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Characterization of the Unfolding Process of the Tetrameric and Dimeric Forms of *Cratylia mollis* Seed Lectin (CRAMOLL 1): Effects of Natural Fragmentation on Protein Stability

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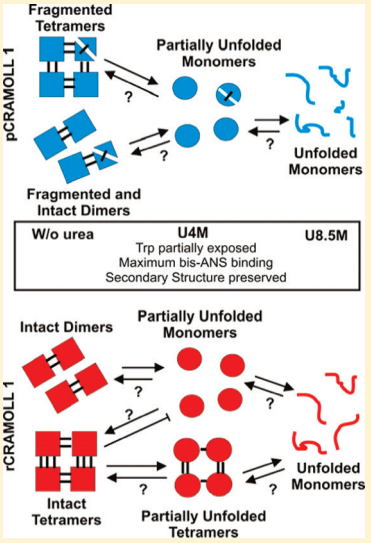
Supporting Information

ABSTRACT: pCRAMOLL 1 is a major, non-glycosylated isolectin found in seeds of *Cratylia mollis*, which belongs to the Leguminosae family and the Diocleinae subtribe. The lectin (~25 kDa) consists of 236 amino acids, sharing 82% identity and virtually identical topological architecture with concanavalin A. Both lectins also share the same pH-dependent dimer−tetramer equilibrium and the ability to recognize Glc/Man moieties. Intricate post-translational events occurring in Diocleinae seed cotyledons result in a mixture of intact and fragmented monomers within the oligomeric assemblies of pCRAMOLL 1. In an earlier report, we demonstrated the production, purification, and characterization of the bacterially expressed form of CRAMOLL 1 (rCRAMOLL 1). The recombinant lectin retained sugar-binding activity and several other biophysical properties of pCRAMOLL 1, but its tetramers, which are composed of intact monomers only, show little enhancement in stability when probed with acidification, high temperatures, or hydrostatic pressure. Here we examined the urea-induced unfolding of the nonfragmented tetramers and dimers of rCRAMOLL 1 and compared this behavior with that of the mixed plant lectin counterparts. Using fluorescence, circular dichroism, size-exclusion chromatography, and chemical cross-linking experiments, we posited that the absence of fragmentation lent greater firmness to tetramers, but not to dimers. Dimeric and tetrameric pCRAMOLL 1 unfolded via a compact monomeric intermediate. In contrast, dimers of rCRAMOLL 1 behaved similarly to the plant dimer counterpart, but its tetrameric form remarkably showed no evidence of such partially unfolded monomers. By analyzing the crystal structure of pCRAMOLL 1, we were able to dissect the importance of the fragmentation to lectin stability.



Lectins are multivalent carbohydrate-binding proteins of nonimmune origin.[1] They exist in almost all living organisms; in leguminous plant seeds, lectins constitute up to 10% of the soluble protein content.[2] The legume lectins form a large family of homologous proteins. Despite great similarity in secondary and tertiary structure, these lectins exhibit considerable diversity in their quaternary assemblies, forming different dimers and tetramers depending on small variations in their amino acid sequences and ambient pH.[3] The existence of stable monomers, dimers, and tetramers of the same lectin makes them an excellent system with which to study the molecular basis of protein folding and oligomerization.[4]

pCRAMOLL 1 is the major and non-glycosylated isolectin found in seeds of *Cratylia mollis*,[5] a plant that belongs to the Leguminosae family and the Diocleinae subtribe. This lectin (~25 kDa) consists of 236 amino acids and shares 82% identity and Glc/Man recognition capability with concanavalin A (Con A).[6] The tertiary fold of pCRAMOLL 1 (Figure 1A), as determined by X-ray crystallography at 1.77 Å, reveals three β-sheets connected by loops to form a topological structure similar to that of Con A, known as the jellyroll domain.[4] In solution, this lectin forms dimers around pH 5.0 and tetramers at pH 7.0 and above (Figure 1B,C). pCRAMOLL 1 has also been used as a tool for biotechnological and biomedical applications.[7−16]

Post-translational modification not only plays a crucial role in generating variability in proteins but also imparts different physicochemical properties to these macromolecules. In Diocleinae lectins, including Con A and pCRAMOLL 1, after global folding, each subunit is post-translationally cleaved at a surface loop into two halves, generating new N- and C-termini and the Ser and Asn residues, originally located at the N- and C-terminus, respectively, are covalently bonded (forming the Asn_{118}−Ser_{119} bond) (ref 17; see Figure 1A). Because the religation step is only ~60% efficient, the monomeric units of these lectins are in fact composed of either a single polypeptide

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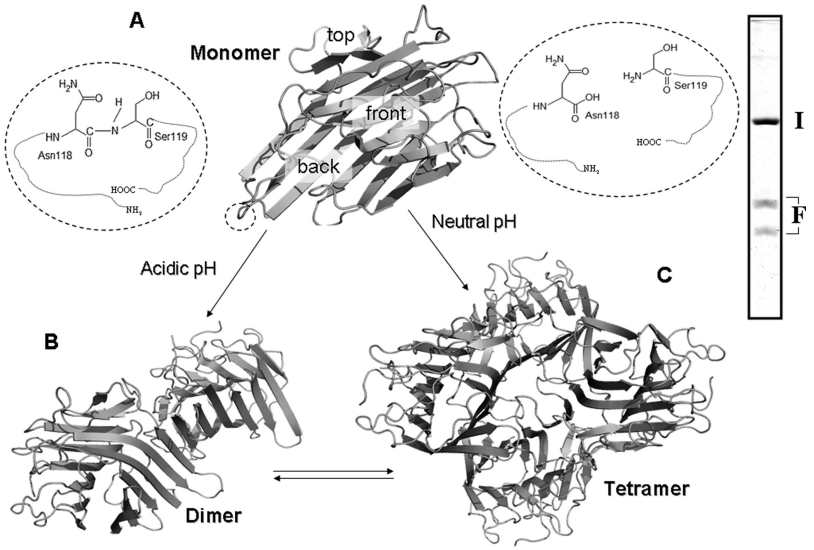

