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ORIGINAL PAPER

Localization and Environment of Tryptophans in Different Structural States of Concanavalin A

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Abstract We have investigated the localization and environment of tryptophan residues in different quaternary and conformational states (tetrameric, dimeric, monomeric and unfolded) of metallized and demetallized concanavalin A (ConA) by selective chemical modification, fluorescence, and phosphorescence. ConA has four tryptophan residues (Trp 40, Trp 88, Trp 109 and Trp 182) per subunit. The pattern of oxidation by N-bromosuccinimide (NBS) shows that NBS modifies, in dimer, only Trp 182 which remains inaccessible in tetramer, two (Trp 88 along with Trp 182) in monomer, all four in unfolded form in presence of EDTA, and three (possibly Trp 40 along with Trp 88 and Trp 182) in unfolded form from native or remetallized ConA. Utilizing wavelength-selective fluorescence approach, we have observed a red edge excitation shift (REES) of 6–8 nm for tetramer and dimer. A more pronounced REES (11 nm) is observed for oxidized monomer compared to REES (3 nm) for unoxidized species. Acrylamide quenching shows the Stern-Volmer constant (K_{SV}) for dimer, monomer, unfolded ConA and unfolded apo-ConA being 3.8, 5.2, 12.8, 14.0 M^{-1}, respectively. Phosphorescence studies at 77 K give more structured spectra, with two (0,0) bands at 406.2 (weak) and 413.2 nm for tetramer. However, a single (0,0) band appears at 413.2 for dimer and 412.6 nm for monomer, while the (0,0) band of the oxidized monomer is red shifted to 414.4 nm. These results may provide important insight into subtlety of organization and environment of tryptophans in the context of folding and structural studies of oligomeric proteins including lectins.

Keywords Concanavalin A · Unfolding/Folding · Fluorescence · Phosphorescence · Tryptophan modification

Abbreviations

ConA	Concanavalin A, lectin from jack bean (*Canavalia ensiformis*)
ANS	8-anilino-1-naphthalenesulfonate
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid
NBS	N-bromosuccinimide
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
DLS	Differential light scattering
REES	Red edge excitation shift

Introduction

Concanavalin A (ConA), the oligomeric lectin protein from *Canavalia ensiformis* (jack bean) is the most widely utilized legume lectin in biology. Its uses include investigation of normal and tumor cell membrane structures and dynamics, probing glycosylation mutants of transformed cells, and isolation of carbohydrates, glycoconjugates and cells on ConA affinity matrixes [1, 2]. ConA is a tetramer at physiological pH, and exists as a dimer around pH 5. Each monomer possesses one carbohydrate (Man/Glc) binding site as well as a transition metal ion (Mn^{2+}) and a Ca^{2+}site [3]. The metal ions are in close proximity to the saccharide binding site, and demetallization of the lectin results in loss of its carbohydrate binding ability [4].

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Studies on lectin structure began with the x-ray diffraction analysis of ConA [5, 6], and the three-dimensional structure of ConA has presently been refined at 0.94Å [7]. The lectin monomer describes a jelly roll motif, a kind of antiparallel β-sandwich comprising a six-stranded flat 'back' β-sheet, a seven-stranded curved 'front', and a five-stranded 'top' β-sheet roofing the other two. The dimeric form of ConA is specified as the 'canonical dimer', which is characterized by a large 12-stranded β-sheet resulting from the antiparallel side-by-side alignment of the two six-stranded back β-sheets, one from each monomer. ConA tetramer is a 'dimer of dimers', formed by two canonical dimers joining the central parts of their back sheets in a perpendicular manner.

Extensive studies on interactions of carbohydrates with ConA have been reported, however, experimental studies on its folding/unfolding including protein stability have appeared in the literature only in past several years [8–10]. We have previously shown that the denaturant-induced equilibrium unfolding of tetrameric ConA involves a structured monomeric intermediate [11], and the kinetics of reconstitution of dimeric ConA from the denatured state reveals the monomer refolding and reassociation along with reactivation [12]. In this paper, we have explored the distinguishing structural aspects of different forms of ConA obtained during unfolding/folding under aqueous conditions, the ubiquitous solvent condition for biological systems. ConA has four tryptophan residues per monomer. Utilizing these tryptophans as intrinsic probes, we have investigated the localization and environment of tryptophans in different conformational states of metallized and demetallized ConA by fluorescence, and phosphorescence at 77 K. Further, the environments of tryptophan residues of ConA have also been studied by selective chemical modification (oxidation) of tryptophans [13] that become accessible in the process of subunit dissociation and unfolding.

Materials and Methods

Materials

Jack bean seeds, 8-anilino-1-naphthalenesulfonate (ANS), N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES) and acrylamide were purchased from sigma. Sephadex G100 was obtained from Pharmacia. Urea (AR, E.Merck) was further crystallized from hot ethanol to remove possible contamination by cyanate ions, and its concentration was determined by refractive index measurements [14]. Concentration of ANS was measured spectrophotometrically using its molar extinction coefficient, ε=5,000 M^{-1} cm^{-1} at 350 nm [15]. The purity of acrylamide was checked from its absorbance at 295 nm using ε=0.23 M^{-1} cm^{-1}, and optical transparency beyond 310 nm [16]. All other reagents used were of analytical grade. Double distilled water was used in all experiments.

