtevant and co-workers [21], yields results for the overall process that are the same as for the reversible process. This less conservative view is supported by others [22,23] and justifies treating the unfolding transition of L-14 from the standpoint of a thermodynamic model.

3.2. *Failure of L-14 to dissociate at the denaturation temperature*

The ratio of $\Delta H_c/\Delta H_v$ (Table 1) for the transition peak with

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Fig. 1. Typical DSC endotherm showing the apparent excess heat capacity for the thermal denaturation of L-14 at a protein (dimer) concentration of 0.05 mM in 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and 10 mM β-mercaptoethanol at a scan rate of 20 K h^{-1}, without (a) and with 100 mM lactose (b). Resolution of the progress baseline subtracted and concentration normalized DSC curve for L-14 into two independent two-state curves: (——) observed curve; (- - -) component curves; (• – • – •) sum of component contributions.

Table 1
Thermodynamic quantities[a] from DSC measurements on the thermal transition of L-14 in PBSM buffer

Scan rate (K h^{-1})	[L-14] (mM)	[Lactose] (mM)	T_{m1} (K)	ΔH_{c1} (kJ mol^{-1})	ΔH_{v1} (kJ mol^{-1})	$\Delta H_{c1}/\Delta H_{v1}$	T_{m2} (K)	ΔH_{c2} (kJ mol^{-1})	ΔH_{v2} (kJ mol^{-1})	$\Delta H_{c2}/\Delta H_{v2}$
10	0.05–0.102	–	333.6	149.4	262.4	0.57	339.9	123.0	513.1	0.24
20	0.05–0.250	–	333.8	139.4	268.3	0.52	340.0	120.5	501.8	0.24
30	0.05–0.101	–	334.1	159.0	270.0	0.59	340.5	120.5	523.5	0.23
60	0.05–0.102	–	334.8	159.5	277.1	0.58	341.0	105.1	477.9	0.22
20	0.05–0.122	100	350.0	421.0	779.7	0.54	–	–	–	–
60	0.05–0.100	100	350.3	404.9	793.9	0.51	–	–	–	–

[a]The errors in the determination of T_m, ΔH_c and ΔH_v values were less than 0.05%, 3.9% and 3.6%, respectively.

a T_m value of 333.8 K is 0.5 whereas it is close to 0.25 for the peak with T_m of 340.0 K at a scan rate of 20 K h^{-1}, indicating that these transitions correspond to L-14 existing as a dimer of dimers and tetramers of dimers (viz. tetramer and octamer of monomers), respectively, at the denaturation temperature. These oligomeric states of L-14 do not undergo dissociation even at the denaturation temperature. Moreover, the T_m values for neither of the transitions are altered with increase in the concentration (0.05–0.25 mM) of the protein.

3.3. Deoligomerization of L-14 by ligands

DSC data for L-14 in the presence of saturating amounts of disaccharides shows a sharp transition with $T_m \sim 350.0$ K (Fig. 1b). This transition is again most consistent with $A \leftrightarrow B$ two-state transition model as shown by the fits of the data. Values of ΔH_c display an increase in the presence of ligand for the transition such that the ratio $\Delta H_c/\Delta H_v$ is in the neighbourhood of 0.5 for the transition. These data thus suggest that ligand binding results in the deoligomerization of the octamer of L-14 monomers to a tetramer of monomers. Interestingly enough, the thermal transition of the tetramer in the presence of sugars is reversible. The increase in protein concentration for even the ligated L-14 did not shift its T_m values to higher temperatures (Table 1). This indicates that the ligated L-14 like its unligated counterpart does not dissociate.

The results of the fit of the data for the unfolding of L-14 in the presence of lactose, lacto-*N*-biose, thiodigalactoside, and *N*-acetyllactosamine are presented in Table 2, which show that both T_m and T_p (which is designated T_{pL} in the presence of sugar) increases with ligand concentration. The increase in T_{pL} arises from preferential binding of the ligand to L-14 in the folded form. The denaturation transition for the liganded form can be expressed as follows [16,24]:

$$A_mL \leftrightarrow mB + L \quad (2)$$

where m is the number of unfolding subunits in an oligomer, which at constant lectin concentration becomes:

$$\ln[L] = -\Delta H_{vL}/(mRT_{pL}) + \text{constant} \quad (3)$$

A typical plot of ln[L] versus $1/T_{pL}$ for lactose is displayed in Fig. 2. The best fits of ln[L] vs. $1/T_{pL}$ yield van 't Hoff enthalpies in the presence of sugar, ΔH_{vL} which are in agreement with ΔH_v values obtained from the DSC fits, when $m = 4$ is used for the transition (Table 2). Thus one molecule of carbohydrate ligand binds to each of the monomers in the tetrameric form of ligated L-14. This also supports the observation that L-14 ligated with saccharides exists as a tetramer (of L-14 monomers), as otherwise different values of 'm' would be required for achieving 1:1 correspondence between ΔH_{vL} using equation 3 and ΔH_v from peak shape analysis. Determination of the binding constants from the DSC results via equation 1 at the denaturation temperature, $K_{b(DSC)}$ are in good agreement with those obtained from ITC measurements which are extrapolated to the value of binding constant at the denaturation temperature [16,17].