Figure 1. (A) Tertiary structure of pCRAMOLL 1 (PDB ID: 1MVQ) monomer, which possesses the classical leguminous lectin fold.[4] It consists of three β-sheets: a relatively flat six-stranded sheet, called the "back" β-sheet; a seven-stranded curved "front"' β-sheet, which is packed against the back β-sheet; and a small five-stranded "top" β-sheet that plays a major role in holding the two large sheets together. In dashed circles we show that the Asn_{118}–Ser_{119} bond is present only in part of the subunits. Therefore, when the lectin is analyzed by SDS-PAGE (right box), three polypeptide bands are observed. Densitometry of these bands indicates that 60% of the protein is intact (I, residues 1–236), the rest being fragmented (F, residues 1–118 and 119–236). pH-dependent oligomeric assemblage of pCRAMOLL 1 proceeds as described for concanavalin A. (B) Dimerization involves antiparallel side-by-side alignment of the flat six-stranded "back" β-sheets, giving rise to the formation of a contiguous 12-stranded sheet, known as the "canonical" mode of dimerization in leguminous lectins. (C) Tetramerization consists of back-to-back association of two dimers, each formed from the side-by-side association of two subunits.[50] Figures were generated using PyMOL.[51]

chain (intact monomer, ~25 kDa) or a noncovalently bound form that contains the N- and C-terminal halves of the polypeptide chain (~12.7 and 12.6 kDa, respectively) and is itself a dimer (see the dashed circles and SDS-PAGE pattern in Figure 1A). Thus far, the physiological role of this type of processing is unknown.[4] Until recently, structure–function relationship studies of pCRAMOLL 1 were limited in this respect because no heterologous expression system was able to carry out this circular permutation. Our group recently surmounted this challenge by cloning and expressing synthetic DNA with the primary sequence of the mature lectin to produce reasonable amounts of a soluble, active protein called rCRAMOLL 1.[18] The recombinant protein shares the biophysical features (molecular mass, charge density, sugar recognition, and secondary and tertiary structures) of its plant-derived counterpart but is not fragmented. rCRAMOLL 1 thus constitutes a homogeneous population of intact monomers, which can also form dimers and tetramers depending on the pH at which they are incubated. At pH 7.0, both r and pCRAMOLL 1 are tetramers. However, 100% of pCRAMOLL 1 is converted into dimers at pH 5.0, while rCRAMOLL 1 becomes 100% dimeric only at pH 4.2, suggesting that the cleavage present in some units of pCRAMOLL 1 weakens tetramer stability.[18]

In the present study, the potential impact of fragmentation on the stability of CRAMOLL 1 was investigated in depth by comparing the urea-induced dissociation and unfolding of tetrameric and dimeric p and rCRAMOLL 1. Spectroscopic techniques (intrinsic and extrinsic fluorescence and circular dichroism) in combination with size-exclusion chromatography and cross-linking studies revealed that while the dimer and tetramer of pCRAMOLL 1 unfold via a compact monomeric intermediate, rCRAMOLL 1 tetramers have a higher stability and unfold without the formation of this monomeric species. On the other hand, the rCRAMOLL 1 dimer dissociates and unfolds like its dimeric plant counterpart, suggesting that there are only differences between the dimer–dimer interfaces of each CRAMOLL 1 and that these make the stability of the two tetramers quite different.

Evaluating the contribution of each specific residue in the crystal oligomeric interfaces of nonfragmented pCRAMOLL 1, we found that Asn_{118}, when covalently bonded to Ser_{119} by the circular permutation process, makes crucial hydrogen bonds and other noncovalent contacts that stabilize the canonical tetramers and that it does not participate in any interactions at monomer–monomer interfaces. Thus, it would be expected that in the subunits where Asn_{118} is not bonded to Ser_{119} several important contacts are not established, rendering the fragmented assemblies less stable. This finding supports our thermodynamic data and leads us to suggest that while dimers of CRAMOLL 1 remain insensitive to the presence of natural fragmentation, tetramers are profoundly affected by this post-translational event. The importance of this fragmentation to plant seeds is discussed.

EXPERIMENTAL PROCEDURES

Purification and Quantification. pCRAMOLL 1 was purified as described.[5] Briefly, an extract of *C. mollis* seeds was separated into two fractions with ammonium sulfate at 0–40% and 40–60% saturation. The 40–60% fraction was dialyzed against 0.15 M NaCl (F2) overnight at 4–6 °C and affinity chromatographed on Sephadex G-75 (GE Healthcare) equilibrated with 0.15 M NaCl. Elution was performed with 0.4 M D-glucose, and the protein was dialyzed against 10 mM sodium citrate phosphate buffer, pH 5.5 (F3). F3 was chromatographed on CM-Cellulose (Sigma-Aldrich) equilibrated with 10 mM sodium citrate phosphate buffer, pH 5.5, and eluted with a 0–0.4 M NaCl linear gradient. rCRAMOLL 1

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was prepared as recently shown by our group;[18] the bacterial extract was applied to a column containing Sephadex G-75 matrix equilibrated with 0.15 M NaCl. After washing off nonadsorbed proteins, the recombinant lectin was specifically eluted with a 0.4 M D-glucose solution. The purity of both lectins was checked by SDS-PAGE and native PAGE for basic proteins,[19,20] and the protein concentration was determined spectrophotometrically at 280 nm using a ε of 33 920 for the monomer of molecular mass of 25 371 Da, as predicted by the Protparam tool.[21,22]

Denaturation and Renaturation Experiments. The unfolding curves were performed by mixing the proteins (1, 5, and 10 μM, final concentration) with increasing concentrations of urea (0−8.5 M) at 25 °C over 20 h to reach equilibrium. The tetramer studies were performed at pH 7.2 (25 mM Tris-HCl, 0.1 M NaCl, pH 7.2, tetramer buffer) while those with the dimers were performed at pH 4.2 (10 mM MES, 0.1 M NaCl, pH 4.2, dimer buffer), where both proteins were 100% tetrameric or dimeric, respectively.[18] To check reversibility, samples incubated at 8.5 M urea for 20 h were dialyzed against urea-free buffers (pH 7.2 or 4.2) at room temperature for 20 h.

Spectroscopic Measurements. The tryptophan and bis-ANS fluorescence spectra were recorded using a Jasco FP-6300 spectrofluorimeter (Jasco Corp., Tokyo, Japan). Tryptophan emission spectra were obtained by setting the excitation wavelength at 280 nm and collecting emission in the 300−400 nm range. These spectra were quantified as the center of spectral mass (ν) according to eq 1:

$$(\nu) = \sum \nu_i F_i / \sum F_i \quad (1)$$

where F_i stands for the fluorescence emission at wavelength ν_i and the summation is carried out over the range of appreciable values of F.[23,24]

The degree of dissociation/denaturation (α) is related to (ν) by eq 2:

$$\alpha = [(\nu_u) - (\nu_i)]/[(\nu_f) - (\nu_i)] \quad (2)$$

where (ν_i) and (ν_f) are the initial and final values of the center of spectral mass, respectively, while (ν_u) is the center of spectral mass in the presence of a given concentration of urea.

The bis-ANS spectra were recorded by exciting the sample at 360 nm and collecting emission from 400 to 600 nm. Bis-ANS binding was evaluated by area of fluorescence intensity in arbitrary units.

Circular dichroism (CD) measurements were performed using a Jasco-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) using a 1.0 mm path-length quartz cuvette. Data were averaged for 10 scans collected at a speed of 100 nm/min with 0.1 nm steps. The baselines (buffer alone) were subtracted from each spectrum.

