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Thermal Stability and Mode of Oligomerization of the Tetrameric Peanut Agglutinin: A Differential Scanning Calorimetry Study[†]

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ABSTRACT: Peanut agglutinin is a homotetrameric legume lectin. The crystal structure of peanut agglutinin shows that the four subunits associate in an unusual manner, giving rise to open quaternary structure [Banerjee, R., et al. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 227−231]. The thermal unfolding of peanut agglutinin has been characterized by differential scanning calorimetry and gel filtration to elucidate its thermal stability and its mode of oligomerization. The unfolding process is reversible and could be described by a three-state model with two transitions occurring at around 331 and 336 K. For the tetramer, the ratio of $\Delta H_c/\Delta H_t$ for the first transition is close to 4 and for the second transition is close to 0.25, suggesting that 4 and 0.25 cooperative unit(s) of the tetramer are involved in the first and second transitions, respectively. The agreement between the model-independent $\Delta H_v(S)$ determined from the values of the temperatures of the peak maximum (T_{p1}) with the protein concentration with the values of ΔH_v obtained from the fit of the data to the transition confirms that the first peak is associated with the dissociation of peanut agglutinin tetramers (A_4) to "folded" monomers (4A), whereas the second peak describes the unfolding (4U) of these monomers. The overall process for the thermal unfolding of peanut agglutinin could therefore be summarized as $A_4 \rightleftharpoons 4A \rightleftharpoons 4U$. Gel filtration studies confirm this process, as peanut agglutinin elutes as a tetramer up to 50 °C, and at and above 56 °C (T_m of first transition), it elutes at a position commensurate with that of the folded monomer of peanut agglutinin. The unfolding behavior of peanut agglutinin in the presence of saturating amounts of carbohydrate ligands is similar to that observed for the unliganded form. The temperature of maximal stability of the peanut agglutinin tetramer at pH 7.4 is calculated to be around 33 °C with a maximal free energy of stabilization of 8.70 kcal/mol. The results demonstrate that unfolding of peanut agglutinin goes through two distinct phases with folded monomer being the intermediate.

Lectins, proteins of nonimmune origin, bind sugars reversibly and with a high degree of specificity (*1*). The recognition of carbohydrate moieties by lectins has important implications in a number of biological processes such as cell−cell interactions (*2*, *3*), signal transduction (*4*, *5*), and cell growth and differentiation (*1*, *6*). Though distributed ubiquitously in nature, lectins from legumes are most often used as paradigms to study the molecular basis of these recognitions because they display a wide repertoire of carbohydrate specificities and the relative ease with which they can be purified. Consequently, a framework for carbohydrate recognition by them is now fairly well understood (*7*). The potential of legume lectins to discriminate exquisitely between oligosaccharide structures has been used to probe the changes that cell surface carbohydrates undergo during growth, differentiation, malignancy, and metastasis as well as in the isolation and characterization of glycolipids and glycoproteins (*1*, *6*, *7*). The high degree of specificity of peanut (*Arachis hypogea*) agglutinin for the tumor-associated T-antigenic structure, Galβ1−3GalNAc (*8*, *9*), for example, has been used extensively for the diagnosis and prognosis of malignancy (*10*, *11*).

Despite striking similarities in their primary, secondary, and tertiary structures, legume lectins display a considerable variation in their quaternary structure (*12*). The first few legume lectin structures exhibited the same (hence also called the canonical) mode of dimerization which consisted of antiparallel side by side alignment of the two flat six-stranded back sheets, one from each monomer, giving rise to the formation of a contiguous 12-stranded sheet. A different mode of dimerization ascribed to the unfavorable consequence of covalently bound carbohydrate and a buried glutamic acid residue was noted in *Griffonia simplicifolia* lectin IV (GS IV)[1] (*13*). Yet another mode of dimerization was observed in *Erythrina corallodendron* lectin (ECorL) (*14*). It was suggested that the handshake kind of interface in EcorL is formed due to the steric hindrance caused to the canonical mode of dimerization by the covalently linked carbohydrate chain. Though the basic lectin from winged

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[1] Abbreviations: CD, circular dichroism; Con A, concanavalin A; DSC, differential scanning calorimetry; ECorL, *E. corallodendron* lectin; GS IV, *G. simplicifolia* lectin IV; ITC, isothermal titration calorimetry; Lac, lactose; LacNAc, *N*-acetyllactosamine; MAG, methyl α-galactopyranoside; MBG, methyl β-galactopyranoside; TAG, T-antigen; WBA I, winged bean agglutinin, basic.

