Differential scanning calorimetric studies of the glycoprotein, winged bean acidic lectin, isolated from the seeds of *Psophocarpus tetrogonolobus*

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Abstract Differential scanning calorimetry of solutions of WBAII and in presence of sugar ligands shows that WBAII dimer dissociates to its constituent monomeric subunits at the denaturation temperature. The thermal denaturation of WBAII consists of the unfolding of two independent domains of WBAII similar to that of basic winged bean lectin and ECorL and in contrast to concanavalin A (conA), pea and lentil lectin, which unfold as single entities. Apparently, the glycosylation reduces the structural integrity of WBAII as compared to conA, pea and lentil lectin. The increase in the denaturation temperature of the sugar-lectin complexes yields binding constants close to the binding constants extrapolated from the ITC results and confirms the mechanism proposed for its thermal unfolding.

Key words: Scanning calorimetry; WBAII; Quaternary association; Denaturation temperature

1. Introduction

Legume lectins exhibit a high degree of similarity in their tertiary structure, with identically positioned carbohydrate recognition and metal binding sites. The tertiary structures of the monomer unit usually consist of two anti-parallel β-pleated sheets, a 6-stranded β-sheet, and a 7-stranded curved β-sheet. In addition, the legume lectins, favin, concanavalin A (conA), pea lectin, *L. ochrus I* and lentil lectin form dimers which result in an extended 12-stranded β-sheet. Despite the very high homology in subunit structure among the legume lectins, there are considerable differences in their thermal stability and thermal unfolding behavior. The intersubunit interface in conA, pea and lentil lectins is extensive and so strong that the subunit dissociation and polypeptide unfolding occur simultaneously at the denaturation temperature [1]. In ECorL the intersubunit interface is reduced due to the steric hindrance caused by the covalently linked oligosaccharide chains. Consequently, its quaternary structure appears in the shape of a 'handshake' [2] and only 700 Å of non-polar surface area is buried at the intersubunit interface of ECorL as compared to 1000 Å for conA. This difference in the quaternary structure is responsible for the differences in its thermal stability and in its mode of thermal unfolding relative to conA and other legume lectins [3]. An earlier DSC study showed that, unlike the single transition exhibited by conA, pea and lentil lectin, denaturation of the basic lectin from winged bean (WBAI) consists of two transitions, with the higher temperature transition resulting in dissociation of the lectin dimer [4]. Like ECorL, WBAI also exhibits an independent unfolding of two domains with the subsequent dissociation of the dimer [4]. Acidic winged bean agglutinin (WBAII), another glycoprotein isolated from the seeds of *Psophocarpus tetrogonolobus* and for which the three-dimensional structure has not been determined, exhibits 53% and 67% identity in its amino acid sequence with EcorL and WBAI respectively [5]. WBAII is also a dimer of two identical subunits with a M_r of 54 000 and isoelectric point of 5.5 [6,7]. Each subunit is glycosylated by N-linked oligosaccharide which is the heptasaccharide Manα6(Manα3)(Xylβ2)Manβ4GlcNAcβ4(Fucα3)GlcNAcβ N-linked to Asn-77 [8]. In this study, the thermal stability of WBAII alone and in the presence of its carbohydrate ligands was investigated by differential scanning calorimetry to determine to what degree the thermal stability properties of WBAII reflect the corresponding properties of ECorL and other legume lectins and perhaps elucidate the role of covalently bound saccharide group on its thermal stability.

2. Materials and methods

2.1. Materials

Galactose (Gal), methyl-α-gal (MeαGal), methyl-β-gal (MeβGal), lactose (Lac) were obtained from Sigma and used without further purification. WBAII was prepared and purified >98% as described previously [7]. Sodium dodecylsulfate polyacrylamide gel electrophoresis performed according to Patanjali et al. [7] showed only one band at a molecular mass of 27 000, the molecular mass of one subunit. Deionized double distilled water was used to prepare all the solutions. The sodium phosphate, phosphoric acid and sodium chloride were obtained from Merck Ltd., Mumbai, India and were of analytical grade.