Size-Exclusion Chromatography. To determine the size of the species of p and rCRAMOLL 1 formed in the presence of urea, size-exclusion chromatography on a Superdex 75 10/300 GL (GE Healthcare) column was performed using a Shimadzu SPD-10A HPLC coupled with a fluorescence detector. Samples of both lectins (1 μM) were prepared as described above. For kinetic measurements, proteins (1 μM) were diluted at pH 7.2 in tetramer buffer containing 4 M urea and incubated over a range of times at 25 °C. Before each injection, the column was equilibrated with dimer or tetramer buffer containing appropriate amounts of urea and 0.4 M D-glucose to avoid interaction of the lectins with the column matrix. The elution profile was monitored by fluorescence emission at 320 nm (excitation at 280 nm).

Cross-Linking Experiments. The oligomeric state of the lectins during denaturation with urea at pH 7.2 was also determined by cross-linking with glutaraldehyde followed by SDS-PAGE.[25] For glutaraldehyde treatment, reaction mixtures with 1 μM of tetrameric lectins in 20 mM phosphate buffer (pH 7.2) in a total volume of 50 μL were treated with glutaraldehyde (0.25% final concentration) for 30 s at 25 °C. The reactions were terminated by addition of Tris-HCl, pH 8.8 (5 μL, 1.6 M). Cross-linked proteins were evaluated by 10% SDS-PAGE as described[19] and stained with silver nitrate.

RESULTS

The urea-induced unfolding of tetrameric and dimeric forms of p and rCRAMOLL 1 at 25 °C was studied by means of several complementary techniques (fluorescence, circular dichroism (CD), size-exclusion chromatography (SEC), and chemical cross-linking followed by SDS-PAGE). At pH 4.2, both p and rCRAMOLL 1 exist in a dimeric state, while at pH 7.2 both proteins are tetrameric.[6,18]

It must be emphasized that pCRAMOLL 1 (as determined by densitometry) is composed of ∼60% nonfragmented monomers mixed with fragmented ones (see SDS-PAGE in Figure 1). As pointed out many years ago by Edelman and co-workers,[26] in concanavalin A preparations there is subunit exchange among assemblies. Thus, since ∼40% of the subunits of pCRAMOLL 1 are fragmented (i.e., about 1.6 monomers per tetramer), we infer that all tetramers have at least one fragmented monomer under equilibrium condition. We investigated the role of this fragmentation in the stability of this lectin.

Urea-Induced Unfolding of Tetrameric p and rCRAMOLL 1. The dissociation and unfolding of the tetramers of p and rCRAMOLL 1 were investigated at pH 7.2. Figure 2A shows the changes in the center of spectral mass (CM) of tryptophan fluorescence emission that take place when 1 μM of p (hollow squares) or rCRAMOLL 1 (filled squares) was incubated in the presence of increasing concentrations of urea at 25 °C for 20 h. CRAMOLL 1 has four tryptophan residues, and the analysis of the three-dimensional crystal structure of pCRAMOLL 1 monomer (PDB ID: 1MVQ) clearly indicates that two of the four tryptophan residues (40 and 109) are located in the protein core.[6,18] It is interesting to note that none of those tryptophan residues are located at the oligomeric interfaces.

The emission spectra of the tryptophan residues of p and rCRAMOLL 1 in the native tetramer are blue-shifted, with a CM of 339 nm. As seen in Figure 2A, there was a progressive shift in the maximum emission to the red (∼351 nm) for both proteins as the concentration of urea was increased to 8.5 M. This change is compatible with the exposure of the tryptophan residues to the aqueous environment as a consequence of the dissociation and denaturation of the proteins induced by urea (Figure 2A). Both curves were virtually identical, exhibiting a sigmoidal shape with a denaturation midpoint around 4.7 M urea. The inset shows as an example the fluorescence emission spectra of rCRAMOLL 1 in the absence (solid line) or presence of 4 (dashed line) and 8.5 M urea (dotted line); the shift to the red of the fluorescence emission in the presence of

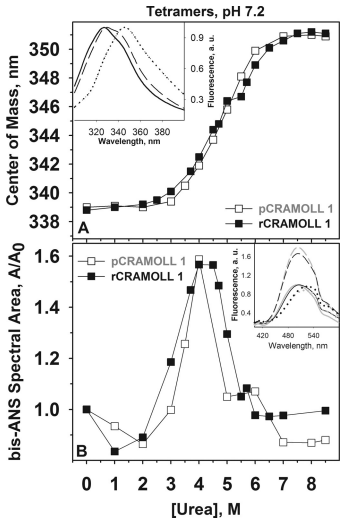

Figure 2. Urea denaturation curves of tetrameric p and rCRAMOLL 1 (open and filled squares, respectively) by means of (A) displacement in the center of spectral mass of tryptophan emission and (B) change in bis-ANS fluorescence spectral area. The measurements were performed after incubation of lectins (1 μM) in 25 mM Tris-HCl buffer (pH 7.2) containing 100 mM NaCl at various urea concentrations at 25 °C for 20 h. After recording intrinsic fluorescence (Exc: 280 nm; Em: 300−400 nm), bis-ANS (10 μM) was added and recorded (Exc: 360 nm; Em: 400−600 nm). Inset: (A) tryptophan and (B) bis-ANS fluorescence emission spectra of p (gray) and rCRAMOLL 1 (black) in the absence of urea, or at 4 and 8.5 M urea (solid, dashed and dotted lines, respectively).

urea is visible. The spectra of pCRAMOLL 1 were identical to those observed with rCRAMOLL 1 (not shown). The reversibility of the denaturation process for both tetramers was confirmed by the complete recovery of the initial value of CM after dialysis (not shown).

Bis-ANS is a suitable fluorescent probe to map partially unfolded states present during the unfolding pathway of several proteins.[27−30] When bound to species that expose hydrophobic patches to the aqueous environment, its fluorescence emission increases considerably. Emission decreases to very low values in the presence of completely unfolded proteins because the probe dissociates into the bulk solution. Tetramers of p and rCRAMOLL 1 bound some bis-ANS in their native state (Figure 2B, and inset solid line spectra), probably on the specific hydrophobic binding site previously observed in other legume lectins.[31] Interestingly, upon addition of urea, both proteins exhibited an enhanced capacity to bind this probe; this enhancement was maximal at 3.5−4.5 M urea (Figure 2B and inset dashed spectra) and decreased to lower values as the concentration of urea increased to 7−8.5 M (Figure 2B and inset dotted spectra).

These findings suggest that an intermediate, partially unfolded species binds bis-ANS in the urea-driven unfolding pathways of p and rCRAMOLL 1 at neutral pH. This intermediate species was not evident when tryptophan fluorescence emission was used as a sensor of the unfolding process. The tryptophan emission data (Figure 2A) generated denaturation curves that instead suggest a two-state unfolding process.