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bean (WBA I) also displays the mode of dimerization akin to that of ECorL, the covalently linked carbohydrate chain is not responsible for it as glycosylation sites in it are far away from its dimeric interface (*15*). What is more, peanut agglutinin, a nonglycosylated homotetrameric protein of 27 kDa monomers, associates in the most unusual manner, giving rise to an open quaternary structure (*16*). It thus appears that the tertiary structure of legume lectin monomers is such that a subtle variation in it is sufficient to cause divergent modes of oligomerization. As the quaternary structure relates to the biological activity of lectins as demonstrated by the formation of highly ordered homogeneous cross-linked lattices between legume lectins and multivalent carbohydrates, leading to a higher form of specificity than what is possible at the level of the monomer, we have been investigating the relationship between their thermal stabilities and their modes of oligomerization. Such studies serve to relate the thermal unfolding behavior of legume lectins with their quaternary structures.

In this paper, we report differential scanning calorimetry (DSC) studies for elucidating the thermal unfolding behavior of peanut agglutinin which is in striking contrast to that observed for the other legume lectins which differ from it in their subunit interfaces.

MATERIALS AND METHODS

Materials. Lactose (Lac), methyl α-galactopyranoside (MαG), methyl β-galactopyranoside (MβG), *N*-acetyllactosamine (LacNAc), and T-antigen (Galβ1−3GalNAc, TAG) were from Sigma and were used without any further purification. Peanut agglutinin was prepared and purified to homogeneity as described previously (*9*). SDS−PAGE showed a single band with an M_r of 27 kDa. All the other chemicals were of analytical grade.

Preparation of Solutions. The peanut agglutinin solutions were prepared in 0.05 M sodium phosphate buffer (pH 7.4) and 0.15 M sodium chloride (PBS), dialyzed against a large volume of the same buffer, and centrifuged to remove any insoluble material. The protein concentrations were determined by using the lectin specific absorbance $A^{1\%}_{280nm}$ of 7.7 (*8*). Sugars were directly added to the protein solutions, when used.

Differential Scanning Calorimetry. DSC scans for measuring the change in excess heat capacity as a function of temperature were performed using a Microcal MC-2 DSC heat conduction scanning microcalorimeter as described previously (*17*). DSC scans were carried out as a function of protein concentration, scan rate, sugar concentration, and pH. All solutions used for DSC were degassed just before being loaded into the calorimeter. DSC scans were carried out with the protein in the sample cell and the respective buffer in the reference cell. Reversibilities of the thermal transitions were checked by rescanning the samples. Data were analyzed using the ORIGIN DSC software (*18*) provided by Microcal Inc., along with the instrument for a two-state, two-peak model. T_{m1} and T_{m2}, and T_{p1} and T_{p2}, are the temperatures of the half of the respective transition peak area, and the temperatures at which the transition peaks are maximum, respectively. The ratio of the calorimetric enthalpy to van't Hoff enthalpy ($\Delta H_c/\Delta H_v$) was used to determine the size of the cooperative unit. The binding constant at denaturation temperature [$K_{b(T_{pL})}$] is obtained from the increase in the denaturation temperature in the presence of ligand (T_{pL}), the concentration of the ligand ([L]), and the calorimetric enthalpy of the tetramer using the relationship in eq 1 (*17*):

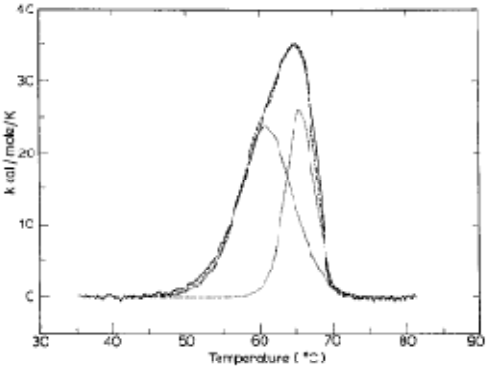

FIGURE 1: DSC scan of 0.160 mM peanut agglutinin in 0.02 M sodium phosphate buffer containing 0.15 M sodium chloride (pH 7.4) at a scan rate of 20 K h^{-1}. The dashed lines are the best fits of the DSC data to the two-state transition model.

$$K_{b(T_{pL})} = \{\exp[(T_{pL} - T_{m1})\Delta H_c(T_c)/nRT_{pL}T_{m1}] - 1\}/[L] \quad (1)$$

where n is the number of binding sites of the ligand in the tetramer.

Gel Filtration. Gel filtration experiments were carried out on a Pharmacia Superdex 75 HR 10/30 column (300 mm × 10 mm) using a Pharmacia FPLC system as a function of temperature. Protein (5 μM) in 200 μL was injected onto the column equilibrated with 50 mM sodium phosphate buffer (pH 7.4) at specified temperatures. The flow rate was 0.5 mL/min, and detection was carried out at 280 nm.

