RESULTS

AND

DISCUSSION
3. Results and discussion

3.1. Denaturation studies of tetrameric concanavalin A

Concanavalin A (ConA) is a Glc / Man-specific legume lectin, and exists as a tetramer at physiological pH. ConA was purified from jack bean seeds by affinity chromatography on Sephadex G-100, and a typical elution profile is shown in Figure 2A. The integrity of tetrameric structure of affinity purified native ConA at pH 7.2 was confirmed from size-exclusion chromatography on Bio-Gel P-100 column when the protein was eluted as a single peak (the elution volume corresponded to a tetrameric molecular mass of 102 kDa) (Figure 2B). The characteristics of solvent denaturation of tetrameric ConA was studied in presence of urea and guanidine hydrochloride (GdnHCl), the two reagents commonly employed as protein denaturants.

3.1.1. Unfolding of ConA at pH 7.2 as monitored by steady-state fluorescence

Steady-state fluorescence is a useful technique for studying the structure and dynamics of proteins (Pace 1986; Eftink 1991). The intrinsic fluorescence of proteins from Trp residues is an excellent built-in reporter (Creighton 1993). There are four Trp residues per subunit of ConA. The wide range of quantum yields (fluorescence intensities) and emission maxima of Trp residues in proteins are attributed to differences in the way the excited indole ring of Trp interacts with its microenvironment in different proteins. For example, a fluorescence red-shift occurs as the microenvironment surrounding Trp residues changes from nonpolar to polar (Burnstein 1973). The fluorescence spectra of ConA in varying concentrations of urea at pH 7.2 are shown in Figure 3A. At 0 M urea the protein exhibits emission maximum at 336 ± 1 nm.
Figure 2. (A) Elution profile from affinity chromatography of ConA on Sephadex G-100 column (3.5 x 24 cm) in PBS containing 0.1 mM Mn$^{2+}$ and 0.1 mM Ca$^{2+}$, pH 7.2. (B) Elution profile of ConA (3 mg) from Bio-Gel P-100 column (1.1 x 100 cm) at pH 7.2. Inset: Molecular weight calibration curve. The column was precalibrated with standard marker proteins (from left to right): bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa) and soybean trypsin inhibitor (20.1 kDa). The elution position of ConA is marked by arrow in the calibration curve and the calculated molecular mass is shown by arrow in the elution profile.
Figure 3. (A) Fluorescence spectra at 25°C of tetrameric ConA (2 μM) in 0 M (a), 2.7 M (b) and 8 M (c) urea in PBS. The spectra were corrected for the buffers containing requisite concentrations of urea. Excitation wavelength, 280 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min. (B) ANS fluorescence spectra of ConA in 0 M (a), 2.7 M (b) and 8 M (c) urea. ANS (50 μM) was present under the same conditions as for (A). Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min.
which gradually red-shifts with increasing concentration of urea, and finally levels off at 351 ± 1 nm in ≥ 6.6 M urea, indicative of protein denaturation and Trp exposure to the aqueous environment. The urea denaturation curve, in terms of relative change of emission maximum as a function of urea concentration, is shown in Figure 4A. The plot reveals two distinct transitions. A stable intermediate appears in 2.2-3.3 M urea with an emission maximum around 339 nm corresponding to ~ 20 % of emission maximum red-shift obtained for complete denaturation.

In order to study these transitions in more detail, we have performed the same experiment in presence of an external polarity-sensitive fluorescent probe, 8-anilino-1-naphthalenesulfonate (ANS), which binds nonspecifically to hydrophobic surfaces in many proteins, with enhancement of fluorescence intensity together with a blue shift of emission maximum (520 → 470 nm) (Slavik 1982). The ANS fluorescence spectra of ConA in different concentrations of urea at pH 7.2 are shown in Figure 3B. A plot of fluorescence intensity at 470 nm as a function of urea concentration (Figure 4B) shows that a significant increase in fluorescence intensity at 470 nm has been observed from 0.6 M urea to ~ 30-fold in 2.1-3.0 M urea, and then the fluorescence has decreased gradually to almost negligible intensity at high concentrations (≥ 6 M) of urea. The ANS fluorescence data thus correlate well with two distinct transitions as observed in the denaturation curve (Figure 4A), and suggest the presence of a hydrophobic equilibrium intermediate at low concentrations of urea.

There is usually a red shift in the emission of a protein upon denaturation, though the emission quantum yield (fluorescence intensity) may either increase or decrease. If the monitoring of the fluorescence intensity at the blue or red edge of the emission envelope gives a
Figure 4. (A) Urea denaturation curve of tetrameric ConA. Relative change of emission wavelength maximum was calculated on the basis of the shift of wavelength maximum (from that in the native state) at different concentrations of urea in PBS. Each data point represents average of three determinations. The protein concentration was 2 μM. (B) ANS fluorescence intensity at 470 nm at various concentrations of urea. ANS (50 μM) was present under the same conditions as for (A). Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min.
Figure 5. The denaturation equilibrium transitions of tetrameric ConA measured in terms of the relative change in fluorescence intensity at 336 nm as a function of urea concentration in PBS. Each data point represents average of three determinations. The protein concentration was 2 μM.
measurable signal change, that becomes the preferred measurable signal for estimating the thermodynamic parameters for denaturation of proteins (Eftink 1994). The relative change of fluorescence intensity at 336 nm as a function of urea concentration is shown in Figure 5. The fluorescence intensity increases to ~ 45 % in 2.1-3.1 M urea, and then decreases gradually to level off in ≥ 5.7 M urea to about the same intensity of the native protein in 0 M urea. This result, coupled with the previous ANS binding data (Figure 4B), further corroborates the evidences for an intermediate structure in the denaturation equilibrium of ConA. The fluorescence intensity data of ConA is, however, in sharp contrast with that observed for the denaturation of tetrameric soybean agglutinin (SBA), when an intermediate state exhibits similar relative intensity as of native lectin followed by an increase in fluorescence at higher concentrations of urea (Ghosh and Mandal 2001). This may be attributed to the differences in the environment of Trp residues in the structures of ConA and SBA.

The denaturation of ConA in GdnHCl exhibits similar characteristics as in urea. Figure 6A shows the fluorescence spectra of ConA in varying concentrations of GdnHCl at pH 7.2. The emission maximum gradually shifts from 336 ± 1 nm in 0 M GdnHCl to 351 ± 1 nm in ≥ 3 M GdnHCl. Since GdnHCl is a stronger denaturant than urea, the molar concentration of GdnHCl required for complete denaturation is significantly lower than in urea. The ANS fluorescence spectra of ConA in different concentrations of GdnHCl at pH 7.2 are shown in Figure 6B. The GdnHCl denaturation process, using emission maximum and ANS fluorescence as parameters, also depicts two distinct transitions (Figure 7) with an intermediate corresponding to an emission maximum around 340 nm and ~ 32-fold increase in ANS fluorescence. Figure 8 shows the denaturation curve in terms of the relative change of fluorescence intensity at 336 nm as a
Figure 6. (A) Fluorescence spectra at 25°C of tetrameric ConA (2 μM) in 0 M (a), 1.8 M (b) and 3 M (c) GdnHCl in PBS. The spectra were corrected for the buffers containing requisite concentrations of GdnHCl. Excitation wavelength, 280 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min. (B) ANS fluorescence spectra of ConA in 0 M (a), 1.8 M (b) and 3 M (c) GdnHCl. ANS (50 μM) was present under the same conditions as for (A). Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min.
Figure 7. (A) GdnHCl denaturation curve of tetrameric ConA. Relative change of emission wavelength maximum was calculated on the basis of the shift of wavelength maximum (from that in the native state) at different concentrations of GdnHCl in PBS. Each data point represents average of three determinations. The protein concentration was 2 μM. (B) ANS fluorescence intensity at 470 nm at various concentrations of GdnHCl. ANS (50 μM) was present under the same conditions as for (A). Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min.
Figure 8. The denaturation equilibrium transitions of tetrameric ConA measured in terms of the relative change in fluorescence intensity at 336 nm as a function of GdnHCl concentration in PBS. Each data point represents average of three determinations. The protein concentration was 2 μM.
function of GdnHCl concentration. As in urea, two distinct transitions have been observed involving an intermediate with increased fluorescence (~50%) in 1.4-2.0 M GdnHCl.

