Conformational Stability of Legume Lectins Reflect Their Different Modes of Quaternary Association: Solvent Denaturation Studies on Concanavalin A and Winged Bean Acidic Agglutinin

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ABSTRACT: Thermodynamic parameters associated with the unfolding of the legume lectin, WBA II, were determined by isothermal denaturation. The analysis of isothermal denaturation data provided values for conformational stability and heat capacity for WBA II unfolding. To explore the role of intersubunit contact in stability, we carried out similar studies under identical conditions on Concanavalin A, a legume lectin of nearly similar size, buried hydrophobic surface area and tertiary structure to that of WBA II but with a different oligomerization pattern. Both proteins showed a reversible two-state unfolding with guanidine hydrochloride. As expected, the change in heat capacity upon unfolding was similar for both proteins at 3.5 and 3.7 kcal mol^{-1} K^{-1} for Concanavalin A and WBA II, respectively. Although the ΔG_{H20} at the maximum stability of both proteins is around 16 kcal/mol, Concanavalin A exhibits greater stability at higher temperatures. The T_g obtained for Concanavalin A and WBA II were 21 °C apart at 87.2 and 66.6 °C, respectively. The higher conformational stability at higher temperatures and the T_g of Concanavalin A as compared to that of WBA II are largely due to substantial differences in the degree of subunit contact in these dimeric proteins. Ionic interactions and hydrogen bonding between the monomers of the two proteins also seem to play a significant role in the observed stability differences between these two proteins.

The unfolding of oligomeric proteins require the disruption of additional molecular interactions over those of monomeric proteins, since both inter- as well as intrasubunit interactions would make distinct and differential contributions to their overall structure and stability. While cooperative unfolding may be expected of single domain monomeric proteins, the relative contributions of the inter- and intramolecular forces would govern the degree of cooperativity, the mechanism of unfolding, and the overall stability of their oligomeric counterparts (*1*, *2*). Equilibrium unfolding studies on oligomeric proteins would provide valuable insights into the relationship of the stability of these proteins vis a vis oligomerization (*3*, *4*). In addition, significant inferences can be drawn on the role of subunit interface and the extent to which their interactions govern the stability and integrity of these proteins.

Legume lectins are a class of oligomeric proteins that bind carbohydrates specifically and reversibly. Their high affinity and specificity for glycoconjugates have found many applications in biological and biomedical research (*5*). Moreover, despite their widely varying specificites, they share a common theme in the architecture of their binding sites and the mechanism of their ligand recognition (*6*). Consequently, they have been intensely investigated so as to serve as paradigms in the study of carbohydrate-protein interactions. Perhaps underlying the common theme to their binding process is the fact that legume lectins are very similar in their tertiary structure (*7*). The tertiary structure of each monomeric unit of legume lectins consists of three antiparallel β-pleated sheets, of which the plane of one is flat and another is twisted by 90° along an axis perpendicular to the chain and the third one is the "top" β sheet which forms a roof like structure above the other two (Figure 1a) (*8*−*10*). This has been described as the "jelly roll" fold. But quite remarkably, despite their very high structural similarity, they differ widely in their quaternary associations. These differences have been attributed to small differences in the amino acids that make up the subunit interfaces of these proteins and/or of glycosylation that may alter their quaternary associations. For example the monomers of Concanavalin A (Con A),[1] pea, and lentil lectin come together so that the two back β-sheets interact to form a 12-stranded contiguous sheet (Figure 1b) (*8*−*10*). This association has been described as the "canonical dimerization". It results in extensive

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[1] Abbreviations: WBA II, winged bean acidic agglutinin; Con A, Concanavalin A; ECorL, *Erythrina corallodendron* lectin; GdnCl, guanidinium Hydrochloride; CD, circular dichroism; ΔC_p, change in heat capacity; ΔASA, change in accessible surface area; a.a., amino acid.

[2] The ΔASA is calculated by subtracting the accessible area of the folded molecule, obtained using the NACCESS (*35*) program, from that of the unfolded molecule. All amino acids are assumed to be completely accessible in the unfolded molecule, and the area exposed in each amino acid is taken to be that in the Ala-X-Ala tripeptide, where X is the amino acid.

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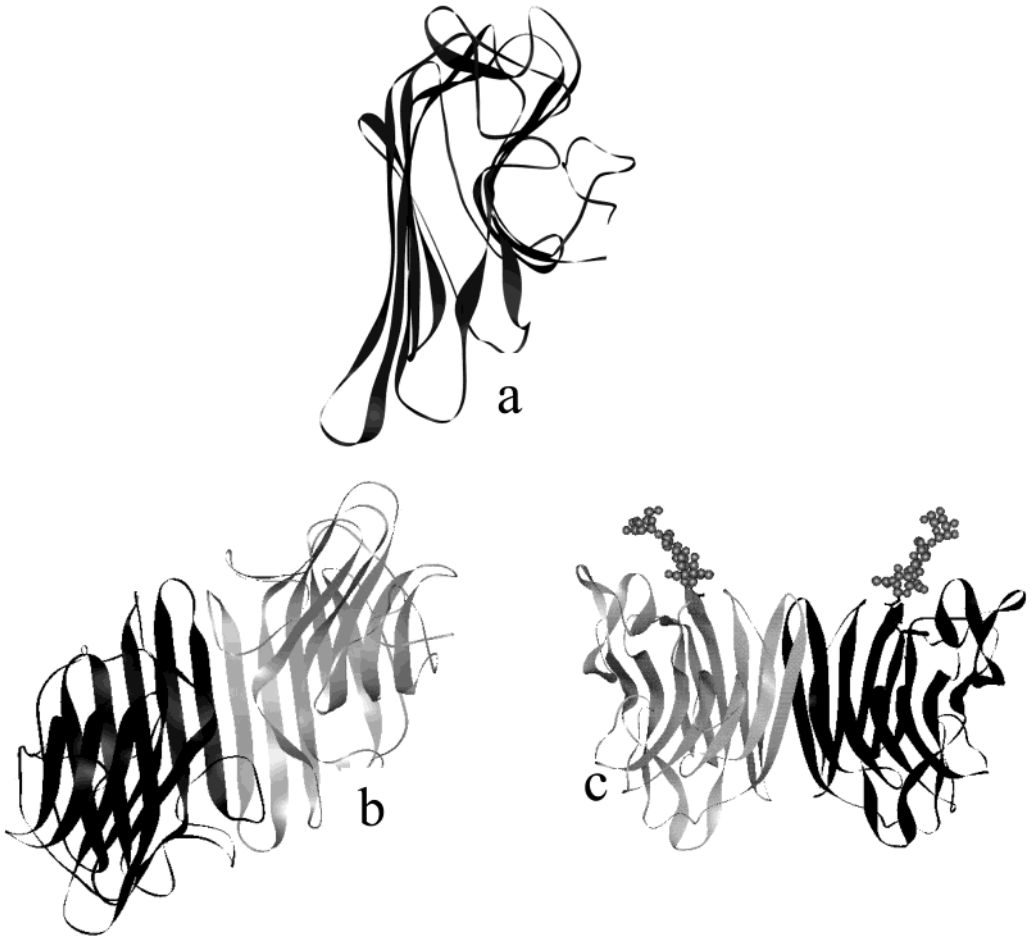