Circular dichroism (CD) measurements were performed to elucidate the secondary structural changes that take place upon urea-induced unfolding of p and r CRAMOLL 1 (Figure 3). The inset of Figure 3 shows the far-UV CD spectra of p (gray lines) and rCRAMOLL 1 (black lines) in the absence (solid

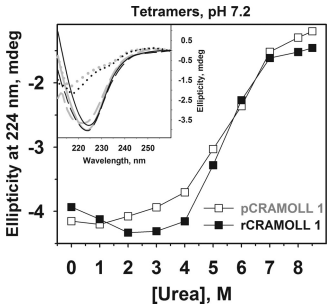

Figure 3. Raw ellipticity values of the tetrameric p and rCRAMOLL 1 at 224 nm (open and filled squares, respectively) in the presence of different concentrations of urea (0−8.5 M). The measurements were performed after incubation of the lectins (5 μM) in 25 mM Tris-HCl buffer (pH 7.2) containing 100 mM NaCl at various urea concentrations at 25 °C for 20 h. Spectra were taken in 1 mm path-length cells using a scan speed of 100 nm min^{-1} and averaged over ten scans. Inset: far-UV CD spectra of p (gray) and rCRAMOLL 1 (black) in the absence of urea, or at 4 and 8.5 M urea (solid, dashed and dotted lines, respectively).

lines) or presence of 4 M (dashed lines) and 8.5 M urea (dotted lines) at pH 7.2. The spectra of the native, tetrameric proteins showed a single negative band at ∼224 nm that is characteristic of β-sheet rich proteins, the secondary-structure hallmark of a typical legume lectin.[4] Figure 3 shows a progressive decrease in the ellipticity at 224 nm as a function of urea addition, which is compatible with a loss of secondary structure. Far UV-CD spectra of the two proteins were nearly identical.

It is interesting to note that up to 4 M urea, a concentration where bis-ANS binding was maximum (Figure 2B), only slight changes in the secondary structure of both p and rCRAMOLL 1 took place. This suggests that in the presence of 4 M urea p and rCRAMOLL 1 retained most, if not all, of their secondary structure, while the tertiary structure was compromised, resulting in partial exposure of the tryptophan residues to the aqueous environment with a concomitant enhancement in bis-ANS binding (Figure 2A,B). At 8.5 M urea, there was an intensive loss of secondary structure of both lectins, suggesting massive denaturation.

The dissociation of oligomeric proteins such as CRAMOLL 1 is expected to be dependent on protein concentration,[32] manifested by displacement of dissociation curves to higher urea concentrations as the protein concentration increases. To investigate whether the reaction under question was the dissociation of tetramers of p and rCRAMOLL 1 into monomers or the denaturation of the monomers within the tetramer (a process that is expected to be concentration independent), we performed urea denaturation curves at 1, 5, and 10 μM p and rCRAMOLL 1, and the changes in CM were evaluated (Figure 4). Curiously, p and rCRAMOLL 1 responded differently to the protein concentration-dependence assays. As seen in Figure 4, while pCRAMOLL 1 (Figure 4A) presented the expected concentration dependence, rCRAMOLL 1 did not (Figure 4B) and the three curves superimposed perfectly. This behavior suggests that tetramers of p and rCRAMOLL 1 dissociate and unfold by different pathways upon urea addition. The lack of protein-concentration

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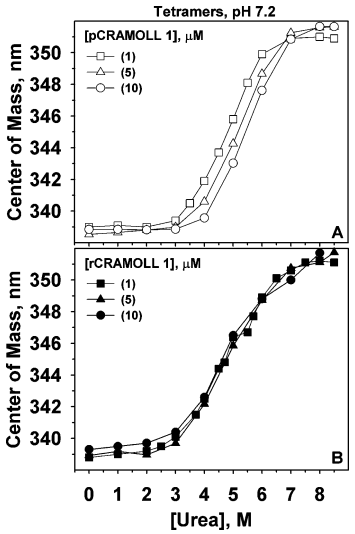

Figure 4. Protein-concentration dependence of urea-induced transitions of tetrameric p (A) and rCRAMOLL 1 (B) evaluated by changes in the center of spectral mass of tryptophan emission. The measurements were performed after incubation of 1, 5, and 10 μM (squares, triangles, and circles, respectively) of the two lectins in 25 mM Tris-HCl buffer (pH 7.2) containing 100 mM NaCl at various urea concentrations at 25 °C for 20 h. Note the absence of concentration dependence in the case of rCRAMOLL 1.

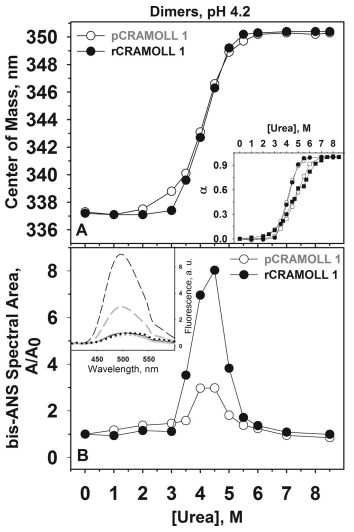

Figure 5. Urea denaturation curves of dimeric p and rCRAMOLL 1 (hollow and filled circles, respectively) by means of (A) displacement in the center of mass of tryptophan emission and (B) relative change of bis-ANS fluorescence spectral area. The measurements were performed after incubation of the lectins (1 μM) in 10 mM MES buffer (pH 4.2) containing 100 mM NaCl at various urea concentrations at 25 °C for 20 h. After recording intrinsic fluorescence (Exc: 280 nm; Em: 300−400 nm), bis-ANS (10 μM) was added and its emission spectrum recorded (Exc: 360 nm; Em: 400−600 nm). Inset: (A) an overlay of the urea denaturation curves of the dimeric (circles) and tetrameric (squares) p and rCRAMOLL 1 at 1 μM, as determined by center of mass of tryptophan emission (pCRAMOLL 1 is in hollow symbols). The data are displayed as fraction unfolded (α), determined as described in eq 2. (B) bis-ANS fluorescence intensity (raw data) in the absence of urea, or at 4 and 8.5 M urea (solid, dashed and dotted lines, respectively). The data from pCRAMOLL 1 are in gray.

dependence in the dissociation process of the tetramers of rCRAMOLL 1 led us to suspect that these assemblies were probably denaturing directly from a tetrameric state, while those of pCRAMOLL 1 were dissociating (probably into monomers) before unfolding.

Urea-Induced Unfolding of Dimeric p and rCRAMOLL 1. At pH 4.2, CRAMOLL 1 exists as a homogeneous population of dimers,[18] which allows us to investigate the dimer ↔ monomer equilibrium more accurately. Figures 5A and 5B show respectively the changes in the CM of tryptophan emission and bis-ANS binding of p (hollow circles) and rCRAMOLL 1 (filled circles) as a function of urea addition (20 h; 25 °C). As shown in Figure 5A, the CM of both lectins as a dimer was 337 nm in the native state; this value shifted to ∼351 nm in the presence of 8.5 M urea, suggesting denaturation of both proteins. Again, up to 3 M urea there was no change in the tertiary structure of the dimeric proteins, as was also observed with the tetramers. Both proteins presented the same stability (transition midpoint at 4.1 M urea). The inset of Figure 5A shows that the dimers (circles) and tetramers (squares) behaved similarly up to 3 M urea, but at greater concentrations of urea the dimers changed more steeply than the tetramers ones, indicating that the species formed at this point were less stable at pH 4.2. The unfolding reactions carried out under acidic conditions for the dimeric species were completely reversible, too (not shown).