Chemical Unfolding. Chemical denaturation experiments were carried out in 50 mM sodium phosphate buffer (pH 7.4) by monitoring the far-UV CD of the protein at 222 nm on a JASCO-J-500A spectropolarimeter. The data were collected with a bandwidth of 1 nm, response time 8 s, and scan speed of 10 nm/min in a 2 mm path length cell. Protein (5 μM) samples were incubated for 8−10 h at the desired denaturant (urea) concentration before the measurements were taken to attain equilibrium.

RESULTS

A representative DSC scan of peanut agglutinin together with the fit of the transition peak data to a two-state model is shown in Figure 1. Deconvolution of the transition peak shows that they consist of two entities melting at different temperatures. A rescan of the sample from 331 K shows a complete reappearance of the peak with a T_m of 331 K; therefore, the first transition is completely reversible. The second transition, however, shows about 60−70% reversibility (not shown). Moreover, the data at higher scan rates exhibit a very small increase in T_{m1}, T_{m2}, T_{p1}, and T_{p2}, while the thermodynamic quantities, ΔH_c and ΔH_v, are almost independent of scan rate. In view of these results, the equilibrium two state transition model was applied to these

Table 1

(a) Thermodynamic Quantities from DSC Measurements of the First Transition of Peanut Agglutinin (tetramer) in PBS at pH 7.4

concentration (mM)	scan rate (K/h)	T_{m1} (°C)	ΔH_{c1} (kcal/mol)	ΔH_{v1} (kcal/mol)	$\Delta H_{c1}/\Delta H_{v1}$	T_{p1} (°C)	$\Delta H_v(S)$ (kcal/mol)
0.05−0.260	20	56.12−60.67	248.14 ± 22.23	75.10 ± 2.12	3.30	57.03−61.11	97.16
0.080	20	57.34	236.93 ± 9.88	73.04 ± 1.98	3.24	58.64	
0.080	45	58.14	255.10 ± 7.50	75.43 ± 2.13	3.38	59.40	
0.080	60	58.64	258.49 ± 10.00	77.03 ± 2.70	3.35	59.04	
0.080	90	58.93	264.20 ± 10.00	81.33 ± 2.23	3.25	59.46	

(b) Thermodynamic Quantities from DSC Measurements of the Second Transition of Peanut Agglutinin (tatramer) in PBS at pH 7.4

concentration (mM)	scan rate (K/h)	T_{m2} (°C)	ΔH_{c2} (kcal/mol)	ΔH_{v2} (kcal/mol)	$\Delta H_{c2}/\Delta H_{v2}$	T_{p2} (°C)	$\Delta H_v(S)$ (kcal/mol)
0.05−0.260	20	63.6 ± 0.5	34.71 ± 4.42	148.86 ± 8.00	0.23	64.1 ± 0.7	139.0
0.080	20	63.17	32.27 ± 1.57	144.92 ± 10.00	0.22	63.54	
0.080	45	62.87	33.46 ± 1.43	150.72 ± 10.00	0.22	64.2	
0.080	60	63.26	34.38 ± 1.75	154.28 ± 21.12	0.22	64.2	
0.080	90	63.33	34.67 ± 1.22	157.77 ± 43.1	0.22	63.8	

data (*20*). The values of T_{m1}, T_{m2}, T_{p1}, T_{p2}, ΔH_{c1}, ΔH_{v1}, ΔH_{c2}, and ΔH_{v2} thus obtained are listed in Table 1.

The ratio of $\Delta H_c/\Delta H_v$ of the first transition, i.e., the peak with a T_m of 331 K, is greater than 3, whereas for the second peak, it is close to 0.25. These data thus suggest that four entities are involved in the first transition, whereas only one entity sufficiently accounts for the occurrence of the second transition. The increases in T_m and T_p with increasing concentrations of peanut agglutinin for the first transition alone are also consistent with such an interpretation., while the second transition peak does not change with protein concentration. Thus, we observe a decreased extent of separation of peaks with increasing concentrations (data not shown). Hence, the two transitions during the thermal unfolding reaction of peanut agglutinin can be summarized as follows

$$A_4 \leftrightarrows 4A \leftrightarrows 4U$$

where A_4, A, and U correspond to the native peanut agglutinin tetramer, either substantially folded or partially folded monomers (henceforth to be called merely "folded" monomers), and the unfolded monomers, respectively, of the lectin. The protein concentration dependence of T_p for an oligomeric protein undergoing dissociation is related to the following equation (*21*):