3.1.2. Dissociation of ConA tetramer and the structural characteristics

Since ConA is a noncovalently associated tetramer at pH 7.2, one possibility is that the intermediate seen in the fluorescence-monitored denaturation, is a monomeric form produced by subunit dissociation in presence of denaturant (Jaenicke 1987). To clarify this issue, size-exclusion chromatography was performed. The gel filtration analysis of native ConA at pH 7.2 on Bio-Gel P-100 column (Figure 2) confirms the tetrameric structure of the protein (the elution volume corresponds to a molecular mass of 102 kDa). Gel filtration size analysis in presence of denaturant was performed on Superdex 75. When ConA denatured in 2.7 M urea (corresponding to the intermediate structure in the denaturation curves (Figures 4 and 5) was loaded onto the Superdex column, a protein peak appeared at a position of molecular mass of 25 kDa (Figure 9) that corresponds to the lectin monomer. It is interesting to note that the completely unfolded monomer in 8 M urea was eluted in the void volume on Superdex column (data not shown). This may be due to the extensive randomly coiled conformations of the denatured subunits leading to an appreciable decrease in elution volume compared with that of a compact globular conformation (Mann and Fish 1972). These results strongly suggest that the denaturation of ConA may be described by a three-state model, in which the tetrameric structure of ConA has been lost in relatively low concentrations of denaturant, resulting in the formation of monomers. The monomeric form then undergoes further perturbation to a completely unfolded state at higher concentrations of denaturant. It is mentioned that native ConA was completely bound to the superdex column in PBS, which served as an affinity matrix. The gel filtration of native
Figure 9. Gel filtration on Superdex 75 column (1.1 x 19 cm) of tetrameric ConA (0.4 mg) denatured in 2.7 M urea in PBS. Inset: Molecular weight calibration curve. The column was precalibrated with standard marker proteins (from left to right): bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa) and soybean trypsin inhibitor (20.1 kDa). The elution position of the dissociated ConA is marked by arrow in the calibration curve and the calculated molecular mass is shown by arrow in the elution profile.
ConA on Superdex 75 was therefore performed in presence of 0.2 M glucose in PBS when the protein was eluted in the void volume because of its tetrameric structure. In contrast, the monomeric intermediate in denaturant did not bind to the Superdex matrix.

Next, in order to study the structural characteristics of the monomer intermediate species, the CD spectra of ConA were measured in absence or presence of denaturant. Figure 10 shows the far-UV CD spectra of ConA in 0 M, 2.7 M and 8 M urea. The native protein exhibits an unusual far-UV CD band centered around 223 nm which probably arises from β-structure with atypical far-UV transitions (Pflumm and Beychok 1974). The spectrum in the presence of 8 M urea shows the loss of secondary structures for the completely unfolded state of the protein. When the spectrum of ConA in 2.7 M urea is compared with those of the native and unfolded proteins, it is clearly different from that in 8 M urea. The dissociated monomer shows the characteristics of β structures but the shift of CD band to 212-215 nm in this case may be associated with a change in atypical geometry of the β-sheet of the native protein (Pflumm and Beychok 1974). The far-UV CD spectra of ConA in presence of GdnHCl, however, show (Figure 11) that the spectrum in 1.6 M GdnHCl closely resembles that of native protein, and is completely different from that of the unfolded form in 3 M GdnHCl. These results demonstrate that the structure of the dissociated monomer retains the secondary conformation of the native protein more in GdnHCl than in urea. Since GdnHCl, as an ionic compound, can render its structure-stabilizing function (Akhtar et al. 2002) at low concentrations (0-2 M), the secondary structure of the dissociated monomer is better preserved in this denaturant. The near-UV CD experiments, however, failed to provide any conclusive evidence in this regard due to the minute
Figure 10. Far-UV CD spectra of tetrameric ConA (2 μM) at 25°C in PBS in presence of 0 M (—), 2.7 M (—- -) and 8 M (-----) urea. The spectra were measured in 1 mm pathlength cell using a scan speed of 20 nm/min, and averaged over five scans. The data are represented in mean residue ellipticities. The spectra were corrected for the buffers containing requisite concentrations of denaturant.
Figure 11. Far-UV CD spectra of tetrameric ConA (2 μM) at 25°C in PBS in presence of 0 M (---), 1.6 M (- - -) and 3 M (-- --) GdnHCl. The spectra were measured in 1 mm pathlength cell using a scan speed of 20 nm/min, and averaged over five scans. The data are represented in mean residue ellipticities. The spectra were corrected for the buffers containing requisite concentrations of denaturant.
intensities and changes of the near-UV signals. ANS-binding experiments shown in Figures 4B and 7B support the notion that the intermediate state resembles a molten globule (Ptitsyn 1995). However, the dissociation of tetramer into monomers from the breakage of the antiparallel β-strand between subunits may also facilitate ANS binding to β-structural conformation due to an increase in accessible surface area in the dissociated state. This may lead to increased fluorescence as the β-structure binds ANS strongly due to its greater hydrophobicity (Semisotnov et al., 1991). Thus it has been concluded tentatively that the ConA monomer in denaturant assumes a structured, partially folded tertiary conformation.

3.1.3. Analysis of equilibrium denaturation of tetrameric ConA

The urea and GdnHCl-induced denaturation of tetrameric ConA at pH 7.2 have been characterized on the basis of the results obtained by the fluorescence properties (Figures 4-8), the elution volume from the size-exclusion column (Figures 2 and 9) and the far-UV CD spectra (Figures 10 and 11). The equilibrium denaturation pathway of ConA may be represented by the following three-state mechanism:

\[ N_4 \leftrightarrow 4N \leftrightarrow 4U \]  

(Scheme 1)

where \( N_4 \) is the native tetrameric state, \( N \) is the structured monomeric state, and \( U \) is the completely unfolded state of the protein. These transitions were found to be completely reversible. The apparent midpoint concentrations (\( C_m \)) of these transitions are summarized in Table 4. In urea, the \( C_m \) values for dissociation and unfolding transitions in terms of fluorescence intensity, are 1.4 M and 4.5 M, respectively. In GdnHCl, which is a stronger denaturant than urea, the corresponding \( C_m \) values are 0.8 M and 2.4 M, respectively. These
observations conform and lend further support to the "2-fold rule" for urea and GdnHCl denaturation of proteins (Mayers et al., 1995; Smith and Scholtz 1996). The apparent midpoints were practically independent of the protein concentration within 0.4 –2.0 μM.

Table 4

Denaturant-induced transitions of ConA

| Denaturant | Transitions (Apparent $C_m$ (M))
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$N_4$ (native tetramer) $\leftrightarrow$ $N$ (structured monomer) $\leftrightarrow$ $U$ (unfolded monomer)</td>
</tr>
<tr>
<td>Urea</td>
<td>1.4</td>
</tr>
<tr>
<td>GdnHCl</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Values at protein concentration of 2 μM from the fluorescence intensity data (Figures 5 and 8).

The nature of the denaturation equilibrium reveals that the dissociation transition can be regarded as distinct and separate from the subsequent unfolding transition (Figure 3). It is therefore assumed that the Scheme 1 can be analyzed by the following two independent dissociation and unfolding reaction steps:

$$N_4 \leftrightarrow 4N$$ (dissociation) \hspace{1cm} (Scheme 2)

$$N \leftrightarrow U$$ (unfolding) \hspace{1cm} (Scheme 3)
3.1.3a. Dissociation reaction of the ConA tetramer

For the dissociation reaction (Scheme 2), the equilibrium constant \( K_{\text{dis}} \) is defined by:

\[
K_{\text{dis}} = \frac{[N]^4}{[N_4]} = \frac{4c^3(f_n)^4}{(1-f_n)^4}
\]

where \([N_4]\) and \([N]\) are the concentrations of native tetrameric and structured monomeric proteins, respectively, \(c\) is the total concentration of monomeric ConA, and \(f_n\) is the fraction of dissociated ConA, i.e., \(f_n = [N] / ([N] + 4[N_4])\).