Preparation of Different Forms of ConA

Native ConA was purified from the crude extract of jack bean seeds by affinity chromatography on Sephadex G 100 according to the published procedure [17]. The purity of the preparation was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [18]. At physiological pH, ConA exists as a tetramer while a dimeric form is obtained at a lower pH around 5. Demetallized ConA (apo-ConA) and the remetallized (Mn^{2+} and Ca^{2+}) form of ConA were prepared according to the procedure described [19]. Studies with apo-ConA were made at pH 5 under high salt concentration (1 M NaCl) to maintain the solubility of the protein. Under conditions of urea-induced equilibrium unfolding, an almost exclusive structured monomer of ConA was generated in 3.5 M urea [12]. Apparently two unfolded forms of ConA were obtained after denaturing native ConA and apo-ConA separately in 8 M urea.

The integrity of tetrameric structure of ConA at pH 7.2 (10 mM HEPES buffer containing 0.15 M NaCl) was determined by gel filtration analysis on a Bio-Gel P-100 column (1.1×100 cm). The size of other forms of ConA was confirmed from size-exclusion chromatography using a Superose-12 10/300 GL column attached to a Waters HPLC system. An aliquot of 200 μL of protein samples (20 μM) prepared at pH 5 (20 mM sodium acetate buffer containing 0.15 M NaCl), and by incubation of native ConA in 3.5 M and 8 M urea at pH 5 was injected into the column. The column was preequilibrated with the same buffer in which the protein samples were prepared. The flow rate was 0.5 mL/min, and eluent was detected on-line by Waters 2489 UV-Visible detector at 280 nm. The column was calibrated with the following marker proteins in appropriate buffer containing requisite concentrations of urea: bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and soybean trypsin inhibitor (20.1 kDa).

Protein concentration was determined spectrophotometrically at 280 nm using $A^{1\%,\ 1\ cm}$=12.4 at pH 5 [4], and 13.7 at pH 7.2 [3], and expressed in terms of monomer (M_r=26500).

Steady-state Absorption and Fluorescence Measurements

Ultraviolet absorption was measured in a Hitachi U 3210 double-beam spectrophotometer using Sigma cuvette (volume: 2 mL; path length: 1 cm).

Steady-state fluorescence measurements were done with a Hitachi F-4010 spectrofluorometer (equipped with a 150 W xenon lamp) using Sigma cuvette (volume: 2 mL; path length: 1 cm). The excitation and emission band pass was 5 nm each, and scan speed was 60 nm/min. All spectra were corrected by subtraction of appropriate blanks without ConA.

ANS binding experiments were performed at pH 5 with native ConA as well as apo-ConA in absence and presence of different concentrations of urea. In a typical experiment, protein sample (8 μM) was incubated with 100 μM ANS for 5 min at 25 °C. Excitation wavelength was fixed at 370 nm, and the emission scanned from 400 to 600 nm.

Chemical Modification

NBS oxidation of tryptophan to oxindolealanine [13] was performed at ambient temperature using different protein samples of ConA, viz., ConA tetramer, dimer, structured monomer in 3.5 M urea, unfolded monomer in 8 M urea in absence and presence of 10 mM EDTA as well as apo-ConA, remetallized ConA, samples of apo-ConA in 3.5 M and 8 M urea, and remetallized ConA in 8 M urea. ConA tetramer was oxidized in 10 mM HEPES buffer, pH 7.2 containing 0.15 M NaCl. For all other samples, the buffer was 20 mM sodium acetate, pH 5, containing 0.15 M NaCl or 1 M NaCl (for samples of apo-ConA). Samples (500 μg/mL) were treated with aliquots of NBS (2 M, freshly prepared) in a 1-cm path length cuvette. The reaction was followed by increase in absorbance at 250 nm (oxindolealanine) and decrease in absorbance at 280 nm (tryptophan). After 5 min incubation, the absorbance at 280 nm was recorded and corrected for dilution. The number of tryptophans oxidized by NBS was determined as described by Spande and Witkop [13].

Dynamic Light Scattering (DLS) Measurement

DLS measurements were done in BI 200SM Research Goniometer system, version 2.0 (Brookhaven Instrument Corporation) equipped with HeNe laser operating at a wavelength of 633 nm. Protein concentration used was 20 μM in 20 mM sodium acetate buffer, pH 5.

Fluorescence Quenching Measurements

Acrylamide quenching of tryptophan fluorescence was measured from the decrease of fluorescence intensity obtained after serial addition of small aliquots of freshly prepared acrylamide solution (2 M) to a sample of ConA taken in a cuvette followed by mixing and incubation for 5 min in the sample compartment in the dark. Excitation wavelength was 295 nm for each sample. Emission intensity was monitored at the respective emission wavelength maximum for each sample (336 nm for ConA tetramer, ConA dimer, apo-ConA; 341 nm for structured monomer in 3.5 M urea, and 352 nm for completely denatured ConA and apo-ConA). The fluorescence intensities obtained were corrected for dilution. Quenching data were analyzed by fitting to the Stern-Volmer equation [20]:

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, [Q] is the molar concentration of the quencher, and K_{SV} is the Stern-Volmer quenching constant.