$$\ln[A_n] = -\Delta H_v(S)/[RT_p(n-1)] + \text{constant} \qquad (2)$$

where n is the number of subunits in the oligomer, $[A_n]$ is the concentration of the protein oligomer in the native state, and $\Delta H_v(S)$ is the van't Hoff enthalpy obtained from the slope of $\ln[A_n]$ versus $1/T_p$. A plot of $\ln[A_n]$ versus $1/T_{p1}$ is shown in Figure 2, and the van't Hoff enthalpies calculated from the slope $[\Delta H_v(S)]$ are listed in Table 1. The values of $\Delta H_v(S)$ obtained using eq 2 are close to those obtained from the two-state fit of the transition data where the value of n is taken to be 4. It should be noted that since T_p is not determined from fits of the two-state transition model to the data, the agreement between ΔH_v and $\Delta H_v(S)$ is an independent conformation that the first peak is associated with the deoligomerization of the peanut agglutinin tetramer to the folded monomers.

DSC scans of peanut agglutinin in the presence of saturating amounts of carbohydrate ligands also reveal unfolding behavior similar to that observed for the unligated form of the protein. The thermodynamic data for the

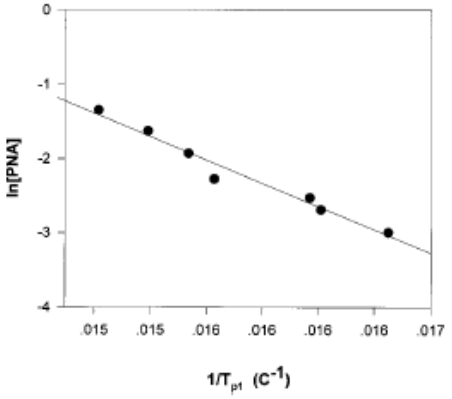

FIGURE 2: Plot of ln[peanut agglutinin] vs $1/T_{p1}$. The line is the best linear least-squares fit of ln[peanut agglutinin] to $1/T_{p1}$.

unfolding of peanut agglutinin in the presence of TAG, Lac, MAG, MBG, and LacNAc are presented in the Table 2. T_{m1}, T_{m2}, T_{p1}, and T_{p2} increase with ligand concentration. The denaturation transition in the presence of bound ligand at a constant protein concentration can be expressed as follows (*21*):

$$\ln[L] = (-\Delta H_v[L]/RT_p m) + \text{constant} \qquad (3)$$

Representative plots of ln[L] versus $1/T_{p1}$ are shown in Figure 3 for TAG, Lac, and MAG. The values of $\Delta H_{v(L)}$, obtained from the slope of the plots of ln[L] versus $1/T_p$ for an m value of 4, are close to ΔH_v and ΔH_c as shown in Table 2. It has been shown (*19*) that an increase in the transition temperature with ligand concentration at [L] ≫ [protein] depends on K_b (binding constant) at the denaturation temperature and the fact that $K_{b(DSC)}$ can be determined from this increase, according to eq 3. In Table 2, values for $K_{b(DSC)}$ are compared with the values for $K_{b(ITC)}$ determined from ITC, on extrapolation to the denaturation temperature. The ratio of $K_{b(DSC)}/K_{b(ITC)}$ shows that the K_b determined from DSC scans is in good agreement with the values obtained by ITC. The increases in T_m, T_p, ΔH_c, and ΔH_v values as a function of ligand concentration arise from the preferential binding of the ligand to the folded form of the protein.

Table 2

(a) Thermodynamic Quantities from DSC Measurements on the First Transition of Peanut Agglutinin (0.08 mM, tetramer) in the Presence of Ligands in PBS at pH 7.4

ligand	[ligand] (mM)	T_{m1} (°C)	ΔH_{c1} (kcal/mol)	ΔH_{v1} (kcal/mol)	$\Delta H_{c1}/\Delta H_{v1}$	T_{p1} (°C)	$\Delta H_v(L)$ (kcal/mol)	$K_{b(DSC)}$/$K_{b(ITC)}$[a]
TAG	0.5–5.0	57.70–61.53	233.20 ± 8.28	78.51 ± 1.26	2.96	58.10–61.01	121.45	0.68
Lac	1.0–20	57.65–59.48	250.20 ± 19.53	80.38 ± 1.47	3.11	57.20–59.19	141.00	0.92
MAG	0.5–10	55.75–59.14	253.00 ± 17.54	76.78 ± 0.93	3.29	57.03–59.60	129.19	1.09
MBG	5.0–30	57.01–59.08	241.79 ± 21.83	79.78 ± 4.33	3.03	58.03–59.79	145.40	0.68
LacNAc	5.0–20	55.80–57.29	257.81 ± 14.91	82.74 ± 5.31	3.23	56.19–57.32	130.20	ND[c]