FIGURE 1: Three-dimensional structures of Con A and WBA II and a representative lectin monomer showing the "jelly roll" fold. (a) A monomer of Con A showing the jelly roll tertiary structure. (b) Dimeric structures of Con A and (c) WBA II showing the canonical and the handshake mode of association, respectively. The ball-and-stick structures in panel c are the covalently attached carbohydrate moieties. The residue Asn 76 in WBA II is shown in the stick form. The structures shown are 1APN (Con A, 39) and 1F9K (WBA II, 36). The figures were generated using WebLab ViewerLite 3.5.

intersubunit interactions with a large buried interface of approximately 1000 Å^2/monomer. On the other hand, the lectin from *Erythrina corallodendron* (ECorL), the winged bean basic agglutinin (WBA I), and the winged bean acidic agglutinin (WBA II) have what is described as a "handshake" kind of quaternary structure, with a relatively reduced buried intersubunit interface of about 700 Å^2/monomer (Figure 1c) (*11*, *12*, *16*). These differences in quaternary associations appear to effect their thermal unfolding properties, with the "canonically associated lectins being much more stable then both ECorL and WBA I" (*13*−*15*). An earlier differential calorimetric study on the winged bean acidic agglutinin (WBA II) has shown it to possess unfolding propensities similar to those of ECorL and WBA I (*15*). This had led to the suggestion that WBA II may possess a quaternary association similar to that of ECorL and WBA I (*15*), and this was subsequently found to be the case when its atomic resolution structure was solved (*36*). Our laboratory has initiated studies on legume lectins to investigate in greater detail the degree to which different modes of quaternary association may plausibly affect the thermodynamic parameters of unfolding and stability of multimeric proteins. In this paper, we report solution denaturation studies on WBA II. The unfolding of WBA II appears to be a two-state process. Also, the thermodynamic parameters, in particular the free energy and heat capacity change upon unfolding, are quite different from those obtained for pea lectin (*18*). To determine, if these differences could be a result of differences in oligomeric structures, we also report similar studies on Con A, which differs from WBA II in its intersubunit interactions. The unfolding studies were carried out at pH 5, where both these lectins exist as dimers. Substantial differences in the folding parameters of the two proteins are observed. Given that both these proteins share the same tertiary structure, these differences appear to be a consequence of differences in their quaternary association, particularly in the extent of their respective intersubunit interface.

MATERIALS AND METHODS

Materials. Ultrapure guanidinium hydrochloride (GdnCl) was purchased from Sigma Chemical Co. All other reagents used for this study were of the highest purity available. Stock GdnCl solutions were prepared freshly in 50 mM acetate buffer pH 5 containing 1 mM $CaCl_2$ and $MnCl_2$, and the molarity of GdnCl was determined by refractive index as described by Pace (*19*, *27*).

Protein Purification. Con A and WBA II were purified by affinity chromatography from jack bean and winged bean seeds, respectively (*20*, *21*). Their purity was checked by sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

The concentrations of the protein solutions were determined from their specific extinction coefficient, $A^{1\%}_{280} \sim 12.4$ for Con A and $A^{1\%}_{280} \sim 7.7$ for WBA II.

Isothermal GdnCl-Induced Denaturation. Equilibrium unfolding as a function of GdnCl concentration was monitored by fluorescence spectroscopy and far-UV CD. The fluorescence measurements were done on a JASCO FP777 spectrofluorimeter in a 1 cm water jacketed cell using a protein concentration of 3μM**.** The excitation and emission wavelengths were fixed at 280 and 323 nm, respectively, for both the proteins with slit widths of 3 and 5 nm for the excitation and emission monochromators, respectively. Each measurement was an average of three readings. The CD measurements were made on a JASCO-J715 spectropolarimeter and were followed at 330 nm in a 0.1 cm path length cell. The spectra were collected with a slit width of 1 nm, response time of 8 s, and a scan speed of 50 nm minute^{-1}. Each data point was an average of 3 accumulations for far-UV CD. The protein concentration used in the far UV CD experiments were 13.3 μM for Con A and 20 μM for WBA II. Reversibility was checked by fluorescence and far-UV CD by monitoring the return of the complete spectrum upon refolding the unfolded protein as GdnCl was diluted from 6.8M to 0.5M or less.

Eight isothermal GdnCl induced denaturation curves were collected between a temperature range 283−318 K. The temperature of the cuvette was monitored using a digital temperature probe. A Julabo water bath was used for maintaining the sample temperature to within ±0.1 K of the set temperature.

Gel Filtration. Gel filtration experiments were performed on Biogel P100 (length, 90 cm; diameter, 1 cm; blue dextran elution volume, 23 mL) on the native protein and in the presence of different concentrations of GdnCl. The column was first preequilibrated with the appropriate buffers (required GdnCl concentration) before the protein was loaded.