Phosphorescence Measurements

Phosphorescence studies at 77 K were made using a Dewar system having a 5 mm o.d. quartz tube. The freezing of the samples at 77 K was carried out at the same rate for all the samples. Triplet state emissions were measured in a Hitachi F-4010 spectrofluorometer equipped with phosphorescence accessories. All the samples were prepared in 40% ethylene glycol in appropriate buffer. The samples were excited at 280 nm using a 10 nm band pass, and the emission band pass was 1.0 nm. The cryosolvent (40% ethylene glycol) used in the experiment was always found to form a clear glass. The low temperature (77 K) spectra were found to be reproducible and free from any polarization artifacts.

Results

Characterization of Different Structural Forms of ConA

Native ConA exhibits pH dependent tetramer-dimer equilibrium in aqueous solution [1]. The integrity of the tetrameric structure at pH 7.2 was confirmed from gel filtration analysis on a Bio Gel P-100 column, and the dimeric ConA was obtained at pH 5 when the protein was eluted as a single peak from a Superose-12 column corresponding to its dimeric molecular mass of 53 kDa (data not shown). The unfolded form of ConA was obtained when the protein was denatured in 8 M urea at pH 5. It was shown previously that the structured monomer of ConA developed under conditions of urea-induced equilibrium unfolding [12], and the monomer species was generated in 3.5 M urea which was eluted as a single peak from a Superose-12 column (data not shown). The steady-state tryptophan fluorescence shows distinguishing and characteristic emission maximum at 334±1 nm for tetramer and dimer, 340±1 nm for monomer and 352±1 nm for unfolded ConA in agreement with previous report [12]. Apo-ConA was obtained by acid demetallization of the protein [19],

and its tryptophan fluorescence at pH 5 (20 mM sodium acetate containing 1 M NaCl) shows similar emission characteristics as for native protein in absence or presence of urea (Fig. 1a). The emission maximum of apo-ConA at 0 M, 3.5 M and 8 M urea occurs at 334±1, 339±1, and 352±1 nm, respectively. Apo-ConA transforms to remetallized form [19] on addition of Mn^{2+} followed by Ca^{2+} at pH 5.

The structured intermediate obtained in 3.5 M urea during unfolding of ConA and apo-ConA at pH 5 was further characterized by ANS binding studies. Free ANS is feebly fluorescent in water, but its spectrum is blue shifted (520→470 nm) and its intensity is dramatically increased when it binds to nonpolar sites of proteins [21]. Figure 1b shows the ANS fluorescent spectra of apo-ConA, intermediate apo-ConA species in 3.5 M urea and unfolded apo-ConA in 8 M urea. As shown, the fluorescence intensity at 470 nm for the ANS-apo-ConA complex is small which, however, increases by ~10 fold for the ANS-intermediate complex whereas the ANS fluorescence for the unfolded

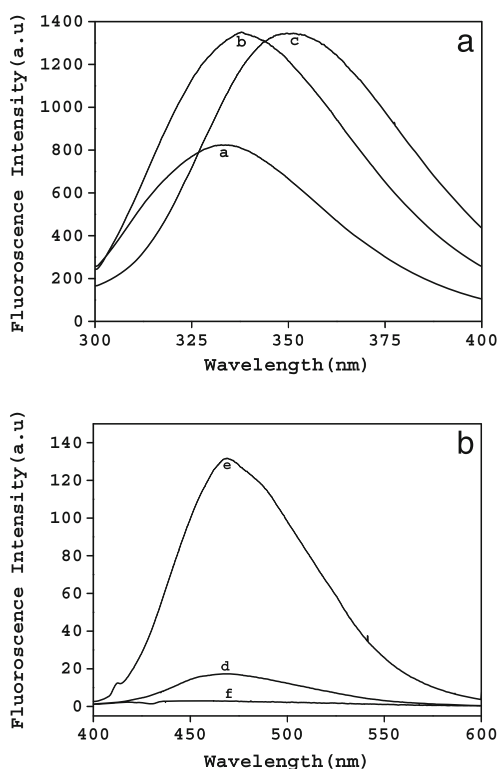

Fig. 1 **a** Fluorescence spectra of apo-ConA (**a**), apo-ConA intermediate in 3.5 M urea (**b**) and unfolded apo-ConA in 8 M urea (**c**). The buffer was 20 mM sodium acetate containing 1 M NaCl, pH 5. Protein concentrations were 8 μM. The spectra were corrected by subtracting the appropriate blank in each case. Excitation wavelength, 280 nm; excitation and emission band pass, 5 nm each; scan rate 60 nm/min. **b** ANS fluorescence spectra at pH 5 of apo-ConA in 0 M (**d**), 3.5 M (**e**) and 8 M (**f**) urea. Protein concentration was 8 μM and ANS was 100 μM in each case. Excitation wavelength, 370 nm; excitation and emission band pass, 5 nm each; scan rate, 120 nm/min

apo-ConA is negligible. These results clearly indicate an increased exposure of hydrophobic surfaces arising from quaternary dissociation to form a structured intermediate. These findings are similar to those obtained from urea-induced unfolding of native dimeric ConA at pH 5 [12]. The results are corroborated by theoretical calculation from the x-ray structure of apo-ConA [22]. The monomer-monomer interface area has been calculated using the program ProFace [23]. Upon subunit dissociation, there occurs an exposure of 2142Å^2 surface area per subunit, of which 1254Å^2 corresponds to non-polar (hydrophobic) patches. In this state, therefore, large clusters of hydrophobic sites are exposed to the solvent and hence impinged by molecules of ANS with a resultant increase in its fluorescence intensity and a blue shift of emission maximum from 520 nm to 470 nm.