The free energy of dissociation \(\Delta G_{\text{dis}}\) at a given concentration of denaturant is then obtained from the equation:

\[
\Delta G_{\text{dis}} = -RT\ln K_{\text{dis}} = -RT\ln\left(\frac{4c^3(f_n)^4}{(1-f_n)^4}\right)
\]

\(f_n\) can be obtained from the fluorescence data as:

\[
f_n = \frac{(\Delta I_{\text{obs}} - \Delta I_{\text{aq}})}{(\Delta I_{\text{N}} - \Delta I_{\text{aq}})}
\]

where \(\Delta I_{\text{obs}}\) is the observed change in intensity at a particular concentration of denaturant in the transition region, and \(\Delta I_{\text{aq}}\) and \(\Delta I_{\text{N}}\) are the values characteristic of the tetrameric and monomeric state, respectively.

The fluorescence intensity data in Figures 5 and 8 were analyzed using Eqs. (1-3) to determine the free energy for the dissociation reaction \(\Delta G_{\text{dis}}\) in urea and GdnHCl, respectively. \(\Delta G_{\text{dis}}\) depends on the denaturant concentration according to the linear extrapolation method (Figures 12A and 12B),

\[
\Delta G_{\text{dis}} = \Delta G_{\text{dis, aq}} - m_{\text{dis}}[D]
\]

where \(m_{\text{dis}}\) is the slope of the plot of free energy of dissociation vs. denaturant concentration \([D]\), and \(\Delta G_{\text{dis, aq}}\) is the free energy of dissociation reaction in aqueous solution in absence of the denaturant.
The thermodynamic parameters for the dissociation reaction are shown in Table 5. For urea as a denaturant, the $\Delta G_{\text{dis}, \text{aq}}$ is estimated to be 7.2 kcal mol$^{-1}$ (monomer mass), which is in good agreement with the value of 7.4 kcal mol$^{-1}$ when GdnHCl is used as a denaturant. From Eq. (2), using the $\Delta G_{\text{dis}, \text{aq}}$ of 28.8 kcal /mol of tetramer in urea-induced denaturation, the dissociation constant of ConA in water ($K_{\text{dis}, \text{aq}}$) is determined to be $7.4 \times 10^{-22}$ M$^3$ (Table 5). The ability of denaturants to denature a protein is more directly reflected in the $m$ values, and the ratio of the $m_{\text{dis}}$ values for the two denaturants, $m_{\text{dis}}$(GdnHCl) / $m_{\text{dis}}$(urea) is 2.0.

3.1.3b. Unfolding reaction of the ConA monomer

For the unfolding reaction (Scheme 3), the equilibrium constant ($K_{\text{unf}}$) is defined by:

$$K_{\text{unf}} = \frac{[U]}{[N]} = \frac{f_U}{(1-f_U)}$$

(5)

where $[N]$ and $[U]$ are the concentrations of structured monomer and completely unfolded monomer, respectively, and $f_U$ is the fraction of unfolded monomer, i.e. $f_U = [U] / ([N] + [U])$.

The free energy of unfolding ($\Delta G_{\text{unf}}$) at a given concentration of denaturant is obtained from the equation:

$$\Delta G_{\text{unf}} = -RT \ln K_{\text{unf}} = -RT \ln \left( \frac{f_U}{(1-f_U)} \right)$$

(6)

$f_U$ can be obtained from the fluorescence data as:

$$f_U = \frac{\Delta I_{\text{obs}} - \Delta I_N}{\Delta I_U - \Delta I_N}$$

(7)

where $\Delta I_{\text{obs}}$ is the observed change in intensity at a particular concentration of denaturant in the transition region, and $\Delta I_N$ and $\Delta I_U$ are the values characteristic of the structured monomer and unfolded monomer, respectively.
Thermodynamic parameters for dissociation and unfolding transitions of ConA

Table 5

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>$K_{\text{dis,\text{aq}}}$</th>
<th>$\Delta G_{\text{dis,\text{aq}}}$</th>
<th>$m_{\text{dis}}$</th>
<th>$K_{\text{unf,\text{aq}}}$</th>
<th>$\Delta G_{\text{unf,\text{aq}}}$</th>
<th>$m_{\text{unf}}$</th>
<th>$\Delta G_{\text{dis}&amp;\text{unf,}\text{aq}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>7.4 x 10^{-22}</td>
<td>7.2 ± 0.2</td>
<td>0.8</td>
<td>5.9 x 10^{-4}</td>
<td>4.4 ± 0.2</td>
<td>1.1</td>
<td>11.6</td>
</tr>
<tr>
<td>GdnHCl</td>
<td>1.9 x 10^{-22}</td>
<td>7.4 ± 0.2</td>
<td>1.6</td>
<td>9.2 x 10^{-5}</td>
<td>5.5 ± 0.2</td>
<td>2.3</td>
<td>12.9</td>
</tr>
</tbody>
</table>

* Expressed in terms of mol of monomer; $\Delta G_{\text{dis,\text{aq}}}$ and $\Delta G_{\text{unf,\text{aq}}}$ are calculated from analysis of the data in Figures 5 and 8 by linear extrapolation method (Figure 12) at $T = 298 \text{ K}$. 
The free energy for the unfolding reaction ($\Delta G_{\text{unf}}$) in urea and GdnHCl has been determined by analyzing the data of fluorescence experiments in Figures 5 and 8, respectively, using Eqs. (6) and (7). $\Delta G_{\text{unf}}$ is found to vary linearly, and the linear extrapolation analyses are shown in Figures 12A and 12B, according to the equation:

$$\Delta G_{\text{unf}} = \Delta G_{\text{unf, aq}} - m_{\text{unf}}[D]$$

(8)

where $m_{\text{unf}}$ is a measure of the dependence of $\Delta G_{\text{unf}}$ on denaturant concentration $[D]$, and $\Delta G_{\text{unf, aq}}$ is the free energy of unfolding reaction in water.

Table 5 summarizes the thermodynamic parameters for the unfolding reaction. Values of 4.4 kcal mol$^{-1}$ ($\Delta G_{\text{unf, aq}}$) and $5.9 \times 10^{-2}$ ($K_{\text{unf, aq}}$) have been determined in urea-induced denaturation, and the values of 5.5 kcal mol$^{-1}$ ($\Delta G_{\text{unf, aq}}$) and $9.2 \times 10^{-5}$ ($K_{\text{unf, aq}}$) in GdnHCl-induced denaturation. The slightly higher free energy value obtained for GdnHCl-induced unfolding may relate to the occurrence of the more native-like structured ConA monomer in GdnHCl than in urea as shown from far-UV CD studies. The ratio of the $m_{\text{unf}}$ values for the two denaturants, $m_{\text{unf}}$ (GdnHCl) / $m_{\text{unf}}$ (urea) is 2.1, being similar to that for the dissociation reaction. The consistency of the results in both denaturants supports the use of linear extrapolation method (Eqs. (4) and (8)) which has been justified on thermodynamic grounds (Schellman and Hawkes 1980) and which has the advantage that no assumption about the binding of denaturants to the native, dissociated and unfolded forms of the protein are needed (Pace 1986).
Figure 12

A

\[ \Delta G_{25} \text{ or } \Delta G_{v} \text{ (kcal mol}^{-1}\text{)} \]

Urea (M)

B

\[ \Delta G_{25} \text{ or } \Delta G_{v} \text{ (kcal mol}^{-1}\text{)} \]

GdnHCl (M)
3.1.3c. Structural stability of tetrameric ConA

The overall structural stability of ConA is obtained from the combined free energy for Scheme 1. The total free energy for dissociation and subsequent unfolding reactions in water ($\Delta G_{\text{dis&unf. aq}}$) of ConA tetramer is therefore determined to be:

$$\Delta G_{\text{dis&unf. aq}} = \Delta G_{\text{dis. aq}} + 4 \Delta G_{\text{unf. aq}} \quad (9)$$