(b) Thermodynamic Quantities from DSC Measurements on the Second Transition of Peanut Agglutinin (0.08 mM, tetramer) in the Presence of Ligands in PBS at pH 7.4

ligand	[ligand] (mM)	T_{m2} (°C)	ΔH_{c2} (kJ/mol)	ΔH_{v2} (kJ/mol)	$\Delta H_{c2}/\Delta H_{v2}$	T_{p2} (°C)	$\Delta H_v(L)$ (kJ/mol)
TAG	0.5–5.0	63.25–66.01	32.79 ± 2.96	155.79 ± 13.25	0.21	62.86–65.01	164.32
Lac	1.0–20	63.21–64.73	34.66 ± 1.32	150.04 ± 9.40	0.23	61.60–63.10	178.70
MAG	0.5–10	62.29–63.73	33.60 ± 2.15	153.93 ± 2.73	0.21	61.03–62.60	161.42
MBG	5.0–30	62.52–63.70	34.11 ± 1.57	156.02 ± 8.71	0.22	60.63–62.70	145.50
LacNAc	5.0–20	62.59–63.15	33.75 ± 1.74	148.75 ± 8.42	0.24	61.19–62.50	150.20

[a] K_b at a T_m of 330.34 K was determined from the eq 2 in the text. [b] G. B. Reddy and A. Surolia, unpublished data. $K_{b(ITC)}$ calculated at the denaturation temperature using the van't Hoff equation $\ln[K_{b(ITC)}/K_{b(Tm)}] = -\Delta H_v[1/T_p - 1/T_m]/R$. [c] Not determined.

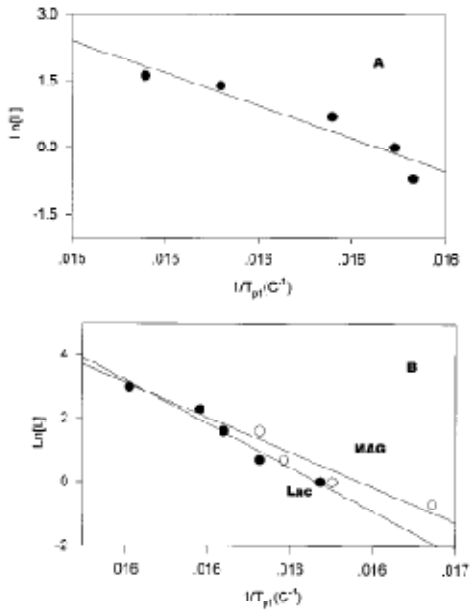

FIGURE 3: Plots of ln[L] vs $1/T_{p1}$ for (A) T-antigen and (B) lactose and MAG. The lines are the best linear least-squares fits of ln[L] to $1/T_{p1}$.

However, the ratios of $\Delta H_c/\Delta H_v$ for both the transitions are the same as in the absence of ligand, indicating that the unfolding behaviors of both the ligated and unligated forms of peanut agglutinin are similar. The lack of an increase in the values of T_m, ΔH_v, etc., as a function of peanut agglutinin concentration attests to the fact that the second peak is related to the complete unfolding of the folded monomers at the denaturation temperature, i.e., it corresponds to the 4A ⇌ 4U type transition.

DSC scans were also carried out as a function of pH in the pH range of 5.5–8.2, and these results are summarized

Table 3: T_m and ΔH_m Values of 0.100 mM Peanut Agglutinin (tetramer) Unfolding as a Function of pH

pH	T_{m1} (K)	T_{m2} (K)	ΔH_{m1} (kcal/mol)	ΔH_{m2} (kcal/mol)
5.5	330.08	334.36	212.12	113.02
6.0	330.68	335.05	218.23	115.13
6.5	331.16	335.53	225.32	120.53
7.0	331.69	336.28	231.34	125.26
7.4	332.13	336.91	236.42	129.15
7.8	333.27	337.58	245.15	132.07
8.2	334.34	338.12	251.43	135.02

in Table 3. There are increases in T_{m1} and T_{m2} with an increase in pH. When ΔH_m is plotted as a function of T_m, as shown in Figure 4, the slope of the plot provides an estimate of ΔC_p (*22*). The plot fits well with a correlation coefficient of of 0.947 for the first transition and 0.983 for the second transition. This indicates that ΔC_p is independent of temperature within the temperature range of this study. The value of ΔC_p obtained from the slope of this plot is 9.30 kcal mol^{-1} K^{-1} for the first transition and 5.07 for the second transition. The moderately high value of ΔC_p for the first transition is perhaps due to the contribution of both the intersubunit interactions and the partial unfolding of the monomers when peanut agglutinin undergoes dissociation. Therefore, the intermediate most likely corresponds to the partially folded monomer. However, for the sake of brevity, their description as a monomeric form is being withheld.