Oxidation of Tryptophan Residues of ConA with N-bromosuccinimide (NBS)

Tryptophans in proteins can be modified selectively with N-bromosuccinimide (NBS), allowing to probe their localization and environment in protein structure. The oxidation reaction is rapid converting the indole side chain to oxindole, and leads to a loss of tryptophan fluorescence and a decrease in absorbance at 280 nm [13]. ConA has four tryptophan residues at positions 40, 88,109, and 182 of a subunit. The number of tryptophan residues oxidized per monomeric chain in different structural forms of ConA is given in Table 1. No tryptophan was susceptible to oxidation in the native tetramer even in presence of excess NBS, whereas only one tryptophan could be oxidized in the native dimer at pH 5, consistent with previous report [24]. After oxidation, aggregation took place quickly with the appearance of turbidity. DLS study has shown that the particle size was increased to ~300 nm from that of 10–12 nm for native dimer. The number of tryptophans oxidized for intermediate monomer in 3.5 M urea is two. The same pattern of tryptophan oxidation is observed for apo-ConA and apo-ConA intermediate in 3.5 M urea, revealing similar tryptophan environment for such metallized and demetallized species (Table 1). However, oxidation reaction of unfolded ConA and unfolded apo-ConA in 8 M urea shows that three tryptophans are modified for the former while all four get oxidized for the latter in agreement with previous literature [24]. Interestingly, the unfolded form obtained from remetallized ConA in 8 M urea shows that maximally three tryptophans could be oxidized as in case of unfolded form of native ConA (Table 1). When the unfolding reaction of native ConA in 8 M urea was carried out in presence of 10 mM EDTA, all four tryptophans of the resulting unfolded species get

Table 1 Oxidation of tryptophan residues of different forms of ConA by NBS

System	No. of Trp Oxidized[a]
Native Tetramer	0
Native Dimer	1
Intermediate Monomer	2
Apo-ConA	1
Apo-ConA Intermediate	2
Unfolded ConA	3
Unfolded Apo ConA	4
Unfolded Form from Remetallized ConA	3
Unfolded ConA in 10 mM EDTA	4

[a] Calculated per subunit of the protein.

oxidized by NBS. It thus appears that the presence of metal ions (Mn^{2+} and Ca^{2+}) during generation of the unfolded protein maintains the environment of one tryptophan residue to the extent so as to make it not accessible to NBS oxidation (Table 2).

Red edge Excitation Shift (REES) of ConA in Different Structural Forms Before and After NBS Oxidation

Red edge excitation shift (REES) refers to a shift in fluorescence emission maximum towards higher wavelength on changing the excitation wavelength towards the red edge of the absorption band. The red edge effects for tryptophans in proteins have been attributed mainly to the slow rates of solvent relaxation around the excited state tryptophan(s) due to motional restriction imposed on the solvent molecules around the fluorophore(s) [25, 26]. Another interpretation for REES centers on the ground-state heterogeneity in tryptophan environments in multi-tryptophan proteins leading to heterogeneity of individual spectra and coupling of red excitation/red emission spectra [27]. REES thus serves as a useful approach to probe the tryptophan environment in different structural states of a protein in its unfolding/folding pathway. Figure 2a shows