2.2. Preparation and analysis of solutions

The WBAII solutions were prepared in the 0.02 M sodium phosphate and 0.15 M sodium chloride buffer pH 7.4 (PBS) by weight, dialyzed overnight in a large volume of the same buffer and centrifuged to remove any insoluble material. The concentrations of the protein solutions were determined by using the lectin specific absorbance $A^{1\%}_{280\ nm} \div 7.7$. Solutions of the carbohydrates were prepared by weight in the dialysate to minimize differences between the protein buffer solution and ligand buffer solution. Milligram quantities of the sugar were added directly to 1.12 ml of lectin solution in the DSC cell for DSC measurements.

2.3. DSC measurements and analysis

DSC measurements were performed with a Microcal MC-2 DSC heat conduction scanning calorimeter which consists of two fixed 1.2 ml cells, a reference cell and a solution cell. The measurements were usually at a scan rate of 30 K h^{-1}. To determine any dependence of the parameters on the scan rate, scans were also performed from 10–60 K h^{-1}. The best least-squares fit of the two-state transition model, $A \Leftrightarrow nB$ where A is the folded state and B the unfolded state, to the data was obtained with $n=2$ by the EXAM Program [9]. This program utilizes a sigmoidal baseline to yield a van 't Hoff enthalpy

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(ΔH_v), a transition temperature (T_m, the temperature at half the peak area), and the transition peak area which when divided by the number of moles of protein in the cell yields the calorimetric enthalpy (ΔH_c). The ratio $\Delta H_c/\Delta H_v$ is the cooperativity of the transition. T_p is the temperature where the transition peak maximum occurs and is independent of application of the two-state model to the data. The ligand binding constants at the transition temperature, $K_{b(T1)}$ were determined from the increase in the T_m of the protein with increase in the ligand concentration using the following equation:

$$K_{b(T1)} = [\exp\{(T_1 - T_m)\Delta H_{c(T1)}/2.0\ RT_1T_m\} - 1]/[L] \quad (1)$$

where T_1 is the transition temperature in the presence of ligand and [L] is the free ligand concentration [2]. [L] is approximated as the difference between the total ligand concentration and twice the protein concentration.

Values for $K_{b(T1)}$ were compared to the values of K_b determined from previous ITC measurements via the van 't Hoff equation:

$$\ln\{K_{b(T)}/K_{b(T0)}\} = -\Delta H_v[1/T_0 - 1/T]/R. \quad (2)$$

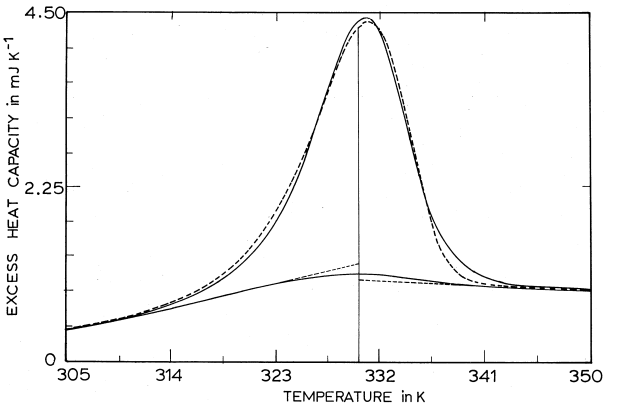

Fig. 1. DSC scan of a 1.0 ml sample of 0.2 mM (dimer) WBAII in 0.02 mol l^{-1} in 0.02 M sodium phosphate and 0.15 M pH 7.4 at a scan rate of 30 K h^{-1}. The solid line is the best least-squares fit of the DSC data to the $A \Leftrightarrow 2B$ two-state transition model. The extrapolated baseline is also shown for the fit as a dotted line.