RESULTS

The unfolding of WBA II and Con A were monitored as a function of GdnCl concentration, by fluorescence and far UV CD. The guanidine-induced unfolding was completely reversible, as monitored by the return of the fluorescence and far-UV CD spectrum, upon refolding of the denatured protein. In dimeric proteins, if the relative contributions of the intersubunit interactions are weaker than the intra subunit ones, then unfolding may be accompanied by the formation of a stable monomeric intermediate, with perhaps a reduced structure. This will be followed by its complete disruption at a higher chaotrope concentration. Such a reaction may be described as $N_2 \leftrightarrow 2I \leftrightarrow 2U$ (*3*, *22*−*24*). The solution denaturation profiles of such a kind of transition may be biphasic and/or the profiles monitored by fluorescence and near- and far-UV CD, nonsuperimposable (*22*). Alternatively, when the intersubunit interactions contribute significantly to the overall stability of the protein, the unfolding may be a simple two-state process: $N_2 \leftrightarrow 2U$ (*3*). Such a transition would have a typical sigmoidal shape. Also, these transitions will be completely superimposable when different spectroscopic probes are used. As can be seen in Figure 2, the denaturation transitions, monitored by fluorescence and far-UV CD, are completely superimposable, evidence against the former possibility. Moreover, on gel filtration (Figure 3a,b), both proteins elute as dimers in GdnCl concentration range in the pretransition region and as unfolded polypeptide above 6 M GdnCl. In the intermediate GdnCl range, varying proportions of dimeric and unfolded polypeptides are seen. Importantly, at no concentration do we observe any species other than the dimeric and monomeric unfolded polypeptides. It may be noted that an outcome of the two-state $N_2 \leftrightarrow 2U$ process, in which the dimer populates the transition zone significantly, is that the fraction of the molecules in the native state increases with protein concentration (equation 1 below) (*3*, *4*, *26*). Indeed, observation of a distinct protein concentration dependence of the unfolding profiles is consistent with the aforementioned suggestion. (Figure 4). Thus, the unfolding of WBA II and Con A may be treated thermodynamically, as a two-state process with only the native dimer and unfolded polypeptide existing as equilibrium species.

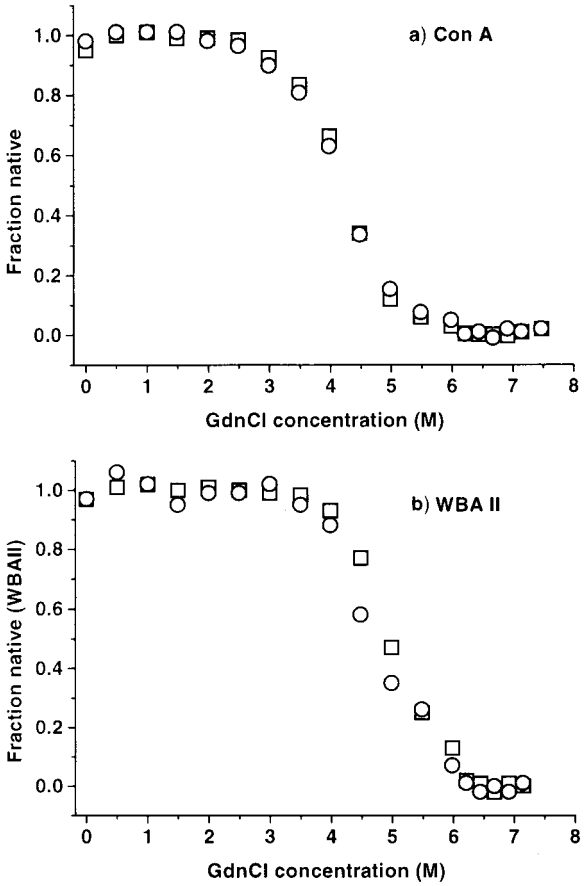

FIGURE 2: Overlap of GdnCl induced unfolding profiles obtained using fluorescence (□) and far UV CD (○). GdnCl induced unfolding transitions of Con A (a) and WBA II (b), monitored by fluorescence and far UV CD. The completely superimposable profiles rule out the non-two-state possibility in the unfolding of these proteins. The melt is done with monomeric protein concentrations of 13.3 and 20 μM for Con A and WBA II, respectively, at 298 K. ΔG (kcal/mol) and *m* and Cm (M) values are as follows: Con A, 14.7, 1.9, 4.3 (Flu) and 14.6, 1.9, 4.3 (CD); WBA, II 16.3, 2.0, 5.0 (Flu) and 15.9, 1.9, 4.9 (CD).

The equilibrium constant for the reaction $N_2 \leftrightarrow 2U$ is

$$K_u = [U]^2/[N_2] = 2P_t[f_u^2/(1 - f_u)] \qquad (1)$$

where f_u is the fraction of protein unfolded and P_t is the monomeric protein concentration.