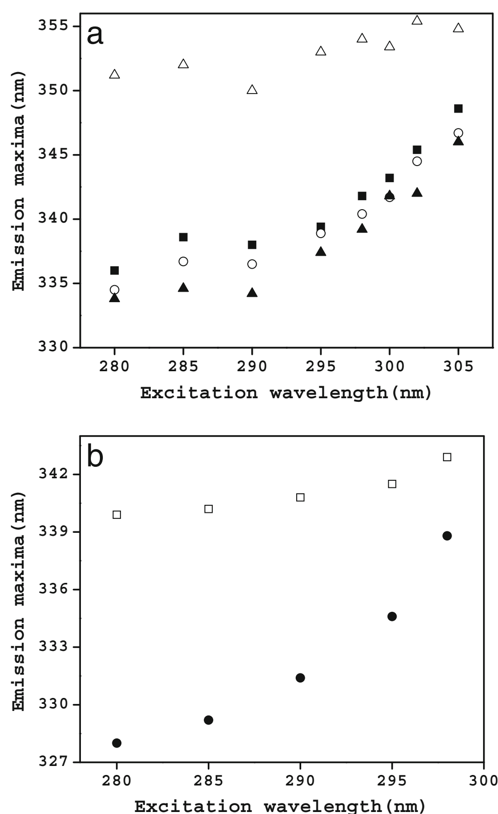

Fig. 2 Effect of changing excitation wavelength on the emission maximum for **a** ConA tetramer (*filled square*) at pH 7.2, ConA dimer (*open circle*) at pH 5, apo-ConA (*filled triangle*) at pH 5 under high salt concentration, and denatured ConA (*open triangle*) in 8 M urea at pH 5; **b** intermediate ConA monomer (*filled circle*) in 3.5 M urea and oxidized monomer (*open square*) at pH 5. Protein concentration was 4 μM. Excitation and emission band pass, 5 nm each; scan rate, 60 nm/min. The buffers used were 10 mM HEPES containing 0.15 M NaCl (pH 7.2), 20 mM sodium acetate containing 0.15 M NaCl or 1 M NaCl (pH 5)

the REES plot for ConA dimer, ConA tetramer, apo-ConA, and denatured ConA. As the excitation wavelength is changed from 280 to 300 nm, the emission maximum of ConA dimer is shifted from 334 to 342 nm, which corresponds to a REES of 8 nm. Similar REES effect (6–8 nm) was observed for ConA tetramer and apo-ConA (336 to 342 nm, and 334 to 342 nm respectively). The unfolded ConA in 8 M urea, however, exhibits no excitation wavelength dependence. ConA is a multitryptophan protein containing four tryptophan residues per monomer. REES effect may therefore be attributed to the average environment experienced by the tryptophans. The observation of analogous REES effect for ConA dimer, tetramer and apo-ConA implies similar average tryptophan environment for these species in which the immediate vicinity of at least some of the tryptophans is highly rigid. No REES is observed for the unfolded ConA due to the high flexibility of the tryptophan residues, when all or most of them become solvent-exposed.

Table 2 Accessible surface area (ASA) of tryptophan residues in different forms of ConA

System	ASA (Å^2) for Tryptophan Residues			
	Trp 40	Trp 88	Trp 109	Trp 182
ConA Tetramer	3.61	18.71	0.55	116.31
ConA Dimer	3.61	18.71	0.55	116.31
ConA Mononer	3.61	127.17	0.55	116.31
Apo-ConA Dimer	7.04	12.0	1.04	52.83
Apo-ConA Monomer	7.04	123.48	1.04	52.83

The shifts in fluorescence emission maxima for the intermediate monomer and the oxidized monomer of ConA as a function of excitation wavelength are shown in Fig. 2b. Interestingly, the intermediate monomer species (emission maximum, 340 nm) after NBS oxidation exhibits an emission maximum at 328 nm when excited at 280 nm, indicative of the buried tryptophans in the interior of the protein as the two exposed tryptophans got oxidized. The REES observed for the oxidized monomer is quite large (11 nm) compared to that for the unoxidized form (3 nm) in the excitation wavelength domain (280 to 298 nm). It may be mentioned that there appears to be a further red shift of emission maximum using excitation wavelength above 298 nm but the results are not included because of low signal-to-noise ratio and possible artifacts. For the oxidized protein, the REES effect is attributable only to the buried tryptophans which offer a highly rigid tryptophan environment. The REES for the oxidized forms of ConA dimer and apo-ConA could not be determined as turbidity appeared quickly in the solution. It is interesting to note that, when excited at 280 nm, the emission maximum of denatured ConA after NBS oxidation occurs at 324 nm characteristic of tryptophan emission whereas that of the oxidized form of denatured apo-ConA does not exhibit tryptophan emission maximum around 320 nm. These results support the occurrence of two distinct unfolded forms obtained from ConA in absence and presence of metal ions in the unfolding reaction, and are in excellent agreement with the studies of NBS oxidation described previously.

Acrylamide Quenching Studies

Acrylamide quenching of tryptophan fluorescence serves as a convenient method to probe tryptophan environments in proteins [28]. The Stern-Volmer plots of acrylamide quenching of tryptophans of the ConA dimer, monomer, denatured ConA and denatured apo-ConA are shown in Fig. 3. The slope (K_{SV}) of such plots is related to the degree of exposure (accessibility) of the tryptophans. Table 3 shows the quenching parameters obtained by analyzing the Stern-Volmer plots. The Stern-Volmer constant (K_{SV}) for the dimer, monomer, unfolded ConA and unfolded apo-ConA was found to be 3.8, 5.2, 12.8, 14.0 M^{-1}, respectively. The progressive increase in the K_{SV} values reflects increased exposure of tryptophans in dimer→monomer→denatured ConA→denatured apo-ConA. These results are in good agreement with the findings obtained from NBS oxidation.