Table 5 shows the combined free energy values of the protein in the two denaturants. For denaturation in urea, the $\Delta G_{\text{dis&unf. aq}}$ value is 11.6 kcal mol$^{-1}$, which agrees fairly well with the value of 12.9 kcal mol$^{-1}$ obtained from GdnHCl-induced denaturation. It is notable that all free energy ($\Delta G_{\text{dis. aq}}, \Delta G_{\text{unf. aq}}$ and $\Delta G_{\text{dis&unf. aq}}$) values are normalized in terms of monomer mass (mol of monomer). Based on this, the free energy of stabilization of structured monomer ($-\Delta G_{\text{unf. aq}}$) of ConA relative to the unfolded monomer is obtained as 4.4-5.5 kcal mol$^{-1}$, and the stabilization free energy for association of tertiary subunits to tetrameric quaternary structure ($-\Delta G_{\text{dis. aq}}$) is estimated to be 7.2-7.4 kcal mol$^{-1}$. Thus the free energy of stabilization of the quaternary structure of ConA relative to the unfolded state ($-\Delta G_{\text{dis&unf. aq}}$) is determined as 11.6-12.9 kcal mol$^{-1}$ (monomer mass). However, Eq. (9) provides, that free energy of unfolding of only 4.4 kcal (per mol of monomer) at the subunit level, leads to overall free energy of 46.4 kcal (per mol of tetramer) for the quaternary structure of the protein, with a contribution of free energy of dissociation of 28.8 kcal (per mol of tetramer) at the quaternary level. Thus the structural stability of ConA is maintained mostly by the formation of the oligomeric structure.
3.2. Denaturation studies of dimeric ConA

ConA is a tetramer at pH 7.2, but exists as a dimer at around pH 5.0. Thus ConA serves as an excellent model system in relation to studies of quaternary dissociation / association, and the role of subunit oligomerization on the conformational stability of oligomeric legume lectins. The denaturant-induced unfolding of dimeric ConA was studied in presence of urea and GdnHCl in 20 mM acetate buffer containing 0.15 M NaCl, pH 5.2.

3.2.1. Equilibrium denaturation of ConA at pH 5.2

The fluorescence spectra of ConA in different concentrations of urea at pH 5.2 are shown in Figure 13A. At 0 M urea the protein exhibits emission maximum at 335 ± 1 nm, which gradually red-shifts with increasing concentration of urea, and finally levels off at 351 ± 1 nm in ≥ 6.9 M urea, indicative of protein denaturation and Trp exposure to the aqueous environment. The relative change of fluorescence intensity at 336 nm as a function of urea concentration is shown in Figure 14A. The fluorescence intensity increases to ~ 50 % in 3.1-4.1 M urea, and then decreases gradually to level off in ≥ 6.9 M urea. This denaturation curve thus depicts two distinct transitions involving an intermediate in 3.1 to 4.1 M urea.

The denaturation of dimeric ConA in GdnHCl at pH 5.2 exhibits similar characteristics as in urea, when the emission maximum gradually shifts from 335 ± 1 nm in 0 M GdnHCl to 351 ± 1 nm in ≥ 3.2 M GdnHCl (spectra not shown). Since GdnHCl is a stronger denaturant than urea, the molar concentration of GdnHCl required for complete denaturation is significantly lower than in urea. Figure 14B shows the denaturation curve in terms of the relative change of fluorescence intensity at 336 nm as a function of GdnHCl concentration. As in urea, two distinct
Figure 13. (A) Fluorescence spectra at 25°C of dimeric ConA (2 μM) in 0 M, 3.5 M and 6.9 M urea in 20 mM acetate buffer containing 0.15 M NaCl, pH 5.2. The spectra were corrected for the buffers containing requisite concentrations of urea. Excitation wavelength, 280 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min. (B) ANS fluorescence spectra of ConA in 0 M (a), 3.5 M (b) and 6.9 M (c) urea. ANS (50 μM) was present under the same conditions as for (A). Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min.
Figure 14. The denaturation equilibrium transitions of dimeric ConA measured in terms of the relative change in fluorescence intensity at 336 nm as a function of (A) urea concentration in acetate buffer, pH 5.2; and (B) GdnHCl concentration in acetate buffer, pH 5.2. Each data point represents average of three determinations. The protein concentration was 2 μM.
transitions have been observed involving an intermediate with increased fluorescence (~ 60 %) in 1.8-2.1 M GdnHCl.

3.2.2. Characterization of intermediate

The intrinsic fluorescence measurements show that the denaturation profile of dimeric ConA at pH 5.2 is biphasic in nature. These observations have been supported by ANS binding studies in presence of varying concentrations of denaturant. The ANS fluorescence spectra of ConA in different concentrations of urea at pH 5.2 are shown in Figure 13B. As shown, ANS does not bind to any significant extent to the native or the fully denatured state of the protein, but it does so very strikingly to the intermediate at 3.5 M urea. A plot of fluorescence intensity at 470 nm as a function of urea concentration (Figure 15A) shows that a significant increase in fluorescence intensity at 470 nm has been observed from 0.9 M urea to 3.2-4.2 M urea, and then the fluorescence has decreased gradually to almost negligible intensity at high concentrations (≥ 6.9 M) of urea. The ANS fluorescence data thus correlate well with two distinct transitions as observed in the denaturation curve (Figure 14A), and suggest the presence of a hydrophobic intermediate at low concentrations of urea. In case of GdnHCl denaturation (Figure 15B), a significant increase in fluorescence intensity at 470 nm has been observed to ~ 25-fold in 1.8-2.3 M GdnHCl, and then the fluorescence has decreased gradually to almost negligible intensity at high concentrations (~ 3 M) of GdnHCl. In contrast to two-state unfolding of dimeric ConA in GdnHCl as reported (Mitra et al. 2002), the ANS data in this study suggest that the unfolding of ConA at pH 5.2 in GdnHCl is not a simple two-state process, as in urea.
Figure 15. (A) ANS fluorescence intensity at 470 nm at various concentrations of urea in presence of dimeric ConA (2 μM) and ANS (50 μM). Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min. (B) ANS fluorescence intensity at 470 nm at various concentrations of GdnHCl in presence of dimeric ConA (2 μM) and ANS (50 μM).
The size of the intermediate species was examined by size-exclusion chromatography. The integrity of dimeric structure of ConA at pH 5.2 was confirmed from gel filtration analysis on Bio-Gel P-100 column (Figure 16A). When ConA denatured in 2.1 M GdnHCl (corresponding to the intermediate structure in the denaturation curve (Figure 14B) was subjected to size-exclusion chromatography on Bio-Gel P-100 column, a protein peak appeared at a position of molecular mass 27 kDa (Figure 16B) that corresponds to the lectin monomer. These results suggest that the denaturation of dimeric ConA at pH 5.2 may be described by a three-state model involving a monomeric intermediate.