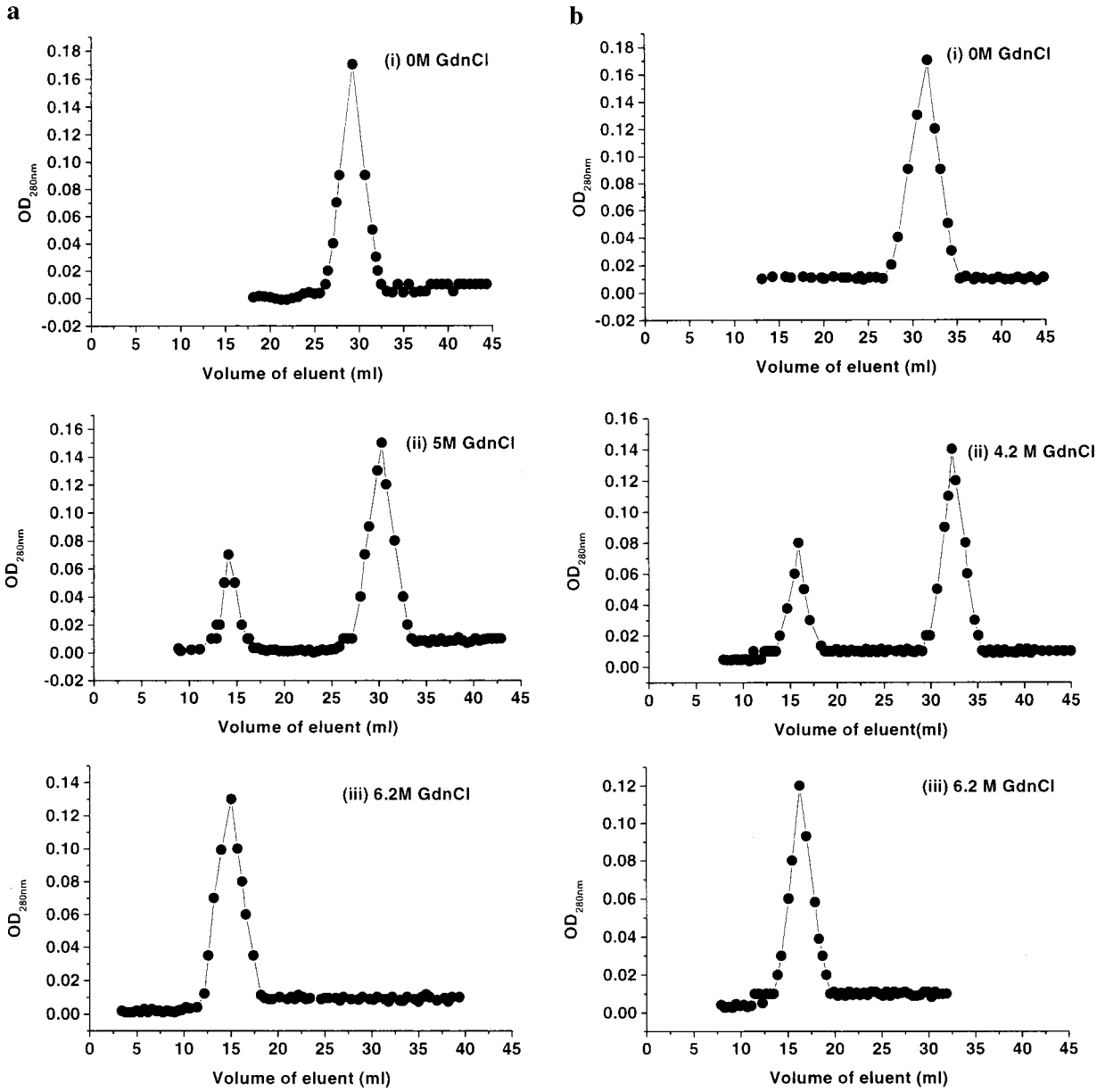

FIGURE 3: (a) Gel filtration profiles of Con A at different GdnCl concentrations at 25 °C. (i) Elution profile of Con A in the absence of any denaturant, (ii) profile in the presence of 5 M GdnCl, and (iii) profile in the presence of 6.2 M GdnCl. (b) Gel filtration profiles of WBA II at different GdnCl concentrations at 25 °C. (i) Elution profile of WBA II in the absence of any denaturant, (ii) profile in the presence of 4.2 M GdnCl, and (iii) profile in the presence of 6.2 M GdnCl.

The free energy change of unfolding, a measure of protein stability, ΔG_{H20}, is calculated from the GdnCl denaturation profiles by the method of Schellman, where the unfolding free energy change in the absence of denaturant is linearly extrapolated from a plot of unfolding free energy change versus the denaturant concentration. (*5*, *25*, *28*−*30*):

$$\Delta G_u = \Delta G_{H20} + m[\text{urea}] \quad (2)$$

The unfolding free energies of Con A and WBA II at various temperatures are presented in Table 1 and Figure 5.

The unfolding of a protein is usually accompanied by a large increase in exposure of hydrophobic regions of the polypeptide chain and, consequently, a large change in heat capacity (ΔC_p) upon unfolding. Thus ΔC_p can be taken as a measure of the extent of the nonpolar surface area buried inside a protein. The value of ΔC_p for the unfolding process may be estimated from solution denaturation studies by the method suggested by Pace, in which the changes in free energy calculated at different temperatures is fitted to the Gibbs−Helmholtz equation (*1*, *25*):

$$\Delta G(T) = \Delta H_g(1 - T/T_g) + \Delta C_p[(T - T_g - T \ln(T/T_g)] \quad (3)$$

where T_g is the temperature at which $\Delta G_{H20} = 0$ and ΔH_g the unfolding enthalpy at T_g.

The values of ΔC_P are estimated to be 3.5 ± 0.8 kcal/mol/K for Con A and 3.7 kcal/mol/K for WBA II, respectively (Table 2, Figure 5). It is seen that the ΔC_p's of both proteins and the ΔG at temperature of maximum stability are nearly the same. But the stability of Con A is higher than that of WBA II as indicated by a higher T_g.

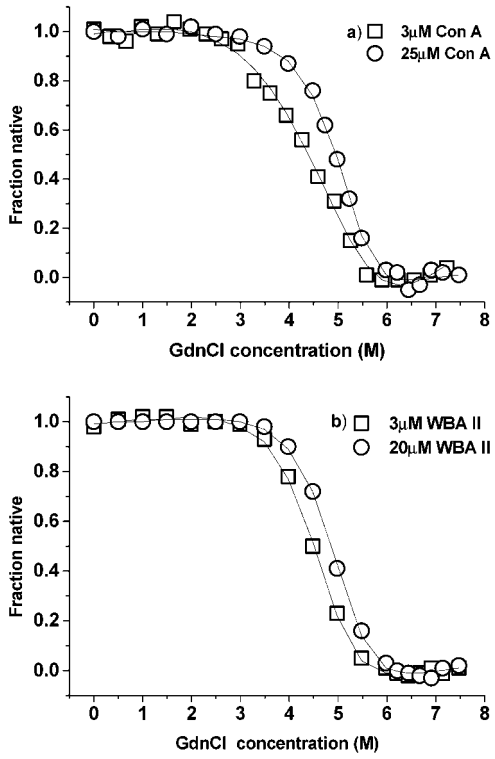

FIGURE 4: Concentration dependence of the unfolding profile of Con A and WBA II. The concentrations used are indicated in the figure. The unfolding was monitored by fluorescence at 298K. ΔG (kcal/mol) and *m* and Cm (M) values are as follows: Con A, 15.8, 1.9, 4.4 (3μM) and 16.3, 2.0, 4.9 (25μM); WBA, II 15.6, 1.8, 4.5 (3μM) and 15.6, 1.9, 4.9 (20μM).