Phosphorescence Studies at Low Temperature (77 K)

Phosphorescence studies of proteins at low temperature give structured spectra with a characteristic (0,0) band, which reveal the immediate environment of the tryptophan

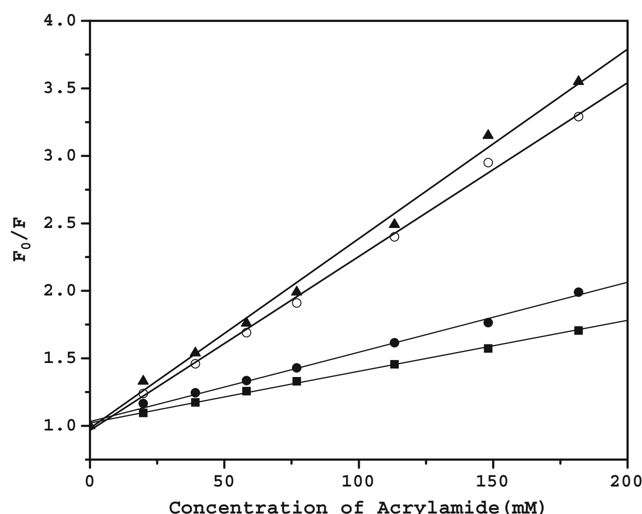

Fig. 3 Stern-Volmer plot of acrylamide quenching of fluorescence at pH 5 for ConA dimer (*filled square*), intermediate monomer (*filled circle*), unfolded ConA (*open circle*) and unfolded apo-ConA (*filled triangle*). F_0 is the fluorescence intensity in the absence of quencher, and F is the corrected fluorescence in the presence of quencher. Protein concentration was 4 μM. Excitation wavelength was fixed at 295 nm for all cases. Emission was monitored at 336 nm for ConA dimer, 341 nm for intermediate and 352 nm for unfolded species

residues [29]. The phosphorescence spectra for ConA tetramer, dimer and unfolded ConA in 40% ethylene glycol at 77 K are shown in Fig. 4a. As shown, the ConA tetramer gave rise to two distinct (0,0) bands located at 406.2 and 413.2 nm at excitation wavelength (λ_{exc}) of 280 nm, the former being weaker than the latter. However, for the ConA dimer, only a single (0,0) peak occurs at 413.2 nm without any splitting, and the unfolded ConA exhibits the (0,0) band at 409.0 nm. It may be mentioned that no significant difference in the phosphorescence was observed for the samples of apo-ConA (data not shown). The observation of multiple (0,0) bands in protein containing more than one tryptophan residue may be attributable to tryptophans located in different local environments. The band at shorter wavelength is due to the solvent exposed tryptophans whereas the band at longer wavelength arises from the buried tryptophans in the protein interior. Figure 4b shows the phosphorescence spectra for the intermediate ConA monomer and its oxidized form. The (0,0) band for the structured monomer was slightly blue shifted to 412.6 nm

Table 3 Acrylamide quenching of tryptophan fluorescence of ConA

System	K_{SV} (M^{-1})
ConA Dimer	3.8±0.2
Intermediate Monomer	5.2±0.3
Unfolded ConA	12.8±0.3
Unfolded Apo-ConA	14.0±0.3

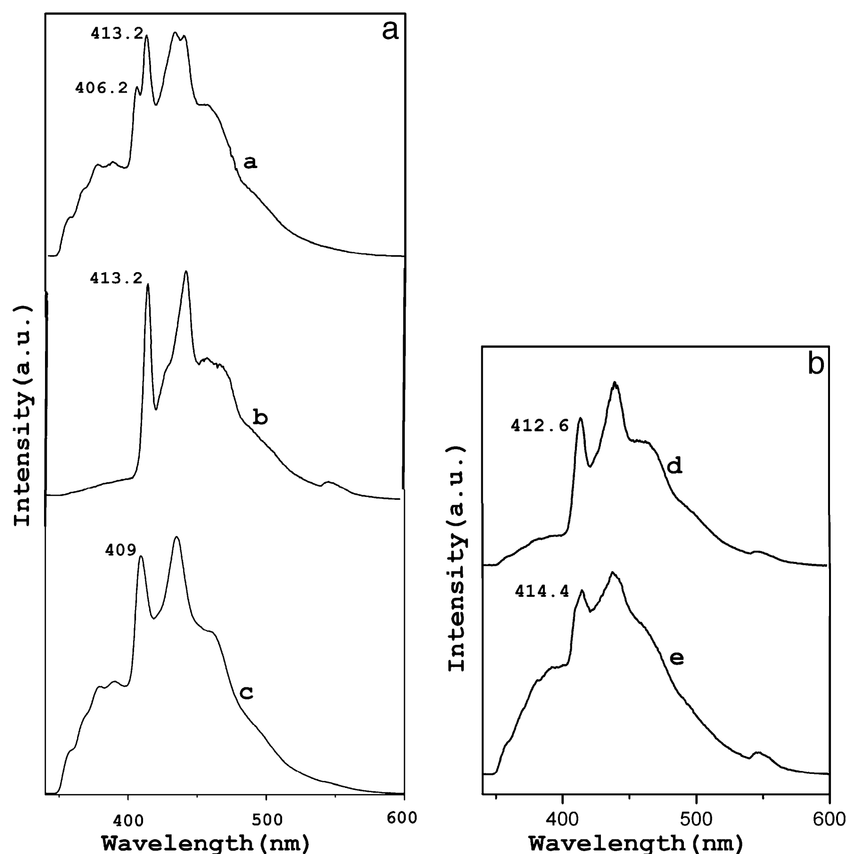

Fig. 4 Phosphorescence spectra for **a** ConA tetramer (**a**) at pH 7.2, ConA dimer (**b**) at pH 5 and unfolded ConA (**c**) in 8 M urea at pH 5; **b** intermediate monomer (**d**) in 3.5 M urea and oxidized monomer (**e**) at pH 5. Protein concentration was 20 μM. Excitation wavelength, 280 nm; excitation and emission band passes were 10 and 1 nm, respectively

compared to that for ConA dimer without any splitting of the band. This probably reflects the average tryptophan environment when two tryptophans are exposed and two remain buried in the monomer. Interestingly, for the oxidized species (when the two exposed tryptophans were modified) the (0,0) band exhibits the red shift to 414.4 nm as a single peak indicating the highly buried environment for the other two tryptophans in the species.