To determine the structural characteristics of the intermediate species, the CD spectra of ConA at pH 5.2 were measured in the absence or presence of denaturant. Figure 17A shows the far-UV CD spectra of ConA in 0, 3.5 and 8 M urea. The spectrum in the presence of 8 M urea shows the loss of secondary structures for the completely unfolded state of the protein. The spectrum in 3.5 M urea is, however, similar to that of native protein. The far-UV CD spectra of ConA in presence of GdnHCl (Figure 17 B) also shows that the spectrum in 2.1 M GdnHCl closely resembles that of native protein, and is completely different from that of the unfolded form in 3 M GdnHCl. These results demonstrate that the structure of the dissociated monomer retains the secondary conformation of the native protein. The near-UV CD experiments, however, failed to provide any evidence regarding the tertiary structure due to the minute intensities and changes of the near-UV signals.
Figure 16. (A) Elution profile of ConA from Bio-Gel P-100 column (1 x 75 cm) in acetate buffer, pH 5.2; (B) Gel filtration on Bio-Gel P-100 column (1 x 50 cm) of ConA denatured in 2.1 M GdnHCl in acetate buffer, pH 5.2. Inset: Molecular weight calibration curves. The columns were precalibrated with standard marker proteins (from left to right): bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa) and soybean trypsin inhibitor (20.1 kDa). The elution positions of the native ConA in (A) and the dissociated ConA in (B) are marked by arrow in the respective calibration curve and the calculated molecular mass is shown by arrow in the respective elution profile.
Figure 17. Far-UV CD spectra of dimeric ConA (2 μM) at 25°C in acetate buffer, pH 5.2 in presence of (A) 0 M (—), 3.5 M (−−) and 8 M (−−−) urea; and (B) 0 M (—), 2.1 M (−−) and 3 M (−−−) GdnHCl. The spectra were measured in 1 mm pathlength cell using a scan speed of 20 nm/min, and averaged over five scans. The data are represented in mean residue ellipticities. The spectra were corrected for the buffers containing requisite concentrations of denaturant.
3.2.3. Three-state analysis of unfolding process

The unfolding of dimeric ConA at pH 5.2 in denaturants may be described as a three-state process:

\[ N_2 \leftrightarrow 2N \leftrightarrow 2U \]

where \( N_2 \) is the native dimeric state, \( N \) is the structured monomeric state, and \( U \) is the completely unfolded state of the protein. These transitions were found to be completely reversible.

The nature of the denaturation equilibrium reveals that the dissociation transition can be regarded as distinct and separate from the subsequent unfolding transition, and can be analyzed by two independent dissociation and unfolding reaction steps:

\[ N_2 \leftrightarrow 2N \quad \text{(dissociation)} \]
\[ N \leftrightarrow U \quad \text{(unfolding)} \]

For the dissociation reaction, the equilibrium constant \( (K_{\text{dis}}) \) is defined by:

\[ K_{\text{dis}} = \frac{[N]^2}{[N_2]} = \frac{2c(f_n)^2}{(1 - f_n)} \quad (10) \]

where \([N_2]\) and \([N]\) are the concentrations of native dimeric and structured monomeric proteins, respectively, \( c \) is the total concentration of monomeric ConA, and \( f_n \) is the fraction of dissociated ConA, i.e. \( f_n = \frac{[N]}{([N] + 2[N_2])} \).

The free energy of dissociation \( (\Delta G_{\text{dis}}) \) at a given concentration of denaturant is then obtained from the equation:

\[ \Delta G_{\text{dis}} = -RT\ln K_{\text{dis}} = -RT\ln[2c(f_n)^2 / (1 - f_n)] \quad (11) \]

\( f_n \) can be obtained from the fluorescence data as:

\[ f_n = \frac{\Delta I_{\text{obs}} - \Delta I_{N_2}}{\Delta I_n - \Delta I_{N_2}} \quad (12) \]
where $\Delta I_{obs}$ is the observed change in intensity at a particular concentration of denaturant in the transition region, and $\Delta I_{n2}$ and $\Delta I_{n}$ are the values characteristic of the dimeric and monomeric state, respectively.

The fluorescence intensity data in Figure 14 were analyzed using Eqs. (10-12) to determine the free energy for the dissociation reaction ($\Delta G_{\text{dis}}$). $\Delta G_{\text{dis}}$ depends on the denaturant concentration according to the linear extrapolation method (Eq 4),

$$\Delta G_{\text{dis}} = \Delta G_{\text{dis, aq}} - m_{\text{dis}}[D]$$

where $m_{\text{dis}}$ is a measure of dependence of $\Delta G_{\text{dis}}$ on the denaturant concentration $[D]$, and $\Delta G_{\text{dis, aq}}$ is the free energy of dissociation reaction in aqueous solution in absence of the denaturant.

For the unfolding reaction ($N \leftrightarrow U$), $\Delta G_{\text{unf, aq}}$ and $m_{\text{unf}}$ could be analyzed according to Eqs 5-8 as discussed in section 3.1.3b.

The total free energy for dissociation and subsequent unfolding reactions in water ($\Delta G_{\text{dis\&unf, aq}}$) of dimeric ConA is obtained as:

$$\Delta G_{\text{dis\&unf, aq}} = \Delta G_{\text{dis, aq}} + 2 \Delta G_{\text{unf, aq}}$$

(13)

For denaturation in urea, the $\Delta G_{\text{dis, aq}}$ and $\Delta G_{\text{unf, aq}}$ are estimated to be 4.5 kcal mol$^{-1}$ and 5.9 kcal mol$^{-1}$ (monomer mass), respectively. Thus the overall free energy of stabilization of the dimeric ConA relative to the unfolded state ($-\Delta G_{\text{dis\&unf, aq}}$) is obtained as 10.4 kcal mol$^{-1}$ (monomer mass). For GdnHCl-induced denaturation, $\Delta G_{\text{dis, aq}}$ and $\Delta G_{\text{unf, aq}}$ are obtained as 4.9 and 7.0 kcal mol$^{-1}$, respectively, with an overall free energy of stabilization ($-\Delta G_{\text{dis\&unf, aq}}$) of 11.9 kcal mol$^{-1}$ (monomer mass). These free energy values are slightly lower than those obtained for tetrameric ConA.
3.3. Denaturation studies of chemically modified derivatives of ConA

Solvent denaturation studies are useful to probe the relationship between chemical structure and the protein stability. These can also provide information as to whether a small change in the chemical structure can affect the mechanism of denaturation of the protein. ConA is an excellent system in relation to studies of the effect of chemical modification on the conformational stability and the pathway of folding and association. Several chemically modified derivatives of ConA which have different quaternary structures (tetrameric / dimeric) have been used in denaturation studies at pH 7.2 for comparative analysis of denaturant-induced unfolding.

3.3.1. Properties of ConA derivatives

Chemical modifications of ConA by reductive methylation, acetylation and succinylation yielded N-dimethyl ConA, acetyl-ConA and succinyl-ConA, respectively. The extent of chemical modification (as estimated by TNBS method) for the derivatives after two cycles of reaction is shown in Table 6. Each derivative retained their capacity to bind to affinity column (Sephadex G-100), and was affinity purified before using in the denaturation studies. The hemagglutinating activity of acetyl and succinyl derivative is similar and ~ 4-fold lower than that of N-dimethyl derivative. The fluorescence properties of the derivatives were also similar to those of the unmodified protein, suggesting that they have similar conformations. The molecular size of each derivative was confirmed from gel filtration analyses and the results are summarized in Table 7A. The N-dimethyl derivative possesses tetrameric quaternary structure while the acetyl and succinyl derivatives exist in dimeric form at pH 7.2.
Table 6

Estimation of the extent of chemical modification for the derivatives of ConA as determined by TNBS method

<table>
<thead>
<tr>
<th>Protein</th>
<th>$A_{335}$ (after 2 cycles)</th>
<th>% Modification $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Dimethyl ConA</td>
<td>0.13</td>
<td>85</td>
</tr>
<tr>
<td>Acetyl-ConA</td>
<td>0.14</td>
<td>84</td>
</tr>
<tr>
<td>Succinyl-ConA</td>
<td>0.12</td>
<td>87</td>
</tr>
</tbody>
</table>