DISCUSSION

Con A and WBA II are two legume lectins that have adopted different kinds of quaternary interactions. As described earlier in the paper, this kind of variation, despite identical tertiary structures, could arise due to small differences in sequences and/or the presence or absence of glycosylation, of which the latter is also ruled out for WBA II, a glycoprotein (*36*). The buried surface area at the interface calculated using NACCESS from the three-dimensional structures (*35*, *36*, *40*) are 912 Å^2/monomer in WBA II and 1151 Å^2/monomer for Con A, respectively. Thus, we see that the interacting area at the dimeric interface is 20% less in WBA II as compared to that in Con A. These differences in the degree of subunit interaction in the oligomeric structure are known to have distinct effects on their stability (*13*−*15*). For instance, Con A, pea, and lentil lectin have significantly higher T_m values than the lectins that associate in a handshake manner. In the case at hand the difference in T_g values is about 24.3 °C. Apparently, the greater intersubunit contact region imparts the canonically associated proteins with greater thermal stability.

The analysis of the denaturation profiles of both Con A and WBA II shows that their unfolding is highly cooperative. In addition, the profiles obtained by using fluorescence and far-UV CD as probes completely overlap (Figure 2). Since these spectroscopic methods, probe different characteristics of the unfolding protein, the overlap of the two profiles rules out the presence of any equilibrium intermediate during their unfolding (*22*). Gel filtration experiments also show the existence of two species during unfolding, folded dimers and unfolded polypeptides (Figure 3a,b). The absence of any folded monomeric intermediate suggests that intersubunit interactions play a dominant role in the stability of these proteins. This seems to be generally true of many oligomeric proteins in that a significant percentage of the conformational stability of the oligomer may be contributed by the quaternary interactions alone and that the isolated monomer is stabilized to a much lesser extent, if at all. For example, though the stability of the tryptophan aporepressor dimer under standard conditions is 22.4 kcal/mol, the dissociated monomer was estimated to have a stability of just 5.4 kcal /mol monomer (*26*, *31*). Yet certain interesting distinctions can be made when the stabilities of Con A and WBA II are compared. It is clear from Figure 5 and Table 2 that Con A in general is more stable than WBA II, with a higher T_g and a greater temperature of maximum stability. This is interesting when we consider that both Con A and WBA II have very similar tertiary structures. One of the reasons for this difference in their stabilities may be attributed to the variation in their quaternary associations.

A very important parameter associated with the unfolding of proteins is the change in heat capacity. A positive change in heat capacity is usually a hallmark of processes that expose nonpolar surfaces to the aqueous environment. The resulting formation of cages of structured water around apolar residues could be the cause of the increase in heat capacity of the solution upon protein unfolding. The change in heat capacity can therefore be taken as a measure of the extent of buried hydrophobic core in the native protein. Spolar et al. were able to show that heat capacity changes for the transfer of hydrocarbons from water to the pure liquid phase and the folding of globular proteins exhibit a similar proportionality to the reduction in water-accessible surface area (ΔASA_{np} and ΔASA_{pol}) (*32*−*34*).

Analysis of thermodynamic data of unfolding of different proteins (usually small monomeric proteins) showed that there was a definite correlation between buried area in a protein (ΔASA) and the change in heat capacity (ΔC_p). Due to the similar number of amino acids in Con A and WBA II (237a.a. and 234a.a.), as expected, the ΔASA's from their three-dimensional structures (*36*, *40*) calculated using NACCESS (*35*) were found to be very similar at 53519.2 and 49932.9 Å^2, respectively. Atom-wise polar and the apolar components of ΔASA in both cases is close to 40% and 60%, respectively. The proportion of this area in the interface of the dimeric molecules is approximately 4.3% (Con A) and 3.6% (WBA II).

The calculation of ΔC_p from ΔASA is done using the equation given by Spolar et al. (*33*) ($\Delta C_p = (0.32 \pm 0.04)$-$(\Delta ASA_{np}) - (0.14 \pm 0.04)$ (ΔASA_{pol})), where the apolar and polar contributions to ΔASA are taken into consideration separately; ΔC_p values of 7.5 and 7.3 kcal/mol/K for Con A and WBA II are obtained. This is not unexpected because despite a difference in the interacting area, in the dimeric interface the ΔASA (both polar and apolar) values are similar in the two proteins. But both values of ΔC_p obtained are much higher than the experimentally obtained values (Table 2). Such disagreements between the calculated and the experimental values of ΔC_p are quite common, especially so with the oligomeric proteins (*37*).

There could be two reasons for this. One is that unfolding in these proteins does not go to completion. Another

Table 1: Unfolding Free Energy Change of Con A and WBA II at Various Temperatures Obtained from GdnCl-Induced Isothermal Melts[a]

temperature (K)	ΔG_{H20} for Con A (kcal/mol)	*m* value for Con A	temperature (K)	ΔG_{H20} for WBA II (kcal/mol)	*m* value for WBA II
283	13.3 ± 0.8	1.4 ± 0.2	283	16.0 ± 0.9	1.6 ± 0.1
288	14.0 ± 0.2	1.4 ± 0.5	288	16.1 ± 0.1	1.8 ± 0.2
293	14.7 ± 0.5	1.5 ± 0.2	293	15.8 ± 1.0	1.8 ± 0.2
298	15.9 ± 0.1	1.6 ± 0.3	298	15.5 ± 0.9	1.8 ± 0.3
303	15.9 ± 1.4	1.9 ± 0.4	303	15.1 ± 0.6	1.9 ± 0.2
308	16.1 ± 0.7	1.9 ± 0.3	308	13.3 ± 0.8	1.6 ± 0.2
313	16.5 ± 0.9	2.3 ± 0.4	313	12.3 ± 1.0	1.7 ± 0.3
318	14.7 ± 0.5	2.0 ± 0.3	320	9.7 ± 0.9	1.7 ± 0.3

[a] The signal monitored was fluorescence in all cases. Each denaturation experiment was performed twice. The profiles from each duplicate were completely superimposable and consequently yielded almost identical values of ΔG_{H20}. The standard deviation for the *m* values of Con A and WBA II are 0.184 and 0.213, respectively.

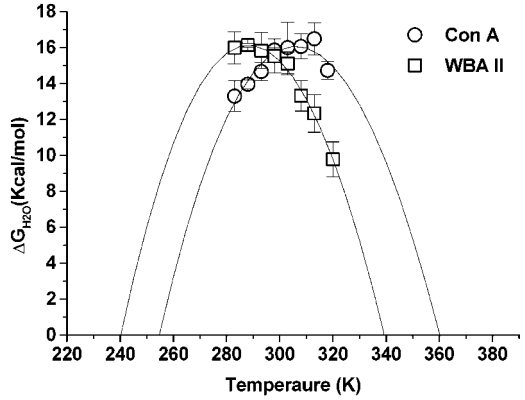

FIGURE 5: Stability plot of Con A and WBA II. Unfolding free energy change (ΔG_{H2O}) of Con A and WBA II as a function of temperature. The continuous line shows the least-squares fit of the points to eq 3. The data points have been obtained using fluorescence signals. The parameters obtained from the fit are shown in Table 2.