Gel filtration studies were performed to identify the species at the denaturation temperature. Figure 6 shows the chromatographic profiles of peanut agglutinin at 15, 26, 37, 50, and 56 °C. It can be observed that the protein elutes at a retention time of 18.6 min up to 50 °C, which can be assigned to the position of the elution of the peanut agglutinin tetramer. At and around 56 °C, it elutes at 39 min which corresponds to the elution time expected for a folded monomer-like species. On the basis of these observations, it is clear that the first transition is associated with the dissociation of the peanut agglutinin tetramer to folded monomers.

The changes in far-UV CD as a function of urea concentration also point to an intermediate (Figure 5). The

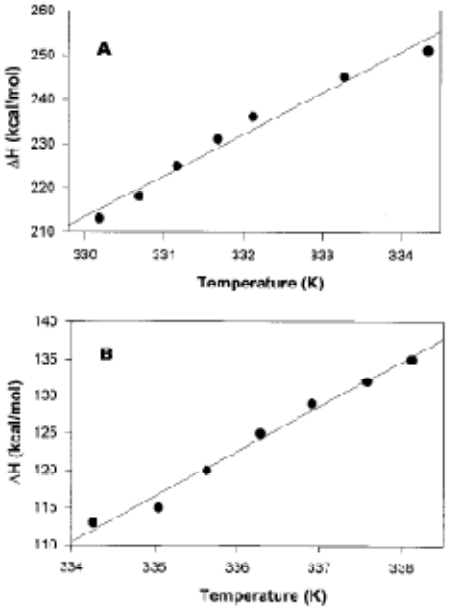

FIGURE 4: Plots of ΔH_m vs T_m for the (A) first and (B) second transitions. The values of ΔH_m and T_m (from Table 3) are a best linear least-squares fit. The slopes yield the ΔC_p values of 9.30 and 6.07 kcal mol^{-1} K^{-1} for first and second transitions, respectively.

intermediate is populated over the 1.6−2.0 M urea concentration range. The occurrence of the intermediate species is consistent with the appearance of the peanut agglutinin monomer (data not shown). Thus, the chaotrope-induced denaturation of peanut agglutinin is qualitatively consistent with the DSC data.

DISCUSSION

Peanut lectin is a homotetramer of 27 kDa monomers, each subunit being 236 amino acids long (*8*, *9*, *23*). The crystal structures of peanut agglutinin (*16*) and its complexes with lactose (*24*) and T-antigen (*25*) have been reported. The structure reveals that the four subunits have the same jellyroll tertiary structural fold common to all the legume lectins, but they associate in an unusual manner, giving rise to an "open quaternary" structure (*16*). In peanut lectin, the interface between monomers 1 and 4, and 2 and 3, consists of back to back association between the two flat six-stranded β sheets, one from each monomer akin to the dimeric interface of GS IV. Two monomers (1 and 4), one from each of these GS IV type of dimers, then associate in a fashion similar but not identical to that of the canonical dimer as the N-terminal strands of the two monomers are held together by six water bridges. Consequently, 1−2 and 3−4 interfaces in peanut agglutinin are not equivalent. The interface between subunits 3 and 4 is unique to peanut agglutinin and is perhaps an incidental consequence of the presence of disparate interfaces between subunits 1 and 2 and 1 and 4 (as well as between subunits 2 and 3). Thus, while peanut agglutinin is made up of two GS IV type dimers both 2-fold symmetric and related

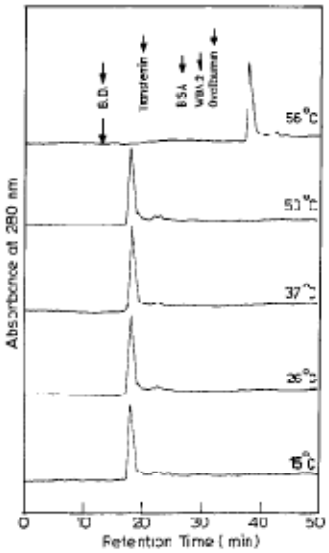

FIGURE 5: Size exclusion chromatographic profiles of peanut agglutinin at specified temperatures. Protein (5 μM) equilibrated at the specified temperatures for 15 min was chromatographed on a FPLC Superdex 75 HR10/30 column equilibrated with 50 mM sodium phosphate buffer at pH 7.4 at the given temperature. The flow rate was 0.5 mL/min. The tetramer peak elutes at 18.5 min and the monomer peak at 39 min. The arrow marks the position (15 min) of elution of the unfolded monomer (above 68 °C).The M_r values of the standards used were as follows: transferrin, 81 kDa; BSA, 67 kDa; WBA2, 54 kDa; and ovalbumin, 43 kDa. Blue dextran (BD) elutes at 13 min.

to each other by a dyad however, as the different dyads fail to intersect the molecule has an open quaternary structure. Since the quaternary structure is related to its biological function, the description of the structural and thermal stability of peanut agglutinin has a bearing on its application as a tool in biological research (*26*−*28*).