3. Results

A typical DSC scan of WBAII is shown in Fig. 1 along with the fit of the single transition peak data to the $A_2 \Leftrightarrow 2B$ two-state transition model. The transition peak did not re-appear upon a re-scan of the sample indicating that the transition is thermally irreversible. Results of the fit of the transition peak data to the two-state transition model are presented in Tables 1 and 2. At any given protein concentration the thermodynamic quantities ΔH_c and ΔH_v, are independent of scan rate. The values of T_p increase by only 2–3 K in increasing the scan rate by more than a factor of 4 and this increase can be easily accounted for by the instrumental response time. Therefore, the equilibrium two-state thermodynamic transition model was applied to these transitions instead of the irreversible kinetic model of Sanchez-Ruiz [10]. The justification for analyzing irreversible thermal transitions which do not exhibit a scan rate dependence in terms of a thermodynamic model has been discussed by Manly et al. [11]. Treatment of such thermal transitions is based on treating the transition as a sequence of two processes, the reversible unfolding of the protein described by the two-state transition quantities followed by a slower irreversible process of aggregation. This approach yields results for the overall process that are the same as for the reversible process [11].

In Table 1, the ratio $\Delta H_c/\Delta H_v = 2.0$ is indicative of the unfolding of two independent portions of the WBAII dimer which may be identified with the independent unfolding of each monomeric unit of the dimeric lectin. Either the WBAII dimer undergoes dissociation first which is followed by unfolding of the monomeric units or the monomeric units unfold independently and this is followed by dissociation of the dimer in the unfolded state. In either case, the unfolded conformation of WBAII exists only as a monomer while in the folded state it exists as a dimer. This is further substantiated by the increase in T_p with increase in the protein concentration from 0.0178 to 0.116 (where the scan rate is fixed at 30 K h^{-1}) as presented in Table 1 and shown in Fig. 2 where ln[WBAII] is plotted as a function of $1/T_p$. The least-squares fit of a straight line to the data points yields a slope of $-6.82 \pm 0.29 \times 10^4$ K. Fukuda et al. [12] have shown that for an oligomer undergoing dissociation, this slope should be $-\Delta H_v(S)/\{R(n-1)\}$ where n is the oligomerization number. For WBAII where $n = 2.0$, $\Delta H_v(S)$ is 567 ± 24 kJ mol^{-1} which is the same as ΔH_v for the transition. Since T_p is not determined from the fits of the two-state transition model to the data, the agreement of $\Delta H_v(S)$ with ΔH_v determined from

Table 1
Dependence of DSC transition quantities on concentration of WBAII at pH 7.4, where the scan rate is fixed at 30 K h^{-1}

Conc. (mM)	T_m (K)	ΔH_v (kJ mol^{-1})	ΔH_c (kJ mol^{-1})	Ratio ($\Delta H_c/\Delta H_v$)	T_p (K)	$\Delta H_v(S)$ (kJ mol^{-1})
0.0178	331.6	512 ± 36.2	1092 ± 39.41	2.13 ± 0.14	331.7	567 ± 24
0.0294	331.7	498 ± 35.17	1010 ± 36.16	2.03 ± 0.135	331.8	
0.0437	330.5	440 ± 31.56	1012 ± 36.64	2.30 ± 0.154	332.5	
0.0870	332.6	525 ± 37.1	1006 ± 39.13	1.92 ± 0.13	333.7	
0.116	332.7	500 ± 35.31	1013 ± 36.47	2.03 ± 0.137	334	

The uncertainty in T_m is ± 0.1 K.

Table 2
Dependence of DSC transition quantities of 0.0294 mM WBAII at pH 7.4 on scan rate

Conc. (mM)	Scan rate (K h^{-1})	T_m (K)	ΔH_v (kJ mol^{-1})	ΔH_c (kJ mol^{-1})	Ratio ($\Delta H_c/\Delta H_v$)	T_p (K)
0.0294	10	326.9	442 ± 31	893 ± 32	2.08 ± 0.16	328.7
0.0294	20	329.9	528 ± 37	806 ± 29	1.53 ± 0.12	330.6
0.0294	30	331.7	498 ± 35.17	1010 ± 36.16	2.03 ± 0.135	331.8
0.0294	60	334.6	521 ± 37	867 ± 31	1.67 ± 0.12	335.3

The uncertainty in T_m is ± 0.1 K.