$a = \text{calculated on the basis of } A_{335} (0.89) \text{ for native conA}$

3.3.2. Denaturation of N-dimethyl ConA, acetyl-ConA and succinyl-ConA

The intrinsic fluorescence spectra of tetrameric N-dimethyl ConA in varying concentrations of urea at pH 7.2 are shown in Figure 18A, and the ANS fluorescence spectra at the same concentrations of urea are shown in Figure 18B. The shift in emission maximum and the relative change of fluorescence intensity at 336 nm were similar as observed for native ConA. The fluorescence spectroscopy using ANS as external probe also suggests the presence of a hydrophobic equilibrium intermediate at relatively low concentrations of urea. As shown in Figure 19, a significant increase in fluorescence intensity at 470 nm has been observed from 0.9 M urea to ~12-fold in 3.2-4.2 M urea, and the fluorescence has decreased gradually to almost negligible intensity at high concentrations ($\geq 5.7 \text{ M}$) of urea.
Figure 18. (A) Fluorescence spectra at 25°C of N-dimethyl ConA (2 μM) in 0 M (a), 3.5 M (b) and 8 M (c) urea in PBS. The spectra were corrected for the buffers containing requisite concentrations of urea. Excitation wavelength, 280 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min. (B) ANS fluorescence spectra of N-dimethyl ConA in 0 M (a), 3.5 M (b) and 8 M (c) urea. ANS (50 μM) was present under the same conditions as for (A). Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min.
Figure 19. ANS fluorescence intensity at 470 nm at various concentrations of urea in presence of N-dimethyl ConA (2 μM) and ANS (50 μM). Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min.
The denaturation profile of dimeric acetyl-ConA at pH 7.2 in increasing concentration of urea is shown in Figure 20. The plots of relative change of emission maximum (Figure 20A) as well as the change of fluorescence intensity at 470 nm in ANS binding experiments (Figure 20B) show that the denaturation profile is biphasic in nature with the involvement of a stable intermediate in 3.0-4.0 M urea. These results agree with the denaturation profile obtained in terms of the relative change of fluorescence intensity at 336 nm as a function of urea concentration (Figure 21). The molecular size of the intermediate species in the urea-induced denaturation of N-dimethyl ConA and acetyl-ConA corresponds to a protein monomer as obtained from size-exclusion chromatography experiments, and the results are summarized in Table 7B.

Surprisingly, the denaturation of dimeric succinyl-ConA in urea occurs in a single step (Figure 22), and further, the unfolding reaction was not completely reversible.
Figure 20. (A) Urea denaturation curve of acetyl-ConA. Relative change of emission wavelength maximum was calculated on the basis of the shift of wavelength maximum at different concentrations of urea in PBS. Each data point represents average of three determinations. The protein concentration was 2 μM. (B) ANS fluorescence intensity at 470 nm at various concentrations of urea. ANS (50 μM) was present under the same conditions as for (A). Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min.
Figure 21. The denaturation equilibrium transitions of acetyl-ConA measured in terms of the relative change in fluorescence intensity at 336 nm as a function of urea concentration in PBS. Each data point represents average of three determinations. The protein concentration was 2 μM.
Figure 22. Urea denaturation curve of succinyl-ConA. Relative change of emission wavelength maximum was calculated on the basis of the shift of wavelength maximum at different concentrations of urea in PBS. Each data point represents average of three determinations. The protein concentration was 2 µM.
### Table 7

**A. Size-exclusion chromatography of derivatives of ConA**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size-exclusion media</th>
<th>pH</th>
<th>Molecular mass determined</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Dimethyl ConA</td>
<td>Bio-Gel P-100</td>
<td>7.2</td>
<td>104 kDa</td>
</tr>
<tr>
<td>Acetyl-ConA</td>
<td>Bio-Gel P-100</td>
<td>7.2</td>
<td>56 kDa</td>
</tr>
<tr>
<td>Succinyl-ConA</td>
<td>Bio-Gel P-100</td>
<td>7.2</td>
<td>56 kDa</td>
</tr>
</tbody>
</table>

**B. Determination of the size of the intermediate species in urea for the derivatives of ConA by size-exclusion chromatography at pH 7.2**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size-exclusion media</th>
<th>Concentration of urea (M)</th>
<th>Molecular mass determined</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Dimethyl ConA</td>
<td>Superdex 75</td>
<td>3.5</td>
<td>27 kDa</td>
</tr>
<tr>
<td>Acetyl-ConA</td>
<td>Superdex 75</td>
<td>3.5</td>
<td>27 kDa</td>
</tr>
</tbody>
</table>
3.3.3. Comparative analysis of conformational stability of native ConA and its derivatives

The thermodynamic parameters for dissociation and unfolding transitions in urea for the tetrameric N-dimethyl ConA are shown in Table 8. The parameters for the native ConA tetramer are also included for comparison. For the dissociation transition, the $\Delta G_{\text{dis}, \text{aq}}$ values for the native ConA and its N-dimethyl derivative are 7.2 and 7.3 kcal mol$^{-1}$ (monomer mass), and the apparent $C_m$ values (1.4 and 1.5 M) are also very similar. These results show that the conformational stability of ConA tetramer at the quaternary level does not alter to any significant extent by reductive methylation. The $m_{\text{dis}}$ values are also similar, suggesting similar dissociating pathways for these proteins. For the unfolding reaction, the $\Delta G_{\text{unf}, \text{aq}}$ for the N-dimethyl derivative is 5.3 kcal mol$^{-1}$ compared to a value of 4.4 kcal mol$^{-1}$ for the native protein. The overall free energy of stabilization ($-\Delta G_{\text{dis}+\text{unf}, \text{aq}}$) of the tetrameric structure of N-dimethyl ConA relative to the unfolded state is 12.6 kcal mol$^{-1}$ (monomer mass) which is similar to that of native protein. Thus the effect of reductive methylation on the conformational stability of ConA tetramer is minimal. Since reductive methylation does not alter significantly the net charge of the protein (Jentoft & Dearborn 1983) and added methyl groups are small compared to other protein modifying reagents, reductive methylation does not affect significantly the conformational stability of native tetrameric ConA:

The thermodynamic parameters for dissociation and unfolding transitions of dimeric ConA at pH 5.2 and dimeric acetyl-ConA at pH 7.2 are shown in Table 9. For the dissociation reaction, the $\Delta G_{\text{dis}, \text{aq}}$ and $m_{\text{dis}}$ values for dimeric ConA and the acetyl derivative do not differ appreciably. However, interestingly, the $\Delta G_{\text{unf}, \text{aq}}$ for the monomer unfolding reaction of acetyl-ConA is much smaller (2.1 kcal mol$^{-1}$) compared to a value of 5.9 kcal mol$^{-1}$ for the native
Table 8
Thermodynamic parameters for dissociation and unfolding transitions for tetrameric ConA and N-dimethyl ConA in urea

<table>
<thead>
<tr>
<th>Protein</th>
<th>$C_m$ (dis)</th>
<th>$\Delta G_{\text{dis, aq}}$</th>
<th>$m_{\text{dis}}$</th>
<th>$C_m$ (unf)</th>
<th>$\Delta G_{\text{unf, aq}}$</th>
<th>$m_{\text{unf}}$</th>
<th>$\Delta G_{\text{dis&amp;unf, aq}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native ConA</td>
<td>1.4</td>
<td>7.2 ± 0.2</td>
<td>0.8</td>
<td>4.5</td>
<td>4.4 ± 0.2</td>
<td>1.1</td>
<td>11.6</td>
</tr>
<tr>
<td>N-dimethyl ConA</td>
<td>1.5</td>
<td>7.3 ± 0.2</td>
<td>1.0</td>
<td>4.8</td>
<td>5.3 ± 0.2</td>
<td>1.1</td>
<td>12.6</td>
</tr>
</tbody>
</table>

* Expressed in terms of mol of monomer.
Table 9

Thermodynamic parameters for dissociation and unfolding transitions for dimeric ConA and acetyl-ConA in urea

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\Delta G_{\text{dis, sq}}$ (kcal mol$^{-1}$)$^a$</th>
<th>$m_{\text{dis}}$ (kcal mol$^{-1}$ M$^{-1}$)</th>
<th>$\Delta G_{\text{unf, sq}}$ (kcal mol$^{-1}$)$^a$</th>
<th>$m_{\text{unf}}$ (kcal mol$^{-1}$ M$^{-1}$)</th>
<th>$\Delta G_{\text{dis&amp;unf, sq}}$ (kcal mol$^{-1}$)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimeric ConA</td>
<td>4.5 ± 0.2</td>
<td>0.5</td>
<td>5.9 ± 0.2</td>
<td>1.1</td>
<td>10.4</td>
</tr>
<tr>
<td>Acetyl-ConA</td>
<td>4.7 ± 0.2</td>
<td>0.6</td>
<td>2.1 ± 0.2</td>
<td>0.4</td>
<td>6.8</td>
</tr>
</tbody>
</table>

$^a$ Expressed in terms of mol of monomer.
dimeric protein. These results indicate that acetylation has a pronounced destabilizing effect on the conformational stability of ConA particularly at the monomer tertiary level. Since acetylation brings about change in overall charge distribution on the protein in the neutral range, it may lead to significant changes in inter- / intramolecular forces and thus may contribute to a substantial decrease in conformational stability of the protein.