Bis-ANS binding studies (Figure 5B) suggested the formation of an intermediate species during the unfolding process of p and rCRAMOLL 1 in the presence of 4−4.5 M urea for both proteins. However, the intermediate species of rCRAMOLL 1 (filled circles) bound much more bis-ANS at these urea concentrations than the intermediate species of pCRAMOLL 1 (hollow circles). It is possible that the fragmentation in some monomers of pCRAMOLL 1 reduces the formation of this intermediate species because when split monomers are released and further denatured by urea, the fragments separate, limiting the formation of the partially unfolded monomers.

The changes in the secondary structure at pH 4.2 for p and rCRAMOLL 1 as a function of urea addition are shown in Figures 6A and 6B, respectively. The dimers of both lectins preserved the β-sheets, presenting a minimum around 222 nm (see the spectra in the absence of urea). Interestingly, increasing the concentration of urea to 4 M caused a progressive increase in the secondary structure content of rCRAMOLL 1 (panel B, inset). In the case of pCRAMOLL 1, this gain in secondary structure in this urea range was much more subtle (panel A, inset). As shown before, bis-ANS binding was maximal at 4 M urea, reinforcing the idea of an intermediate species that retains or even increases its secondary structure content.

Monitoring the Dissociation of p and rCRAMOLL 1 at pH 7.2 and 4.2 by SEC Experiments. Because we did not observe any protein concentration dependence in the dissociation of the tetramers composed of rCRAMOLL 1, we took advantage of SEC experiments to unravel the oligomeric state of the species populated in the presence of different urea concentrations at pH 7.2 (Figure 7) and 4.2 (Figure 8). The same experiments were performed with pCRAMOLL 1 for comparison. It must be emphasized that the proteins were injected onto a column previously equilibrated with the same urea concentrations used during the incubation of the proteins.

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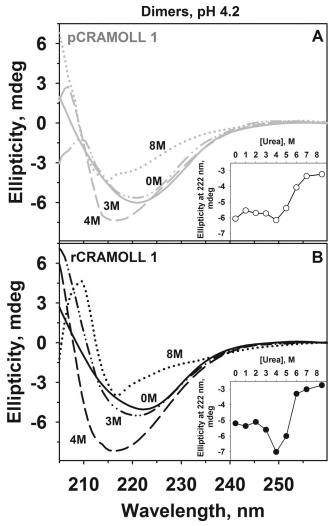

Figure 6. Far-UV circular dichroism spectra of dimeric pCRAMOLL 1 (A) and rCRAMOLL 1 (B) (5 μM) at 25 °C in MES buffer (pH 4.2) in the absence of denaturant (solid lines) or at 3 M (dashed dotted lines), 4 M (dashed lines), or 8 M (dotted lines) urea. Spectra were taken in 1 mm path-length cells using a scan speed of 100 nm min^{-1} and averaged over ten scans. The insets show the changes in ellipticity at 222 nm as a function of urea addition.

The column buffers also contained 400 mM glucose to avoid interaction of the proteins with the column matrix.

The upper panel of Figure 7A shows the elution profile of the tetramers of p and rCRAMOLL 1 (1 μM, pH 7.2), which eluted as a single peak at 9.3 mL, compatible with a 100 kDa protein. (We show only the elution profile of pCRAMOLL 1, which is superimposable with that of rCRAMOLL 1.) After 20 h in the presence of 3 M urea (panel 3M), the majority of pCRAMOLL 1 (70%) eluted at 10.7 mL, compatible with the molecular weight of the monomer (25 kDa, dashed line). Another population eluted at 8.8 mL, close to the elution volume of native tetramers. Interestingly, at this concentration of urea, rCRAMOLL 1 eluted as a single peak at 8.8 mL (solid line), and the peak corresponding to monomers was not present. Although native tetramers of CRAMOLL 1 eluted at 9.3 mL, the species with a retention volume of 8.8 mL is probably a tetramer that is structurally altered by the presence of 3 M urea.

While pCRAMOLL 1 formed a homogeneous monomeric species (10.7 mL, dashed line) in the presence of 4 M urea (panel 4M), this species was still all but absent from the chromatogram of rCRAMOLL 1 incubated at this urea concentration (solid line) or even at 5 or 6 M urea (see the corresponding panels). We conclude from these data that the monomeric species that formed during the unfolding pathway of pCRAMOLL 1 was not populated in the unfolding process of rCRAMOLL 1, suggesting they undergo different processes of dissociation and unfolding, and that fragmentation likely dictates these differences.

Nevertheless, it must be noted that in the presence of 6 M urea (lower panel of Figure 7A) a peak at 7.9 mL appears in addition to the original peak corresponding to the monomer of pCRAMOLL 1 that eluted at 10.7 mL in the presence of 3, 4, and 5 M urea. Although we are not sure of the precise nature of this peak, we hypothesize that it could be the expanded, unfolded monomer. Curiously, rCRAMOLL 1 incubated in the