Table 2: Thermodynamic Parameters of Con A and WBA II from the Analysis of the Stability Curves (Table 1 and Figure 5)

protein	ΔH_g (kcal/mol)	T_g (K)	ΔC_p (kcal/mol/K)	ΔH_{gc} (kcal/mol)	T_{gc} (K)
Con A	207.6 ± 23.8	360.2 ± 8.3	3.5 ± 0.8	−163.7	254.7
WBA II	209.2 ± 9.9	339.3 ± 1.4	3.7 ± 0.3	−157.4	240.2

factor that probably plays a part is the difference in pH during the experiment, pH5 and the crystallization conditions, pH7 (*36*, *40*). If we use the PDB coordinates for Con A at pH 5 (*39*), it is seen that now the ΔC_p value obtained using the equation of Spolar et al. is 7.0 kcal/mol/K. This value is less than that derived from the pH 7 structure and thus closer to that obtained experimentally (Table 2). A corresponding structure at pH 5 is not available for WBA II.

Although a smaller area in the interface could be responsible for the reduced stability of WBA II, it is possible that there could be other differences between the two proteins as well. The differences could be in the amino acid composition, hydrogen bonding, and other interactions and structural parameters.

Although the major interaction determining the stability of a protein is thought to be hydrophobic interactions, many mutagenesis studies have shown that hydrogen bonds also play an important role in stabilizing a protein (*41*−*45*). Using the HBPLUS program, we determined the number of hydrogen bonds in the two proteins under study (Table 3) (*46*).

Table 3: Hydrogen Bond Distribution between the Main Chain and the Side Chains of Con A and WBA II

protein	main chain−main chain (MM)	main chain−side chain (MS)	side chain−side chain (SS)
Con A	235	89	52
WBA II	233	49	37

As seen in Table 3, the number of main chain−main chain (MM) hydrogen bonds are similar in number in both the proteins, and they have nearly identical secondary structure. This is expected because the number of amino acids per monomer are similar in both proteins and they have nearly identical secondary structures. But the number of main chain−side chain (MS) and side chain−side chain (SS) interactions are very different in the two lectins, with Con A being involved in a much higher number of these interactions. In an analysis of the number of amino acids, it is seen that there is a dramatic increase in the number of serine residues from 14 in WBA II to 31 in Con A. Thus serine is involved in 44 MS and SS interactions in Con A but only in 21 such interactions in WBA II. There is also a great increase in the number of positively charged amino acids and a small increase in the negatively charged amino acids. All these changes in the amino acid level is reflected in an increase in the number of hydrogen bonds. There is also a rise in the interchain hydrogen bond number from 6 to 14 in Con A. This is probably due to an increased area of contact at the interface.

It is also seen that in the case of Con A, a greater number, 161, of the hydrogen bonds have a hydrogen−acceptor separation less than 2 Å as compared to just 146 in WBA II (Figure 6).

Analysis of structures of ferredoxin from thermophiles and mesophiles led Perutz to point out that the additional stability in the thermophilic proteins is a result of the salt bridges between external polar groups and hydrogen bonding (*47*). The recent surge in genomic information regarding thermophiles have shown that these organisms have a higher proportion of charged residues as compared to mesophilic organisms (*48*−*50*), thus implicating them in protein stability. On taking a look at the amino acid composition of the two proteins of interest, we see that the number of charged residues in Con A is 94 whereas there are only 67 charged groups in WBA II; the number of charged residues in Con A is approximately 40% higher than that in WBA II. But it is not just the presence of the charged groups but also their

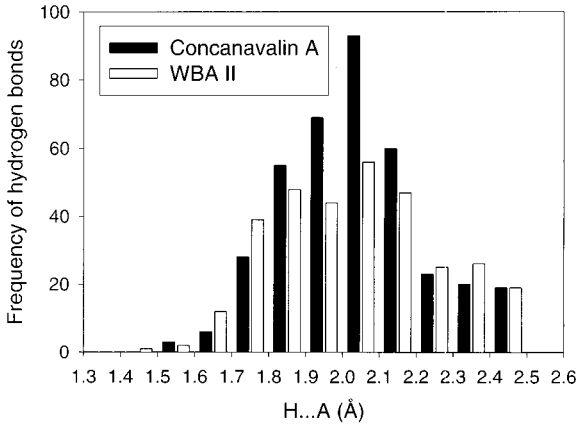

FIGURE 6: Plot of the number of hydrogen bonds as a function of hydrogen−acceptor distance in Con A and WBA II. The number of hydrogen bonds in Con A are much greater in number as compared to WBA II. The strong hydrogen bonds, i.e., those with a hydrogen−acceptor distance less than 2 Å, are also higher in number in Con A.

position in the final three-dimensional structure of the protein that affects its stability. Recent work has shown that the optimization of charge−charge interactions on the surface of proteins promotes their stabilization (*51*−*54*). This led us to look for positions of charged residues and charge−charge interactions in these proteins. It was observed that 58% of the charged residues in WBA II were more than 70% buried, as compared to only 34% in Con A (Figure 7). In actual numbers, this corresponds to 49 and 48 buried charged residues in Con A and WBA II, respectively. But when we take a look at the residues that are exposed, Con A has 23 charged residues less than 40% buried, but WBA II has only 8. Thus, the charged groups in Con A are more exposed or closer to the surface as compared to those in WBA II, i.e., Con A has stabilizing surface residues. In an analysis of the charge−charge interactions, it is seen, as expected, that the number of ionic interactions in Con A are higher by a significant amount and most of these lie on the surface. In the less than 4 Å and 4−6 Å interionic distance category, the number of salt bridges were nearly the same in both protein dimers. But a greater number of these bridges were buried in WBA II. This kind of charge burial tends to reduce protein stability (*52*, *58*). The main difference, however, occurred in the group that are within 6−8 Å, where Con A had nearly 7 times the number of salt bridges as compared to WBA II. The distances of the residues from the surface of the protein molecule were obtained using the surface depth program (*55*). Thus, we see that favorable ionic interactions are probably responsible for increased stabilization of Con A at higher temperatures.