These data show that upon thermal denaturation, the peanut agglutinin tetramer dissociates into its constituent monomeric subunits which retain their ability to bind to their complementary ligands. This is followed by their complete unfolding. The main asymmetrical peak (centered around 58 °C) is best fitted to the dissociation transition of the tetramer to four units, as the ratio of $\Delta H_v/\Delta H_v$ was 4, and could be described as a $A_4 \rightleftharpoons 4A$ type of transition. The symmetrical peak (T_m is 63 °C) is best fitted to the unfolding of monomer alone as the ratio of $\Delta H_c/\Delta H_v$ (for tetramer) for this transition is close to 0.25. Hence, the later peak could be described in terms of $4A \rightleftharpoons 4U$. Therefore, the thermal unfolding of peanut agglutinin appears to occur in two discrete steps, $A_4 \rightleftharpoons 4A \rightleftharpoons 4U$, where A refers to the monomeric intermediate.

Gel filtration data confirm further the occurrence of the folded monomer as an intermediate in the thermal unfolding of peanut agglutinin. The peanut agglutinin elutes at 18.6 min on gel filtration column. The elution time of peanut agglutinin up to a temperature of 50 °C remains the same. On the other hand around 56−57 °C, it elutes largely at 39 min which corresponds to the position of the retention time for the folded monomer (Figure 5). The gel filtration data

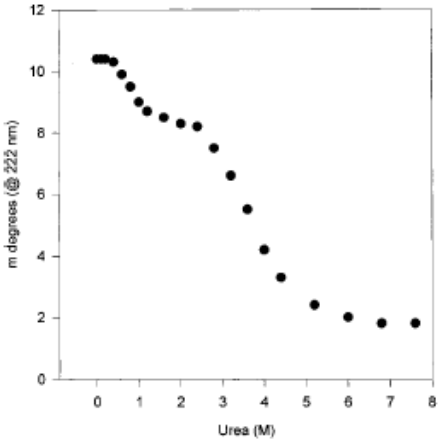

FIGURE 6: Urea-induced unfolding of peanut agglutinin (5 μM) at 30 °C, monitored by far-UV CD. Unlike any other legume lectin, peanut agglutinin is best described as a dimer of two back to back dimers each with 2-fold symmetry. Though the two dimers, viz. subunits 1 and 4, and 2 and 3, in the tetramer are also related by a dyad, the different dyads neither intersect nor are mutually perpendicular. Consequently, the molecule has an open quaternary arrangement. The interactions between the two dimers involve two distinct kinds of interfaces. One is a side by side antiparallel alignment of the flat six-stranded back β sheets, between subunits 1 and 2, like the highly extended "canonical" dimeric interface in legume lectins. However, the two sheets at this interface do not come close enough as in Con A, pea lectin, etc. Instead, the association between the two back β sheets from subunits 1 and 2 are stabilized by six water bridges. The other interface, viz. between subunits 3 and 4, is an incidental consequence of the presence of the two back to back (1 and 4, and 2 and 3) and one side by side (1 and 2). The back to back association between the two flat back β sheets across subunits 1 and 4 and subunits 2 and 3 of the peanut agglutinin tetramer appears to be intrinsically less stable compared to the canonical dimeric interfaces of the legume lectins. The 1−2 interface in peanut agglutinin which is common to 1−4 and 2−3 dimers does not link up to give a contiguous 12-stranded sheet, as it does in Con A, pea lectins, etc.; hence, it is unable to confer stability to the extent observed in the latter class of lectins and, with a slight perturbation, dissociates into monomers readily.

therefore confirm that the first transition in DSC is associated with dissociation of the peanut agglutinin tetramer to the monomeric form, while the second transition being symmetrical is best assigned to the unfolding of the folded monomeric species. Solution denaturation studies of peanut agglutinin by far-UV CD spectroscopy also show that unfolding of peanut agglutinin is a biphasic process with a well-defined intermediate being predominantly populated at a urea concentration of 1.8 M (Figure 6).

Upon addition of sugar ligands to peanut agglutinin, there is some increase in the thermal stability of the two thermodynamic domains, indicating that ligand binding occurs preferentially to the folded state of the protein. Yet the multidomain structure remains the same as the ratios of $\Delta H_c/\Delta H_v$ remain unaltered. Moreover, the van't Hoff enthalpies calculated from plots of ln[L] versus $1/T_{p1}$, according to eq 3, for each transition are qualitatively and quantitatively similar to those in the absence of the ligand (Table 2). Thus, like that of other legume lectins (*29*, *30*), and unlike that of the vertebrate galectin-1 (*31*, *36*), the state of the oligomerization of peanut agglutinin is not influenced by the presence of ligands.