Discussion

Tryptophan residues in proteins serve as intrinsic probe, and can be characterized by selective chemical modification, fluorescence, and phosphorescence. Based on these techniques, we have examined the localization and environment of tryptophan residues in different quaternary and conformational states (tetrameric, dimeric, monomeric and unfolded) of metallized and demetallized ConA that are involved in the unfolding pathway of the protein. The pattern of modifications of the tryptophans by NBS (Table 1) has been analyzed from the x-ray crystal structure of the protein [22, 30]. ConA has four tryptophan residues (Trp 40, Trp 88, Trp 109 and Trp 182) per monomer (Fig. 5a). NBS oxidizes exposed but not buried indole side chains in folded proteins. For example, only two of the six tryptophan residues in soybean agglutinin (SBA) tetramer [31] and no tryptophan in pea lectin (PSL) dimer [32] are oxidized by NBS. The accessible surface area (ASA) of each tryptophan in ConA tetramer, dimer and monomer as well as apo-ConA dimer and monomer was calculated using NACCESS [33], and the results are shown in Table 2. It is seen that Trp 40, Trp 88, Trp 109 and Trp 182 in ConA tetramer and in dimer have same ASA values with a high ASA value of 116.3 Å^2 for Trp 182. Therefore the only Trp oxidized for ConA dimer appears to be Trp 182. However, no Trp is oxidized for tetramer although Trp 182 shows the same large ASA value as in dimer (Table 2). It thus appears that Trp 182 remains inaccessible to NBS in the quaternary tetrameric state probably due to the complex topology arising from the association of two canonical dimers in perpendicular fashion (Fig. 5c). On subunit dissociation, Trp 88 exhibits a large increase in ASA (ΔASA being 108.5 Å^2) (Table 2). It is seen that Trp 88 lies at the monomer-monomer interface of the dimer (Fig. 5b). Thus Trp 88, in addition to