The unfolding of succinyl-ConA occurs in a single step, and it may be probable that the charge state of the succinylated protein exerts even stronger destabilizing effect on the tertiary monomer so that no significant equilibrium population of monomeric intermediate was obtained. As the unfolding reaction of the succinyl derivative was not completely reversible, the thermodynamic analysis of the unfolding reaction was not performed.
3.4. **Renaturation studies of ConA (tetramer / dimer) and its tetrameric and dimeric derivatives**

Refolding of monomeric proteins is determined by sequential or parallel first-order folding reactions as rate-limiting steps. In the process of reconstitution of oligomers, second-order association steps may be rate-determining besides folding. Folding steps must, on the one hand, precede association in order to provide the surface areas required for correct inter-subunit recognition; on the other hand, they must succeed association, as a consequence of exclusion of water molecules from the subunit interfaces that serve to stabilize the native quaternary structure.

The renaturation studies of ConA at pH 7.2, and at pH 5.2 as well as the reconstitution of its tetrameric and dimeric derivatives have been performed using fluorescence, ANS binding, far-UV CD and activity assay. It has been previously shown that the denaturation of ConA and its derivatives (N-dimethyl ConA and acetyl-ConA) involves a structured monomeric intermediate characterized by fluorescence and CD spectroscopy, and size-exclusion chromatography. During renaturation, the restoration of 'structure' at the monomeric level, that is, the formation of structured monomer from the completely denatured state, takes place in a rapid reaction within the dead time of measurement (<30 s). Thus the process of subunit association which occurs in a much longer time scale (h) has been examined after manual mixing of the unfolded protein in denaturant with the appropriate reconstitution buffer (PBS containing 0.1 mM Mn$^{2+}$ and 0.1 mM Ca$^{2+}$, pH 7.2 or 20 mM acetate buffer containing 0.15 M NaCl, 1 mM Mn$^{2+}$ and 1 mM Ca$^{2+}$, pH 5.2).
3.4.1. Reconstitution of proteins as monitored by fluorescence spectroscopy

The fluorescence spectra of native tetrameric ConA, the unfolded protein, and the renatured protein at pH 7.2 are shown in Figure 23A. While the unfolded protein in 8 M urea exhibits an emission maximum at 351 ± 1 nm, the emission maximum of the renatured protein almost coincides with that of native protein at 336 ± 1 nm. When the denatured protein is subjected to reconstitution by dilution with renaturation buffer, the emission maximum shifts immediately (< 30 s) to 339 ± 1 nm (Figure 23A), which shows an increase in intensity at 336 nm relative to the native protein. The relative change of fluorescence intensity at 336 nm as a function of time during reconstitution is shown in Figure 23B. The relative intensity remained constant up to about 80 min, and then gradually decreased to that of the native state after 3 h. The reconstitution process of tetrameric conA was also monitored by ANS binding experiments. Figure 24A shows the ANS fluorescence spectra at 30 s and 3 h of reconstitution along with that of the native state of the protein. The results show that a pronounced ANS fluorescence was observed at the early stage of renaturation, and the fluorescence intensity decreased to almost negligible value after ~ 3 h. The plot of ANS fluorescence intensity at 470 nm as a function of time is shown in Figure 24B. These results indicate that an intermediate similar in properties of the structured monomer as observed in equilibrium unfolding of the protein, is formed from the unfolded state immediately on dilution with the reconstitution buffer, and thereafter the process of renaturation involves the association of monomers in a time period of ~ 3 h to form the native quaternary structure.

The reconstitution studies of dimeric ConA was carried out from its unfolded state in 8 M urea or 6 M GdnHCl after dilution with appropriate renaturation buffer at pH 5.2. In these
Figure 24. (A) ANS fluorescence spectra at pH 7.2 of native tetrameric ConA (a), reconstituting protein (b) within 30 s of reconstitution and renatured ConA (c). The protein concentration was 0.8 μM and ANS was 50 μM. Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min. (B) ANS fluorescence intensity at 470 nm as a function of time during reconstitution.
studies also, the ANS binding experiments provided convincing evidence for the formation of a structured monomer at the early stage of reconstitution. Figure 25A shows the ANS fluorescence spectra at the initial stage (30 s) of reconstitution along with those of native dimeric ConA, and the renatured protein at pH 5.2. As shown, during the initial period of reconstitution, a significant ANS fluorescence was observed which is characteristic of the structured monomer of the protein. A plot of ANS fluorescence intensity at 470 nm as a function of time is shown in Figure 25B. The ANS fluorescence showed ~8-fold increase in intensity during the period up to 80 min followed by the decrease of ANS fluorescence with time to almost negligible value as of native protein after ~3 h, implying the formation of a fully constituted protein.

The renaturation of chemically modified tetrameric derivative, N-dimethyl ConA and dimeric derivatives, acetyl- and succinyl-ConA was attempted by dilution of the denatured protein in 8 M urea with appropriate renaturation buffer at pH 7.2. ANS fluorescence intensity at 470 nm as a function of time during reconstitution is represented in Figure 26A for N-dimethyl ConA and in Figure 26B for acetyl-ConA. These results are similar as observed for renaturation studies of tetrameric and dimeric ConA, and in agreement with the results of equilibrium unfolding experiments with these derivatives. The reconstitution studies of succinyl-ConA, however, yielded interesting results. The ANS fluorescence spectra of succinyl-ConA during the initial period and after 3 h of reconstitution are shown in Figure 27A, and a plot of ANS fluorescence intensity at 470 nm as a function of time is represented in Figure 27B. The results show that a significant ANS fluorescence remained practically constant with time, and the reconstitution could not be achieved even after 24 h. It may be possible that the charge state of the succinylated monomer contributes to the repulsive interaction in the reassembly of the
Figure 25. (A) ANS fluorescence spectra at pH 5.2 of native dimeric ConA (a), reconstituting protein (b) within 30 s of reconstitution and renatured ConA (c). The protein concentration was 0.8 μM and ANS was 50 μM. Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min. (B) ANS fluorescence intensity at 470 nm as a function of time during reconstitution.
Figure 26. (A) ANS fluorescence intensity at 470 nm as a function of time during reconstitution at pH 7.2 for N-dimethyl ConA. The protein concentration was 0.8 μM and ANS was 50 μM. Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min. (B) ANS fluorescence intensity at 470 nm as a function of time during reconstitution at pH 7.2 for acetyl-ConA. The protein concentration was 0.8 μM and ANS was 50 μM.
Figure 27. (A) ANS fluorescence spectra at pH 7.2 of succinyl-ConA (a), reconstituting protein (b) within 30 s of reconstitution, and reconstituting protein (c) after 3 h. The protein concentration was 0.8 µM and ANS was 50 µM. Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min. (B) ANS fluorescence intensity at 500 nm as a function of time.
subunits, and thereby prevents the formation of the quaternary structure of the lectin derivative.

The fluorescence parameters in terms of emission maxima, and the ANS fluorescence intensity at 470 nm in the renaturation studies of ConA and its derivatives have been summarized in Tables 10 and 11, respectively, for comparison.