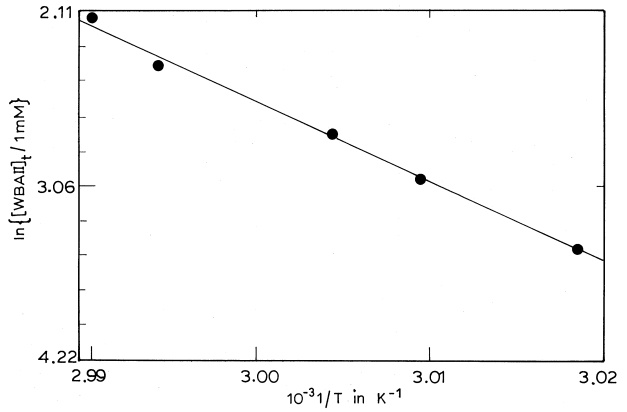

Fig. 2. A plot of $\ln\{[WBAII]_t/[1mM]\}$ vs. $1/T_p$. The line is the linear least-squares fit of $\ln\{[WBAII]_t/[1mM]\}$ to $1/T_p$.

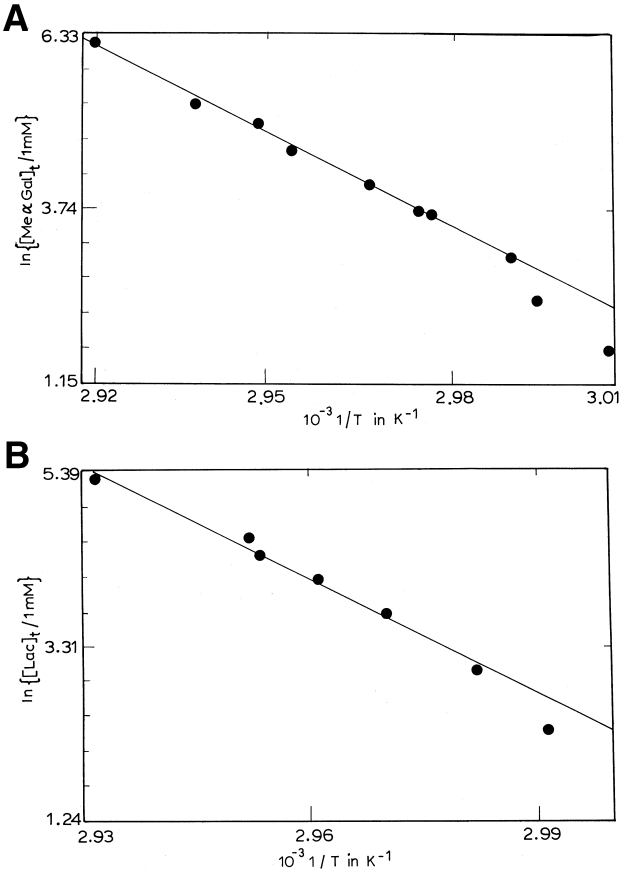

Fig. 3. A: A plot of $\ln\{[L]_t/[1mM]\}$ vs. $1/T_p$ for MeαGal. The line is the linear least-squares fit of $\ln\{[L]_t/[1mM]\}$ to $1/T_p$. B: A plot of $\ln\{[L]_t/[1mM]\}$ vs. $1/T_p$ for Lac. The line is the linear least-squares fit of $\ln\{[L]_t/[1mM]\}$ to $1/T_p$.