The total electrostatic free energy ($\Delta\Delta G_{tot}$) contribution on the formation of a salt bridge is the sum of the free energies due to desolvation of the salt bridge ($\Delta\Delta G_{dslv}$), the electrostatic interactions between the side chains ($\Delta\Delta G_{brd}$), and the interaction of the salt bridge with the rest of the protein ($\Delta\Delta G_{prt}$)(*56*). The desolvation of the salt bridge is an unfavorable contribution, and the electrostatic interaction is a favorable contribution to the free energy. But the low dielectric constant of the protein interior strengthens the electrostatic interaction. At higher temperatures, the desolvation penalty is reduced, and the salt bridge becomes more stabilizing thus increasing the stability of the protein. We

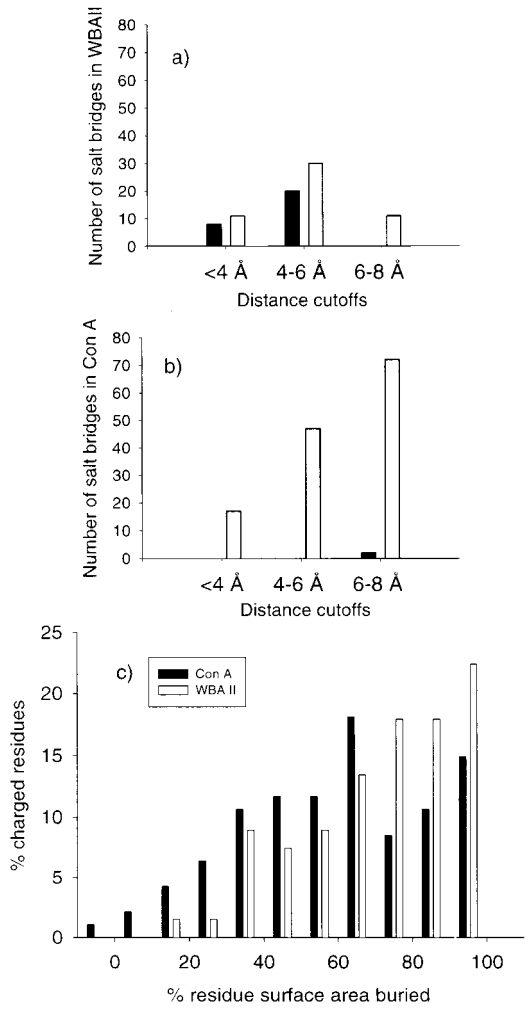

FIGURE 7: Plots a and b show the salt bridges in WBA II and Con A as a function of the distance between the charge centers. The bridges which on average are more than 5.5 Å from the surface of the protein are assumed to be buried. The white boxes show external salt bridges, and the black ones show buried salt bridges. Plot c shows the percentage of charged residues vs buried area. A completely exposed residue (0% buried) has the same amount of area exposed as that in the Ala−X−Ala tripeptide. Residue accessibility was calculated using the NACCESS (35) program.

see this in Con A, which is more stable than WBA II at higher temperatures (Figure 5).

We also calculated the ξ_h and ξ_p, the optimization parameters for protein−solvent interactions; ξ_h is the hydrophobic optimization parameter, and ξ_p is the hydrophilic optimization parameter (*57*). This is a ratio of solvent accessibility of the protein in the native state and a reference state, which is the unfolded state of the molecule (ξ_h = solvent accessible area of hydrophobic atoms in the folded state/ solvent accessible area of hydrophobic atoms in the unfolded state, ξ_p = solvent accessible area of hydrophobic atoms in the folded state/ solvent accessible area of hydrophobic atoms in the unfolded state). $\xi_h = \xi_p = 1$ is the reference unfolded state, which is the most deoptimized hydrophobic and the most optimized hydrophilic interactions. In the case of Con A and WBA II dimers, there was not much difference between the ξ_p values. But it was seen that hydrophobic interactions are more optimized in the case of Con A as compared to WBA II. Also, the monomers were less hydrophobically optimized as compared to the dimers,

which shows that dimerization indeed leads to stabilization of these oligomeric proteins. Thus, both the monomer and dimer of WBA II are hydrophobically less stabilized as compared to their counterparts in Con A.

In conclusion, we have shown that the two dimers, Con A and WBA II, show differences in stability. These differences may reflect different modes of subunit associations which are probably a result of differences in amino acid composition (*36*). This difference also leads to a variation in hydrogen bonding and ionic interactions among the two protein molecules. Thus, we see that two proteins from the same family of plants, with nearly identical tertiary structure differ dramatically in stability due to differences in quaternary structure, ionic interactions, and hydrogen bonding patterns. More detailed studies on the folding pathways of these proteins would further serve to delineate the differences inherent among these proteins and in addition, establish model systems for the study of the stability of oligomeric proteins in general.