Table 10

Fluorescence parameters (emission maxima) for ConA and its derivatives in renaturation studies

<table>
<thead>
<tr>
<th>Protein</th>
<th>Emission maximum in absence of denaturant (nm)</th>
<th>Emission maximum in unfolded state (nm)</th>
<th>Emission maximum during renaturation after 4 h (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrameric ConA</td>
<td>336</td>
<td>351</td>
<td>337</td>
</tr>
<tr>
<td>Dimeric ConA</td>
<td>334</td>
<td>351</td>
<td>335</td>
</tr>
<tr>
<td>N-Dimethyl ConA</td>
<td>335</td>
<td>351</td>
<td>336</td>
</tr>
<tr>
<td>Acetyl-ConA</td>
<td>334</td>
<td>352</td>
<td>335</td>
</tr>
<tr>
<td>Succinyl-ConA</td>
<td>334</td>
<td>352</td>
<td>342</td>
</tr>
</tbody>
</table>
Table 11

ANS fluorescence intensity at 470 nm ($I_{470}$) for ConA and its derivatives in renaturation studies

<table>
<thead>
<tr>
<th>Protein</th>
<th>$I_{470}$ (A.U) in absence of denaturant</th>
<th>$I_{470}$ (A.U) in unfolded state</th>
<th>$I_{470}$ (A.U) during renaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 s</td>
</tr>
<tr>
<td>Tetrameric ConA</td>
<td>1.8</td>
<td>0.7</td>
<td>16</td>
</tr>
<tr>
<td>Dimeric ConA</td>
<td>1.5</td>
<td>1.0</td>
<td>8.5</td>
</tr>
<tr>
<td>N-Dimethyl ConA</td>
<td>1.0</td>
<td>1.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Acetyl-ConA</td>
<td>2.0</td>
<td>1.0</td>
<td>13.5</td>
</tr>
<tr>
<td>Succinyl-ConA</td>
<td>2.5</td>
<td>1.0</td>
<td>11.3</td>
</tr>
</tbody>
</table>
Figure 28. (A) Far-UV CD spectra of tetrameric ConA (0.8 μM) in native, unfolded and renatured states in PBS containing 0.1 mM Mn²⁺ and 0.1 mM Ca²⁺, pH 7.2. The spectra were measured in 1mm pathlength cell using a scan speed of 50 nm / min, and averaged over five scans. The data are represented in mean residue ellipticities. (B) Far-UV CD spectra of dimeric ConA (0.8 μM) in native, unfolded and renatured states in 20 mM acetate buffer containing 0.15 M NaCl, 1 mM Mn²⁺ and 1 mM Ca²⁺, pH 5.2. The data are represented in molar ellipticities.
3.4.2. Circular dichroism studies

The far-UV CD spectra of ConA at pH 7.2 in native, unfolded and renatured states are shown in Figure 28A. While the spectrum in the presence of 8 M urea shows the loss of secondary structures for the completely unfolded state of the protein, the spectrum of native ConA tetramer and that of renatured protein are very similar. It may be mentioned that at the initial stage of reconstitution, the far-UV CD spectrum of the reconstituting protein closely resembled that of native protein, implying that the secondary conformation was restored immediately. Thus the intermediate formed during the process of reconstitution was characterized as structured monomer. The far-UV CD spectra of dimeric ConA, its unfolded monomer and the renatured protein at pH 5.2 are shown in Figure 28B. The results show that the reconstituted protein exhibits similar secondary structures as of native protein. The near-UV CD experiments, however, failed to provide any conclusive evidence about the restoration of tertiary structure due to the minute intensities and changes of the near-UV signals under conditions of renaturation of the protein.

3.4.3. Hemagglutinating activity of the renatured proteins

The regain of activity of ConA and its derivatives after denaturation in urea or GdnHCl was assayed by measuring the hemagglutinating activity of the reconstituted proteins vis-à-vis the native lectins. The assay was done by serial 2-fold dilution technique with visual estimation of agglutination in microtiter plates, and the lowest concentration of lectin giving visible agglutination was determined. Table 12 shows the hemagglutinating activity of ConA and its
derivatives in renaturation studied at pH 7.2. The results show that the hemagglutinating activity of the fully reconstituted protein was similar to that of native lectin.

**Table 12**

Hemagglutinating activity for ConA and its derivatives in renaturation studies by 2-fold serial dilution technique at pH 7.2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Native state</th>
<th>Renatured state</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hemagglutination titer</td>
<td>Minimal hemagglutinating concentration (µg/mL)</td>
</tr>
<tr>
<td>Native ConA</td>
<td>16</td>
<td>1.0</td>
</tr>
<tr>
<td>N-Dimethyl ConA</td>
<td>8</td>
<td>2.0</td>
</tr>
<tr>
<td>Acetyl-ConA</td>
<td>4</td>
<td>6.5</td>
</tr>
<tr>
<td>Succinyl-ConA</td>
<td>4</td>
<td>6.0</td>
</tr>
</tbody>
</table>

3.4.4. **Kinetics of reactivation of dimeric and tetrameric ConA**

The hemagglutination technique for the assay of lectin activity does not entail quantitative analysis of reactivation kinetics, and a more sensitive and reliable method would be necessary to monitor the activity quantitatively at the low concentrations of protein which need to be employed for reconstitution studies. To this end, a method using a combination of affinity
technique and OPA fluorometric procedure of protein estimation (which makes possible to quantitate as little as 100 ng of protein) has been developed for the quantitative analysis of lectin activity during reconstitution.

A plot of percent reactivation vs. time in the reconstitution studies of dimeric ConA at pH 5.2 is shown in Figure 29A. A small activity (~10%) persists up to about 80 min followed by an increase in activity to ~90% in 180 min. After 24 h, the yield of reactivation is close to 100%, that is, the activity of the native protein. These and previous results (based on fluorescence and CD) for the reconstituting protein suggest that the process of reactivation during 80-180 min involves the subunit association of the structured monomers to form the dimeric quaternary structure at pH 5.2. The reactivation data can be fitted to a second-order kinetics by the simple model involving monomers (N) and native dimers (N₂):

\[
k \quad 2N \rightarrow N₂
\]  

This may be analyzed mathematically as follows. The formation of dimer is given by

\[
d[N₂] / dt = k[N]^2
\]  

If \([N₀]\) is the total initial concentration of monomeric protein, then from mass balance \([N₀]\) can be expressed as

\[
[N₀] = [N] + 2[N₂]
\]  

Eq 15, after substitution of Eq 16 into it, can be integrated to

\[
- \frac{2}{[N₀]} + \frac{2}{([N₀] - 2[N₂])} = kt
\]  

Since the increase in percent reactivation is due to the formation of dimer, it is apparent that

\[
\frac{2}{([N₀] - 2[N₂])} = \frac{2}{[N₀]} \left(1 - \frac{A_t}{A_∞}\right)^1
\]  

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Figure 29. (A) A plot of percent reactivation vs. time during reconstitution of dimeric ConA at pH 5.2 as determined by a combination of affinity technique and OPA fluorimetric method of protein estimation. The symbol (■) represents the percent activation after 24 h. The data are shown for a final protein concentration of 0.8 μM.

(B) Second-order kinetic plot for the data given in (A) according to Eq 18, using the least squares analysis of the data.
where $A_1$ and $A_\infty$ represent the percent reactivation at time $t$ at infinite time, respectively.

Hence a plot of $(2 / [N_0]) (1 - A_1/A_\infty)^{-1}$ vs. $t$ should yield a straight line whose slope is directly related to the rate constant ($k$). Such a second-order plot (after correcting the reactivation data for approximately 10% of activity present up to 80 min) is shown in Figure 29B. The value of $k_1$ is obtained as $2.6 \times 10^2$ M$^{-1}$ s$^{-1}$.

The kinetics of reactivation after denaturation of tetrameric ConA at pH 7.2 is shown in Figure 30. The percent reactivation increases from ~10% to ~90% during 80 - 180 min, and the maximum yield of reactivation is close to 100% after 24 h. ConA tetramer is a "dimer of dimers" (Figure 1). Since no direct evidence was obtained so far about the population of dimeric intermediates in the reconstitution pathway, and further, the dimeric intermediate may contribute to the observed activity, it was not possible to analyze the reactivation data in terms of a simple bimolecular reaction, and the reactivation in this case may reveal a more complex kinetic pattern, which may be addressed in future studies.
Figure 30. A plot of percent reactivation vs. time during reconstitution of tetrameric ConA at pH 7.2 as determined by a combination of affinity technique and OPA fluorimetric method of protein estimation. The symbol (■) represents the percent activation after 24 h. The data are shown for a final protein concentration of 0.8 μM.