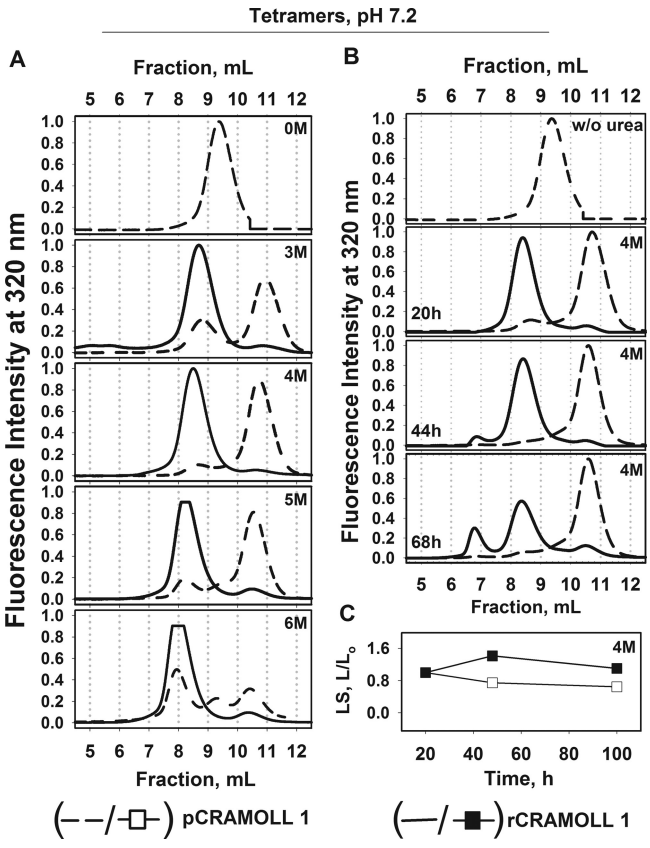

Figure 7. Molecularity of p and rCRAMOLL 1 at pH 7.2 in selected urea concentrations addressed by size exclusion HPLC elution profiles. (A) Protein samples (1 μM) were incubated with different concentrations of urea at 25 °C for 20 h. (B) Protein samples (1 μM) were incubated with 4 M urea at 25 °C for 20−188 h. In both cases, a 250 μL aliquot was chromatographed on a Superdex 75 10/300 GL column pre-equilibrated with the mentioned concentration of urea in 25 mM Tris-HCl, 100 mM NaCl, pH 7.2 containing 400 mM glucose. Plant and recombinant lectins are denoted by dashed and solid lines, respectively. (C) Changes in the light scattering (LS) in the presence of 4 M urea over time (filled squares = rCRAMOLL 1 and hollow squares = pCRAMOLL 1). Other settings are described in the Experimental Procedures.

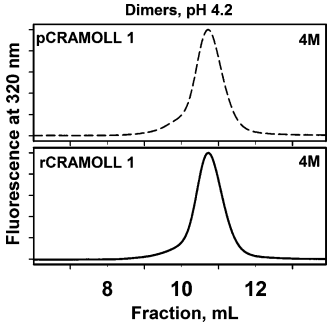

Figure 8. Size exclusion HPLC elution profiles of p (top) and rCRAMOLL 1 (bottom) in the presence of 4 M urea at pH 4.2. Protein samples (1 μM) were incubated with 4 M urea at 25 °C for 20 h, and a 250 μL aliquot was chromatographed on a Superdex 75 10/300 GL column pre-equilibrated with 4 M urea in 10 mM MES buffer (pH 4.2), 100 mM NaCl, containing 400 mM glucose.

presence of 6 M urea eluted as a single peak exactly at 7.9 mL, which suggests that at this high urea concentration the

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recombinant protein also forms this unstructured monomer. Taken together, it seems that rCRAMOLL 1 initially denatures as a tetramer (up to 5 M urea), followed by the release of denatured monomers from the tetramer to give completely unfolded, monomeric species. The data presented in Figure 9 give further support to this hypothesis.

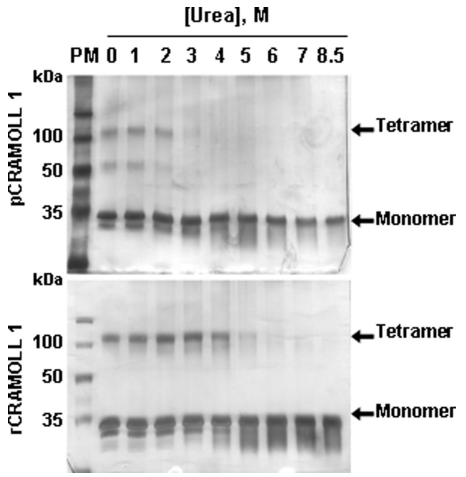

Figure 9. SDS-PAGE of p (top) and rCRAMOLL 1 (bottom) after covalent cross-linking in the presence of various concentrations of urea at pH 7.2. Lectin samples (1 μM) were preincubated 20 h at the indicated urea concentrations and then mixed with glutaraldehyde as described in Experimental Procedures. Proteins were dissolved in SDS sample buffer and electrophoresed on a 10% acrylamide SDS slab gel. Standard markers (lane 1); CRAMOLL 1 after cross-linking without urea (lane 2). Lectins incubated in urea at 1, 2, 3, 4, 5, 6, 7, and 8.5 M (lanes 3−10, respectively) after cross-linking. Note the persistence of tetramers up to 4−5 M urea in the rCRAMOLL, while 3 M urea was sufficient to completely dissociate the tetramers of pCRAMOLL 1.

Next, we tested whether the tetramer ↔ monomer transition of rCRAMOLL 1 would achieve equilibrium more slowly in the presence of urea. We incubated both lectins in the presence of 4 M urea, and aliquots were injected onto the column pre-equilibrated with 4 M urea (Figure 7B). While the plant lectin formed monomers after 20 h in the presence of 4 M urea (panel 20 h, dashed lines) with no further major change in its elution profile after longer incubation times (see the other panels), the recombinant protein never eluted as a typical monomer (10.7 mL), even after 68 h in the presence of 4 M urea; its retention volume was compatible with the altered tetramer mass (8.8 mL, solid lines). After 44 h, this peak decreased, giving rising to a species, most likely aggregated, that eluted at 6.8 mL.

Time-elapsed Rayleigh light scattering was used to determine whether rCRAMOLL 1 formed aggregates in the presence of 4 M urea (Figure 7C). There was no change in scattering signal at 320 nm over 100 h of incubation in the presence of 4 M urea, indicating that no massive aggregation of the protein was taking place.

The dissociation of the dimers present at pH 4.2 was also investigated by SEC (Figure 8). Dimers of p and rCRAMOLL 1 eluted as a single peak at 10.7 mL when incubated in the presence of 4 M urea for 20 h. This elution volume is compatible with a 25 kDa monomer (see also Figure 7A, panel 4M). This result suggests that starting with the dimers both lectins followed the same urea dissociation−unfolding pathway: in a first step (e.g., 4 M urea), the dimers dissociated into a partially unfolded monomeric intermediate that bound bis-ANS (Figure 5B), began to expose tryptophan residues to the solvent (Figure 5A), and exhibited an increased content of secondary structure (Figure 6); at higher concentrations of urea this species completely denatured.

Intermediate Species Populated during the Unfolding of rCRAMOLL 1 Was a Nonnative Tetramer: Evidence from Cross-Linking Experiments. The data presented thus far suggest that tetramers of p and rCRAMOLL 1 followed different dissociation and unfolding pathways. While tetramers of the plant lectin dissociated into a partially unfolded monomer at 4 M urea, the tetramer from the recombinant protein at this concentration remained tetrameric, but with altered structure.

Cross-linking experiments were performed at different urea concentrations using the bifunctional agent glutaraldehyde to better understand these different pathways. We attempted to create a snapshot of the species present at each urea concentration to complement the SEC experiments. Figure 9 shows two SDS-PAGE runs in which p and rCRAMOLL 1 (top and bottom, respectively) were incubated at increasing concentrations of urea (from 0 to 8.5 M) at pH 7.2 and 25 °C for 20 h before being cross-linked with glutaraldehyde. As seen, in the absence of urea (lane 0 M), a population of intact tetramers was cross-linked and thus resistant to SDS (see arrow). However, the majority of proteins appeared as monomer because they were not cross-linked (interchain cross-linking, see arrow). In this experiment we did not see the bands related to the fragments of pCRAMOLL 1, probably because they were cross-linked (intrachain), mainly in the presence of low urea concentration, where the monomers are not completely unfolded.