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REFERENCES

1. Agashe, V. R., and Udgaonkar, J. B. (1995) *Biochemistry 34*, 3286−3299.
2. Alber, T., and Matthews, B. W. (1987) *Methods Enzymol. 154*, 511−533.
3. Neet, K. E., and Timm, D. E. (1994) *Protein Sci. 3*, 2167−2174.
4. Bowie, J. U., and Sauer, R. T. (1989) *Biochemistry 28*, 7139−7143.
5. Sharon, N., and Lis, H. (1990) *FASEB J. 4*, 3198−3208.
6. Sharma, V., and Surolia, A. (1997) *J. Mol. Biol. 267*, 433−445.
7. Loris, R., Hamelryck, T., Bouckaert, J., and Wyns, L. (1998) *Biochim. Biophys. Acta 1383*, 9−36.
8. Hardman, K. D., and Ainsworth, C. F. (1972) *Biochemistry 11*, 4910−4919.
9. Vijayan, M., and Chandra, N. (1999) *Curr. Opin. Struct. Biol. 9*, 707−714.
10. Einspahr, H., Parks, E. H., Suguna K., Subramanian, E., and Suddath, F. L. (1986) *J. Biol. Chem. 261*, 16518−16527.
11. Shaanan, B., Lis, H., and Sharon, N. (1991) *Science 254*, 862−866.
12. Schwarz, F. P., Puri, K. D., Bhat, R. G., and Surolia, A. (1993) *J. Biol. Chem. 268*, 7668−7677.
13. Surolia, A., Sharon, N., and Schwarz, F. P. (1996) *J. Biol. Chem. 271*, 17697−17703.
14. Schwarz, F. P., Puri, K. D., and Surolia, A. (1991) *J. Biol. Chem. 266*, 24344−24350.
15. Srinivas, V. R., Singha, N. C., Schwarz, F. P., and Surolia, A (1998) *FEBS Lett. 425*, 57−60.
16. Prabu, M. M., Sankaranarayanan, R., Puri, K. D., Sharma, V., Surolia, A., Vijayan, M., and Suguna, K., (1998) *J. Mol. Biol. 276*, 787−796.
17. Manoj, N., Srinivas, V. R., Satish, B., Singha, N. C., and Suguna, K. (1999) *Acta Crystallogr., Sect. D 55*, 564−565.
18. Ahmad, N., Srinivas, V. R., Reddy, G. B., and Surolia, A. (1998) *Biochemistry 37*, 16765−16772.
19. Pace, C. N. (1990) *Trends Biochem. Sci. 15*, 14−17.
20. Agrawal, B. B., and Goldstein, I. J. (1967) *Biochim. Biophys. Acta 147*, 262−271.
21. Acharya, S., Patanjali, S., Sajjan, S. U., Gopalkrishnan, B., and Surolia, A. (1990) *J. Biol. Chem. 265*, 11586−11594.
22. Barrick, D., and Baldwin, R. L. (1993) *Protein Sci. 2*, 869−876.
23. Robertson, A. D., and Baldwin, R. L. (1991) *Biochemistry 30*, 9907−9914.
24. Kim, P. S., and Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459−489.
25. Nicholson, E. M., and Scholtz, J. M. (1996) *Biochemistry 35*, 11369−11378.
26. Gittelman, M. S., and Matthews, C. R. (1990) *Biochemistry 29*, 7011−7020.
27. Pace, N. C., and Tanford, C. (1968) *Biochemistry 7*, 198−208.
28. Becktel, W. J., and Schellman, J. A. (1987) *Biopolymers* 26, 1859−1877.
29. Schellman, J. A. (1990) *Biophys. Chem. 37*, 121−140.
30. Schellman, J. A. (1987) *Biopolymers 26*, 549−559.
31. Matthews, C. R. (1993) *Annu. Rev. Biochem. 62*, 653−683.
32. Livingstone, J. R., Spolar, R. S., and Record, M. T., Jr. (1991) *Biochemistry 30*, 4237−4244.
33. Spolar, R. S., Livingstone, J. R., and Record, M. T., Jr. (1992) *Biochemistry 31*, 3947−3955.
34. Ganesh, C., Shah, A. N., Swaminathan, C. P., Surolia, A., and Varadarajan, R. (1997) *Biochemistry 36*, 5020−5028.
35. Hubbard, S. J. (1996) *NACCESS*, Version 2.1.1Department of Biomolecular Sciences, UMIST, Manchester, U.K.
36. Manoj, N., Srinivas, V. R., Surolia, A., Vijayan, M., and Suguna, K. (2000) *J. Mol. Biol. 302*, 1129−1137.
37. Backmann, J., Schafer, G., Wyns, L., and Bonisch, H. (1998) *J. Mol. Biol. 284*, 817−833.
38. Myers, J. K., Pace, N. C., and Scholtz, J. M. (1995) *Protein Sci. 4* 2138−2148.
39. Bouckaert, J., Loris, R., Poortmans, F., Wyns, L. (1995) *Proteins Struct. Funct. Genet. 23*, 510−514.
40. Naismith, J. H., Emmerich, C., Habash, J., Harrop, S. J., Helliwell, J. R., Hunter, W. N., Raftery, J., Kalb, A. J. Gilboa and Yariv, J. (1994) *Acta Crystallogr., Sect. D 50*, 847−858.
41. Shirley, B. A., Stanssens, P., Hahn, U., and Pace, C. N. (1992) *Biochemistry 31*, 725−732.
42. Pace, C. N. (1995) *Methods Enzymol. 259*, 538−554.
43. Pace, C. N., Shirley, B. A., McNutt, M., and Gajiwala, K. (1996) *FASEB J. 10*, 75−83.
44. Myers, J. K., and Pace, C. N. (1996) *Biophys. J. 71*, 2033−2039.
45. Pace, C. N. (2001) *Biochemistry 40*, 310−313.
46. McDonald, I. K., and Thornton, J. M. (1994) *J. Mol. Biol. 238*, 777−793.
47. Perutz, M. F., and Raidt, H. (1975) *Nature 255*, 256−259.
48. Haney, P. J., Badger, J. H., Buldak, G. L., Reich C. I., Woese, C. R., and Olsen, G. J. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 3578−3583.
49. Chakaravarty, S., and Varadarajan, R. (2000) *FEBS Lett. 470*, 65−69.
50. Kumar, S., Tsai, C.-J., and Nussinov, R. (2000) *Protein Eng. 13*, 179−191.
51. Perl, D., Mueller, U., Heinemann, U., and Schmid, F. X. (2000) *Nat. Struct. Biol. 7*, 380−383.
52. Kajander, T., Kahn, P. C., Passila, S. H., Cohen, D. C., Lehtiö, L., Adolfsen, W., Warwicker, J., Schell, U., and Goldman., A. (2000) *Structure 8*, 1203−1214.
53. Martin, A., Sieber, V., and Schmid, F. X. (2001) *J. Mol. Biol. 309*, 717−726.
54. Fukuchi, S., and Nishikawa, K. (2001) *J. Mol. Biol. 309*, 835−843.
55. Chakravarty, S., and Varadarajan, R. (1999) *Structure Fold. Des. 7*, 723−732.
56. Kumar, S., and Nussinov, R. (1999) *J. Mol. Biol. 293*, 1241−1255.
57. Spassov, V. Z., Karshikoff, A. D., and Ladenstein, R. (1995) *Protein Sci. 4*, 1516−1527.
58. Hendsch, Z. S., and Tidor, B. (1994) *Protein Sci. 3*, 211−226.

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