The thermal stability of peanut agglutinin was also characterized as a function of pH using DSC scans. At all the pH values listed in Table 3, the thermal unfolding transitions of peanut agglutinin could be fit to a two-state, two-peak model. Peanut agglutinin is known to dissociate into dimers at pH $\leq$3.5. The results presented in Figure 4 and Table 3 reassert that peanut agglutinin maintains its tetrameric state over the pH range of 5.5−8.2. The correlation coefficients of 0.974 and 0.988 for the estimates of ΔC_p from ΔH_m versus T_m plots (Figure 4) indicate that the ΔC_p values are pH-independent over the selected pH range. The value of ΔC_p obtained from this method is considered a better estimate (*32*) than that obtained directly as the difference between the native and unfolded baseline of a single DSC scan because of the errors associated with arbitrariness in determining the unfolded baselines (*33*).

Although the constituent subunits of the legume lectins concanavalin A (Con A), ECorL, pea, lentil and winged bean basic lectin, and the galectins from bovine spleen (galectin 1) and sheep spleen (L-14) have the same tertiary structure (*12*, *14*−*16*, *31*, *34*, *36*), their thermal denaturation occurs in differing ways and at different temperatures (*29*, *30*, *35*, *36*, *38*). Thermal denaturation of Con A, for example, occurs at 363 K, while that of ECorL and peanut agglutinin occurs around 333 K, reflecting the greater stability of the former. Pea and lentil lectin also display greater stability than peanut agglutinin. Greater stabilities of these lectins are also reflected in the $\Delta H_c/\Delta H_v$ ratios which are close to 1, indicating that they unfold as single entities as a consequence of strong interactions between their subunits (*30*). ECorL ($\Delta H_c/\Delta H_v = 2$) which has a smaller monomer−monomer interface than Con A exhibits independent unfolding of its constituent subunits (*29*). WBA I displayed two distinct thermal unfolding transitions which were ascribed to its less tenacious interface between its subunits in the dimer (*35*). The structural solution at atomic resolution has subsequently confirmed the insights from DSC studies which suggested that the intersubunit interface in WBA I dimer is indeed less extensive (700 Å as compared to 1000 Å for Con A) (*14*). The thermal unfolding behavior of WBA II is similar to that of ECorL, in that the $\Delta H_c/\Delta H_v$ of WBA II is 2 (*37*). It may thus seem that the quaternary association of WBA II may be similar to that of ECorL, with a monomer−monomer interface smaller than that of Con A. On the other hand, DSC studies show that the bovine spleen galectin exists as a tetramer at the denaturation temperature and does not dissociate upon denaturation ($\Delta H_c/\Delta H_v < 1$) (*36*). A similar behavior is observed for L-14, which also does not dissociate upon unfolding (*31*). In galectin, the two β sheets of the monomers extend continuously across the dimer interface. This continuity of the two β sheets across the dimer interface, compared to the only one β sheet being continuous across the monomer interface of Con A, pea, and lentil lectins and the even lesser degree of subunit association of ECorL and WBA I, stabilizes the galectin dimer such that they do not unfold even at the denaturation temperature (*36*). The peanut agglutinin displays yet another mode of thermal unfolding behavior wherein the constituent subunits of this tetrameric protein first dissociate into the folded monomers which is then followed by their unfolding. This we believe is largely

due to the unusual and open quaternary structure of the protein. This open quaternary structure hence seems to be responsible for the lower structural stability of peanut agglutinin compared to those of Con A, pea and lentil lectin, and the galectins. The somewhat larger ΔC_p for the first transition compared to that for the second transition suggests that the dissociation of the tetramer results in a degree of unfolding of each monomeric unit greater than, perhaps, that of a basically monomeric protein of comparable size and compactness. Thus, the stabilization and formation of the quaternary structure and the maintainence of its compactness may be of paramount importance for the stability of each tertiary unit itself, in peanut agglutinin in particular and perhaps for other oligomeric proteins in general. The mode of intersubunit association in legume lectins, therefore, appears to have a striking influence on their thermal unfolding behavior. More so, it also emphasizes that the tertiary structure of legume lectins is such that minor alterations in it are sufficient to accommodate a wide variety of intersubunit interfaces and quaternary structures.

In summary, these results quite explicitly show another fascinating facet of peanut agglutinin, viz., its unfolding through dissociation to its constituent subunits which retain their ligand binding activity, which sets it apart from the other highly homologus legume lectins. This we believe is a consequence of its unusual and open quaternary structure.

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