No major differences were detected in the gels as urea concentration increased to 2 M (lanes 1 and 2 M). In the presence of 3 M urea (lane 3 M urea), tetramers of pCRAMOLL 1 almost completely disappeared. This result is consistent with SEC data (Figure 7A, panel 3M). rCRAMOLL 1 in the presence of 3 or 4 M urea (lanes 3 and 4 M, lower gel) remained in a tetrameric form, again confirming SEC data (Figure 7A, panels 3 and 4M). From 5 to 8.5 M urea, the tetramer of rCRAMOLL 1 gradually vanished from the gel (see arrow in lower gel), reinforcing the previous hypothesis that tetramers of the recombinant protein were being dissociated only at these high urea concentrations.

DISCUSSION

Subunit association plays an important role in stabilizing the structure of large proteins and in generating new functions, ligand-binding sites, or sites for regulation.[33−35] Acidification converts most legume lectins from native tetramers into dimers; CRAMOLL 1 responds to pH changes in the same way. This feature is of interest because important biological activities displayed by lectins, such as mitogenic stimulation, are dependent on their oligomeric state.[36−39]

In general, the unfolding of oligomeric proteins first requires the disruption of interchain interactions and then the breakage of intrasubunit contacts within those monomeric units.[40] The type and number of bonds that hold the oligomer and the folded monomer in their native form govern the degree of cooperativity, the mechanism of unfolding, and the overall stability of the oligomeric species.[41,42] Legume lectins serve as

an excellent model for the study of protein folding and oligomerization. They share at least 35% sequence identity and have similar secondary and tertiary structures, yet differ in their modes of oligomerization, and hence are correctly described as "natural mutants" of quaternary structure.[43] As mentioned, changes in pH allow the conversion of native tetramers into native dimers, as in the case of CRAMOLL 1.

In the present work, we have studied the urea-induced dissociation and unfolding process of p and rCRAMOLL 1 at two different pH values using fluorescence, circular dichroism, gel filtration chromatography, and chemical cross-linking. As previously shown by our group,[6,18] CRAMOLL 1 extracted from plants forms dimers or tetramers composed of both intact (60%) and cleaved (40%) monomers, a process that takes place during the post-translational processing of the lectin in the plant cell. The role of the lectins in plant seeds is controversial, as is the impact of this partial proteolytic process on lectin function and stability. Recently, we successfully cloned and expressed CRAMOLL 1 in a soluble, active form that is able to agglutinate red cells and trypanosomatids.[18] As expected, the monomer of this recombinant protein is intact and produces dimers and tetramers composed of nonproteolytically cleaved chains. This allowed us to compare the stability of naturally fragmented lectin extracted from the plant seeds with that displayed by a homogeneous population of full-length recombinant protein.

Our data showed that tetrameric p and rCRAMOLL 1 dissociate and unfold through different intermediate species, depicted in Figure 10. We chose the concentration of 4 M urea to show the species we think are populated during the unfolding processes investigated here. In the case of pCRAMOLL 1 (upper panel) in the presence of 4 M urea, tetramers (composed of intact and fragmented monomers) dissociate into partially unfolded monomers that begin to expose tryptophan residues, retain secondary structure, bind bis-ANS, and elute at 10.7 mL. On the other hand, in the presence of 4 M urea the tetramer of rCRAMOLL 1 (composed of intact monomers, lower panel) first forms partially unfolded tetramers that begin to expose tryptophan residues, retain secondary structure, bind bis-ANS, and elute at 8.8 mL. Both species completely unfold at 8.5 M urea.

The absence of monomers in the dissociation process of rCRAMOLL 1 was confirmed by a lack of protein concentration dependence in its dissociation process (Figure 4B) as well as SEC experiments (Figure 7A, solid lines) and SDS-PAGE after glutaraldehyde treatment (Figure 9, bottom). We wondered whether the lack of perceptible monomers depended on limited incubation time in the presence of 4 M urea. However, even when rCRAMOLL 1 was incubated for 68 h in the presence of 4 M urea, tetrameric rCRAMOLL 1 was not converted into monomers (Figure 7B, solid lines), suggesting that this species is not populated at this urea concentration.

Starting with the dimer species, the dissociation into monomers seems to follow the same pathway for both lectins (Figure 10). At 4 M urea, both proteins were converted into partially unfolded monomers (elution volume of 10.7 mL, tryptophan residues partially exposed, maximum bis-ANS binding, and gain in secondary structure that was more drastic for rCRAMOLL 1).

Is it possible that the absence of a single peptide bond (Asn_{118}–Ser_{119} bond) changes the stability of the tetramers

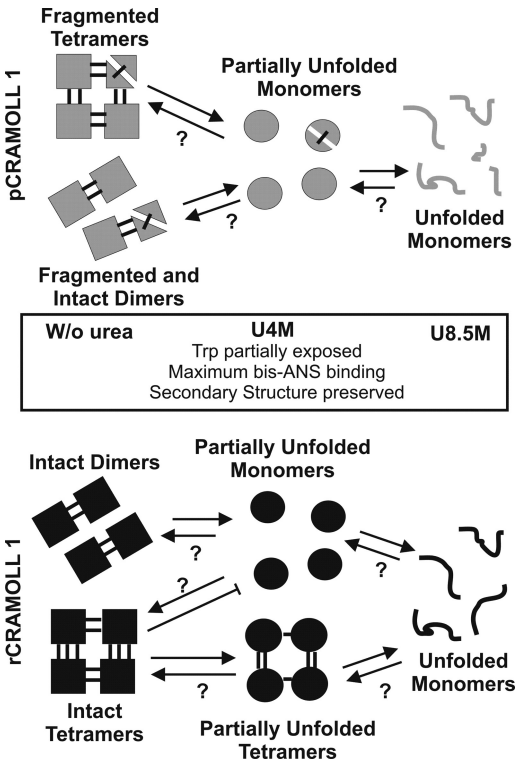

Figure 10. Schematic summary of the dissociation/unfolding pathways of dimers and tetramers of CRAMOLL 1. The dimers and tetramers of the plant lectin are composed of a mixture of intact and fragmented subunits, while the recombinant lectin is composed only of intact subunits. Starting with the dimers, the two lectins in the presence of 4 M urea dissociate into partially unfolded monomers, which are subsequently unfolded in the presence of 8.5 M urea. However, the tetramers dissociate–unfold by different mechanisms: while the tetramer of the plant lectin in the presence of 4 M urea dissociates into partially unfolded monomers, the recombinant tetramer forms initially a partially denatured tetramer, which only releases unfolded monomer in the presence of higher urea concentrations. The question marks indicate that the reversibility of these specific reactions was not examined step by step.

and thus the unfolding pathways of p and rCRAMOLL 1? In order to find an answer to this question, we analyzed all the contacts established at both dimeric and tetrameric interfaces in the nonfragmented crystallized form of pCRAMOLL 1 (PDB ID: 1MVQ) using the PDBsum database[44] and PISA[45] for calculating the salt bridges (both from European Bioinformatics Institute) (Figure 11). Figure 11C depicts all contacts established between the monomers A and B (or C and D) to form a dimer (Figure 11A). Dimers are stabilized by 12 H-bonds and 127 nonbonded contacts (van der Waals and hydrophobic), and neither Asn_{118} nor Ser_{119} (the site of circular permutation) makes any contact at the dimeric interface. Thus, the absence of the Asn_{118}–Ser_{119} bond is not expected to compromise the stability of dimers. This explains why dimers of p and rCRAMOLL 1 dissociate and unfold through the same pathway.