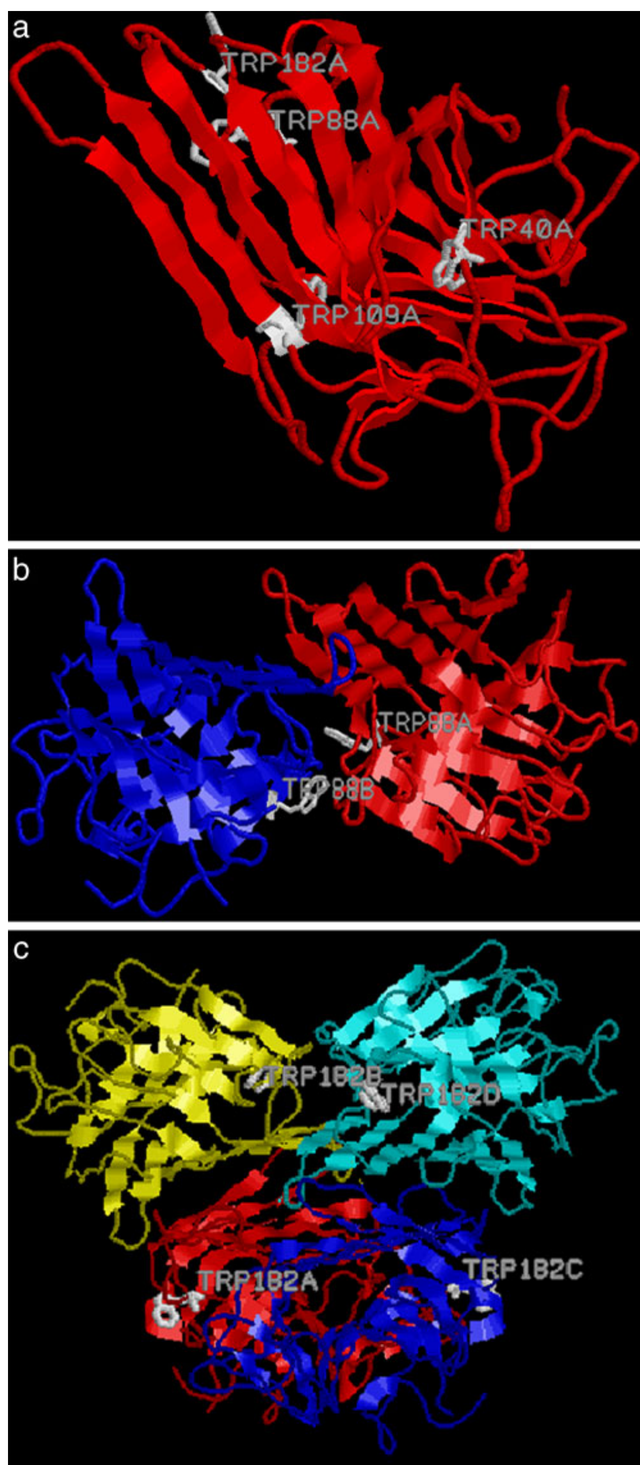

Fig. 5 **a** Ribbon representation of ConA monomer (PDB entry 2cna). The monomer is shown as subunit A in which four tryptophan residues are labeled and marked as white stick. **b** Ribbon representation of ConA dimer showing one tryptophan residue (Trp 88) of each subunit at the interface. Other tryptophan residues for a subunit are not shown for clarity. **c** Ribbon representation of ConA tetramer highlighting only Trp 182 of each subunit

Trp 182, of the monomer is exposed to the solvent, as well as NBS reagent, and therefore get oxidized. The ASA values calculated for apo-ConA dimer and monomer (Table 2) also exhibit similar pattern as for native ConA, and the same tryptophan residues are involved in the oxidation of apo-ConA and its intermediate in 3.5 M urea. For the unfolded apo-ConA, all four tryptophans are accessible to NBS, however, one of them remains resistant to oxidation in the unfolded form obtained from both native ConA and remetallized ConA. This tryptophan, however, becomes accessible to NBS in the unfolded form generated from native ConA in presence of EDTA that complexes with the metal ions during unfolding. Examination of the crystal structure of ConA shows that between Trp 40 and Trp 109, each having a very low ASA value (Table 2), Trp 40 is close to the compact network of amino acid residues [30] bonded to Ca^{2+}. The relative localization and rigidity of Trp 40 environment may possibly be less perturbed or at least partially maintained in the unfolded form obtained from native ConA or remetallized ConA, which may render this tryptophan inaccessible to NBS. This is apparent, as in presence of EDTA which sequesters the metal ions, Trp 40 along with other three are oxidized by NBS.

Using fluorescence, the magnitude of REES could be employed to ascertain the relative rigidity of the region surrounding the tryptophans [25–27]. The tetrameric ConA as well as the dimeric forms of native Cona and apo-ConA exhibit similar REES (6–8 nm). As the REES effect may be attributed to the average environment experienced by the tryptophans, it is likely that the tryptophans that are shielded from bulk solvent would contribute predominantly to the REES due to the presence of restricted water molecules in their vicinity. This implies that all or most tryptophans of the above species are localized in a rigid environment, and hence buried, which agrees fairly well with the studies of chemical modification. When two tryptophans become exposed in the monomer, REES diminishes to 4 nm in the excitation wavelength from 280 to 300 nm. However, oxidized monomer (in which the exposed tryptophans were modified) displays a remarkable increase in REES to a value of 11 nm even in the smaller excitation wavelength domain (280–298 nm). Considering that the buried tryptophans contribute largely to the REES effect, the above results clearly depict the localization and environment of tryptophans in the different structural states of ConA. With all or most tryptophans being exposed in the unfolded forms of native and apo-ConA, no REES effect is obtained. These results regarding solvent exposure of tryptophans have been further supported by acrylamide quenching experiments.

Compared to the fluorescence spectra of tryptophan residues which are generally broad, phosphorescence studies

at 77 K provide more structured spectra, characteristic of tryptophan environment. The position of the (0,0) band could be correlated with the solvent exposure of the tryptophan residue, though the local charges and the rigidity of the environment may also play their role. The blue-shifted phosphorescence can be attributed to the lower polarizability of the environment and the less stabilization of the triplet state by rigid solvation geometry for the exposed tryptophans whereas the red-shift of phosphorescence emission occurs due to the tryptophan residues located in a buried polarizable environment that stabilizes the triplet state more than the ground state [34]. For the ConA dimer, the (0,0) band appears as a single peak at 413.2 nm showing red-shifted phosphorescence for the buried tryptophans though one of the tryptophans (Trp 182) is solvent exposed. The tryptophans in this case are not optically resolved. In sharp contrast, the (0,0) band for the ConA tetramer is found to split at 406.2 nm (weak) and 413.2 nm showing two distinct kinds of tryptophan environments. Such spectral resolution in case of tetramer may possibly arise due to the complex topology resulting from the dimer-dimer association in perpendicular fashion. For the structured monomer (in which Trp 88 and Trp 182 are exposed), the (0,0) band exhibits a small blue shift to 412.6 nm, though no splitting was obtained. When the two exposed tryptophans are modified, the (0,0) band of the oxidized monomer is red shifted to 414.4 nm confirming the highly buried environment for the two tryptophans (Trp 40 and Trp 109) in the species.

In conclusion, the tryptophan localization and environment of ConA is characterized by unique structural features that are maintained in specific quaternary, tertiary and denatured states during unfolding of native as well as metal free protein. These results provide novel insight into the subtlety of organization and environment of tryptophans, which may assume major significance in the context of investigation of structure and folding of oligomeric proteins in general and lectins in particular.

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