application of the two-state model to the data constitutes an independent confirmation that not only does the denaturation of WBAII result in dissociation, but the application of the two-state transition model to the data is justified. The thermodynamic transition quantities are unaffected by reducing the salt concentration from 0.15 mol l^{-1} to 0.0 mol l^{-1}. DSC scans of WBAII in the presence of saturating amounts of ligands also exhibit a single transition which is best fitted to the same two-state transition model, $A_2 \Leftrightarrow 2B$. The results of fitting the model DSC data for the unfolding of WBAII in the presence of Gal, MeαGal, MeβGal and Lac are presented in Table 3. T_m and T_p increase with ligand concentration while ΔH_v and ΔH_c remain approximately the same. The increase in both T_m and T_p with ligand concentration is shown in Fig. 3A and B and arises from preferential binding of the ligand to the lectin in the folded form. Linear least-squares fits of ln[L] to $1/T_p$ as shown by the Fig. 3A and B yield slopes of the ratio, $\Delta H_v(L)/\{RT_p m\}$ where m is the number of binding sites per WBAII dimer. Values of $\Delta H_v(L)$ (Table 2) are close to ΔH_v for $m = 2$.

In Table 3, values for $K_{b(T1)}$ determined from Eq. 1 are compared to values for K_b determined from independent isothermal titration calorimetry, $K_{b(ITC)}$, using Eq. 2 in terms of the ratio, $K_{b(T1)}/K_{b(ITC)}$. This comparison shows that the two methods for determining K_b at the transition temperature are in good agreement.

4. Discussion

A change in the quaternary structure is evident from the thermal transition of WBAII relative to conA. For WBAII and ECorL with covalently linked carbohydrates, the thermal stability is lower than that of conA which is similar in size and tertiary structure [1,3,4]. Furthermore, conA, pea and lentil lectin dimers unfold as single entities ($\Delta H_c/\Delta H_v$ are close to one) indicating that in the native state, the intersubunit interactions in these lectins are stronger than those in WBAII. The thermal unfolding of WBAII involves the independent unfolding of two domains as indicated by the $\Delta H_c/\Delta H_v$ ratio of 2. These domains may be identified as the two monomeric subunits, similar to the unfolding of ECorL [3]. In this respect, WBAII is different from WBAI which exhibits independent unfolding of the domains at different temperatures [4]. Also, the lower unfolding transition of WBAI is about 10 K higher than the thermal transition of WBAII. Perhaps, the presence of a covalently linked oligosaccharide alters the quaternary structure of WBAII so that the lectin is less thermally stable than the non-glycosylated lectins such as conA, pea and lentil lectins. The glycosylation, perhaps, reduces the intersubunit

Table 3
DSC transition quantities of 0.0294 mM WBAII at pH 7.4 in the presence of various carbohydrate ligands

[Ligand] (mM)	T_m (K)	ΔH_v (kJ mol^{-1})	ΔH_c (kJ mol^{-1})	Ratio ($\Delta H_c/\Delta H_v$)	T_p (K)	$\Delta H_v(L)$ (kJ mol^{-1})	$K_{b(T)}/K_{b(ITC)}$
Gal: 50–100	334.2–341.9	614 ± 52	1008 ± 98	1.64 ± 0.02	334.8–342.3		0.62 ± 0.3
MeαGal: 5.0–500	332.0–341.9	611 ± 29	971 ± 81	1.67 ± 0.2	332.9–342.4	791 ± 20	1.39 ± 0.33
MeβGal: 5.0–91.8	332.2–337.7	562 ± 31	932 ± 92	1.65 ± 0.15	332.5–338.4	899 ± 369	1.63 ± 0.3
Lac: 5.0–200	332.3–341.3	595 ± 49	887 ± 27	1.61 ± 0.11	333.2–341.5	704 ± 1	1.4 ± 0.47

The uncertainty in T_m is ± 0.1 K.

interactions in the protein, making it less stable as an integrated structure which is reflected in its lower thermal denaturation temperature relative to these legume lectins. For all three glycoproteins, WBAI, ECorL and WBAII, the thermal denaturation transition consists of the independent unfolding of two domains which reflects a less integrated structure in the native state than that of conA, pea and lentil lectin which unfold as single entities [1,3,4]. Though the exact role of the covalently linked sugar in the thermal stability of WBAII, vis a vis other legume lectins is not clear, the DSC studies seem to suggest that the intersubunit interface in it is less extensive as compared to conA, pea and lentil lectins, making it less thermally stable and causing the two subunits to unfold independently.

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