Under conditions that promote approximation of two dimers to form the tetramer (Figure 11A), the residue Asn_{118}, absolutely conserved in legume lectins that adopt the ConA-type tetrameric association,[46] which is located at the dimer–dimer interface (Figure 11B, located at red line loops within the boxes), participates strongly in tetramer stability. The four

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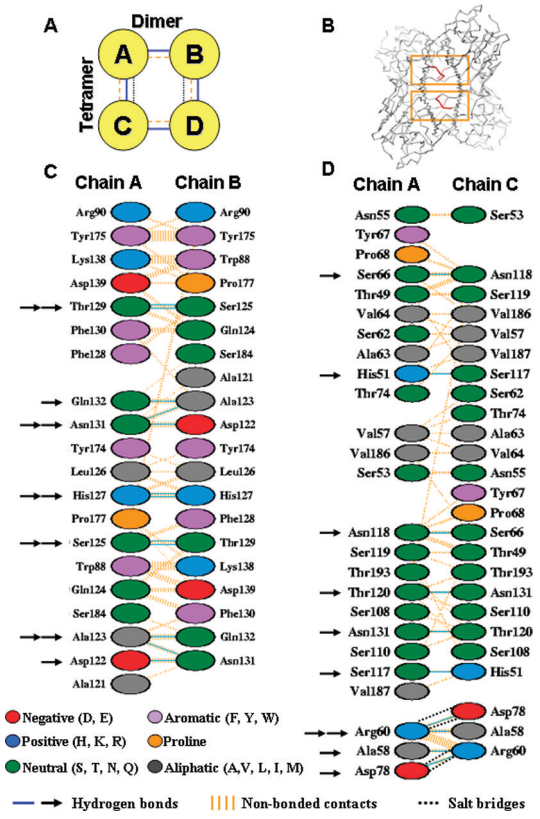

Figure 11. Noncovalent stabilizing interactions between atoms in oligomeric assemblies of intact pCRAMOLL 1. (A) Schematic arrangement of the four identical protomers (A, B, C, and D); (B) crystal structure of pCRAMOLL 1 (Cα view) and spatial disposition of central loops (red line loops within the boxes) that contain Asn_{118}–Ser_{119} peptide bonds at the tetrameric interface. Residues involved in atomic contacts at (C) dimeric and (D) tetrameric interfaces. The hydrogen bonds are indicated by boldface lines and arrows and salt bridges by dotted lines. For nonbonded contacts, which can be plentiful, the width of the striped line is proportional to the number of atomic contacts. Amino acid residues are colored as neutral (green), aliphatic (gray), aromatic (purple), positive (blue), negative (red), and proline (orange). Calculations were performed using the PDBsum and the PISA database[44,45] using the pCRAMOLL 1 PDB file (ID: 1MVQ) after symmetry operations were carried out as described.[6]

Asn_{118} residues (one from each monomer) make 76 out of the 248 nonbonded contacts (ranging in length from 2.74 to 3.84 Å) and 4 out of 20 hydrogen bonds (each ∼2.74 Å in length) (Figure 11D). Thus, it is clear how important the contacts established by Asn_{118} are for tetramer stability. Only Arg_{60} contributes as effectively to tetramer stability. Also, structural studies performed by other groups have shown that the organization of central loops (residues 117–120 in each subunit; Figure 11B, red line loops within the boxes) are strongly implicated in stabilization of interdimer interface of the canonical pH-dependent tetramer of *C. floribunda* seed lectin (CFL), a lectin that has 95% identity with CRAMOLL 1.[47] When the Asn_{118}–Ser_{119} peptide bond is not formed, the side chain of Asn_{118} may be able to move from its original position, impeding the formation of contacts that are necessary to stabilize the tetramers. This would render a fraction of the tetramers of pCRAMOLL 1 less stable and able to form partially unfolded monomers at intermediate concentration of urea. The complete list of contacts established at the interfaces of the dimer and tetramer are found in Tables S1 and S2, respectively.

It has been demonstrated that glycosylation of soybean agglutinin (SBA) increases its stability. This plant legume lectin is tetrameric, and each subunit is glycosylated. As pointed out elsewhere,[48] this protein shows exceptionally high stability in terms of free energy of unfolding when compared to other proteins from the same family. This increase in stability is due to the glycan moiety because the recombinant, non-glycosylated protein is less stable. Interestingly, the two unfolding pathways are different. While the glycosylated form, which presents a greater number of interactions at the tetrameric interface (as does tetrameric rCRAMOLL 1), follows a two-state unfolding process, the non-glycosylated species unfolds via a compact monomeric intermediate.

In conclusion, we speculate that the absence of this specific peptide bond decreases tetramer stability considerably, making monomers more accessible. Although the precise role of lectins in the plant cell is not known at present, it has been postulated that this protein could serve as a source of nutrition for the growing seed embryo.[49] This decrease in stability would be very important in making monomers available to proteases. We thus suggest that fragmentation of pCRAMOLL 1 and probably of other Diocleinae leguminous lectins has an important role in avoiding the formation of this extremely tight tetramer. For this reason, it may modulate protein activity by affecting the oligomerization state of the lectin and therefore its sugar-binding capacities.

■ ASSOCIATED CONTENT

Supporting Information

Tables S1 and S2: list of interatomic contacts established in the monomer–monomer and dimer–dimer interfaces of the non-fragmented subunits of pCRAMOLL 1 (PDB ID: 1MVQ). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

ANS, 8-anilinonaphthalene-1-sulfonate; bis-ANS, bis(8-anilinonaphthalene-1-sulfonate); Man, mannose; MES, 4-morpholineethanesulfonic acid; pCRAMOLL 1, plant CRAMOLL 1 (extracted from *Cratylia mollis* seeds); rCRAMOLL 1, recombinant CRAMOLL 1 (expressed in *Escherichia coli* cells); Glc, glucose; SEC, size-exclusion chromatography.

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