DSC results show that in the absence of the disaccharide ligand, L-14 exists both as a dimer of dimers and as a tetramer of dimers assuming a 28 000 M_r i.e. L-14 dimer, as the cooperative unit. Moreover both the oligomers undergoing transitions are very stable and maintain their oligomeric integrity even at the denaturation temperature. In other words, the dissociation of L-14 oligomers does not occur upon denaturation. These interpretations are also supported by a lack of

Table 2
Thermodynamic quantities[a] from DSC measurements on the thermal transition of L-14 (0.1 mM) in the presence of ligands in PBSM buffer at a scan rate of 20 K h^{-1}

Ligand[b]	[Ligand] (mM)	T_m (K)	T_{pL} (K)	ΔH_c (kJ mol^{-1})	ΔH_v (kJ mol^{-1})	$\Delta H_c/\Delta H_v$	ΔH_{vL} (kJ mol^{-1})	$K_{b(DSC)}/K_{b(ITC)}$[e]
LAC[c]	10–100	348.6–352.1	348.4–352.2	423.5	784.2	0.54	842.3	0.84
LNC[c]	5.6–20	343.4–348.1	343.2–348.1	413.5	751.6	0.55	860.4	0.91
TDG[c]	20–60	347.7–350.1	347.7–350.0	397.2	749.9	0.53	857.9	0.86
LNB[d]	5–10	346.3–348.7	346.4–348.5	380.0	690.9	0.55	822.7	0.95

[a]The errors in the determination of T_m, ΔH_c and ΔH_v values were less than 0.05%, 2.5% and 2.7%, respectively.
[b]LAC, lactose; LNC, *N*-acetyllactosamine; TDG, thiodigalactoside; LNB, lacto-*N*-biose.
[c]$K_{b(ITC)}$ from ref. [15].
[d]$K_{b(ITC)}$ was determined to be 23 418 M^{-1} at 281.2 K by titration calorimetry (Ramkumar, R., Swaminathan, C.P., Podder, S.K., Surolia, A., unpublished results).
[e]$K_{b(DSC)}$ is the binding constant at the denaturation temperature determined by DSC measurements whereas $K_{b(ITC)}$ represents the extrapolated value of binding constant determined from ITC.

the effect of protein concentration on T_m or T_p values. This behaviour of L-14 is in stark contrast with that exhibited by legume lectins, all of which dissociate to monomeric or submonomeric units upon denaturation [16,17]. This is, however, not very surprising if one examines the dimeric interfaces of L-14 and legume lectins. In L-14, both the β-sheets of the monomers extend continuously across the dimer interface while in concanavalin A, pea lectin and lentil lectin only one β-sheet is contiguous and in *Erythrina corallodendron* lectin the β-sheets of the two monomers are inclined in a roughly perpendicular orientation while in *Griffonia simplicifolia* isolectin IV and peanut agglutinin the two monomers associate in a back-to-back manner through their flat β-sheets [11,12,26–28]. The contiguous β-sheets in L-14 apparently stabilize the molecule so that the oligomer does not dissociate to monomers. Moreover, L-14 complexed with sugar, in contrast to soybean agglutinin, displays a variety of architecture ranging from roughly parallel motif, bent arrays and helicoidal arrangements which attest to its propensity for formation of different kinds of assemblies [29–31]. The ligand dependent deoligomerization of L-14 suggests that ligand perturbs the oligomeric interface in a significant manner permitting the formation of the infinite arrays observed in all the three crystal forms of 14-kDa galectins [30,31]. Given that L-14 has a fold similar to that of legume lectins it is instructive to compare their thermal stabilities. The denaturation temperature of L-14 is 20 K lower than that of concanavalin A but close to that of lentil lectin [16,17]. These differences in T_m values for concanavalin A (50 kDa) and L-14 (28 kDa) are easily explained on the basis of the differences in their molecular masses.

In the presence of ligand, oligomerization of the dimers is altered strikingly so much so that the octamer peak ($T_m = 340.0$ K, $\Delta H_c/\Delta H_v = 0.24$) totally disappears and instead a peak for the tetramer ($T_m = 333.8$ K, $\Delta H_c/\Delta H_v = 0.52$) is retained and shifted to higher temperature ($T_m \sim 350.0$ K). The state of the oligomerization of legume lectins in contrast is not influenced by the presence of ligand. Ligand induced change in the oligomeric state of L-14 is again in tune with its remarkable ability to assemble in a variety of different arrays. The tetramer in the presence of ligand also does not dissociate upon denaturation. The $K_{b(DSC)}$ for the association of disaccharides to L-14 is in agreement with ITC results indicating that ligand binding to it occurs preferentially in the folded state unlike for ribonuclease where some binding to the unfolded state is also observed [32].

In conclusion these results quite explicitly show a fascinating property of galectins viz. the structural stability of their oligomeric assembly even at their denaturation temperature that sets them apart from the related legume lectins. The example of the thermal denaturation of L-14 also suggests that a small perturbation such as the addition of ligand can lead to different mode of quaternary associations in galectins hitherto not observed in their leguminous counterparts.

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