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Research paper

Organization and dynamics of tryptophan residues in tetrameric and monomeric soybean agglutinin: Studies by steady-state and time-resolved fluorescence, phosphorescence and chemical modification

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ABSTRACT

We have investigated the organization and dynamics of tryptophan residues in tetrameric, monomeric and unfolded states of soybean agglutinin (SBA) by selective chemical modification, steady-state and time-resolved fluorescence, and phosphorescence. Oxidation with N-bromosuccinimide (NBS) modifies two tryptophans (Trp 60 and Trp 132) in tetramer, four (Trp 8, Trp 203 and previous two) in monomer, and all six (Trp 8, Trp 60, Trp 132, Trp 154, Trp 203 and Trp 226) in unfolded state. Utilizing wavelength-selective fluorescence approach, we have observed a red-edge excitation shift (REES) of 10 and 5 nm for tetramer and monomer, respectively. A more pronounced REES (21 nm) is observed after NBS oxidation. These results are supported by fluorescence anisotropy experiments. Acrylamide quenching shows the Stern–Volmer constant (K_{SV}) for tetramer, monomer and unfolded SBA being 2.2, 5.0 and 14.6 M^{-1}, respectively. Time-resolved fluorescence studies exhibit biexponential decay with the mean lifetime increasing along tetramer (1.0 ns) to monomer (1.9 ns) to unfolded (3.6 ns). Phosphorescence studies at 77 K give more structured spectra, with two (0,0) bands at 408.6 (weak) and 413.2 nm for tetramer. However, a single (0,0) band appears at 411.8 and 407.2 nm for monomer and unfolded SBA, respectively. The exposure of hydrophobic surface in SBA monomer has been examined by 8-anilino-1-naphthalenesulfonate (ANS) binding, which shows ~20-fold increase in ANS fluorescence compared to that for tetramer. The mean lifetime of ANS also shows a large increase (12.0 ns) upon binding to monomer. These results may provide important insight into the role of tryptophans in the folding and association of SBA, and oligomeric proteins in general.

1. Introduction

Oligomeric proteins comprise lectins as an important class that bind carbohydrates with high specificity, and are involved in numerous cellular processes such as cell–cell and host–pathogen interactions, targeting proteins within cells, lymphocyte homing, and tissue development [1–3]. Lectins form a highly diverse group of proteins comprising many protein families [4]. The plant lectins [5], and the animal lectins such as the galectins [6], the C-, I- and P-type lectins [7–9] provide classic examples of such families. However, the richest source of lectins is the seeds of leguminous plants, and legume lectins are among the most thoroughly studied families of proteins. The members of this family display an oligomeric state as dimer or tetramer. The distinct quaternary structures of these proteins dictate well-defined spacing between the carbohydrates' binding sites, and thus, the quaternary organization of these lectins assumes prime importance for their function. Dimerization of legume lectin occurs in various modes involving the six-stranded β sheets of the two monomers: i) a side-by-side 'canonical' mode forming a continuous 12-stranded antiparallel β sheet as exemplified by concanavalin A [10], and ii) two back-to-back modes, one represented by *Erythrina corallodendron* lectin [11] and the other by *Griffonia simplicifolia* lectin [12]. The tetrameric forms can, however, be considered as 'dimers of dimers' involving different modes of dimer–dimer association [1]. The easy availability of the legume lectins and their known three-dimensional structures have turned members of this family into excellent model systems for investigation of 'protein folding problem' of oligomeric proteins.

Soybean agglutinin (SBA) is a GalNAc/Gal-specific legume lectin. It is a glycoprotein with one Man-9 oligomannose type chain per

Abbreviations: SBA, soybean agglutinin, lectin from soybean (*Glycine max*); HEPES, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; ANS, 8-anilino-1-naphthalenesulfonate; NATA, N-acetyl-L-tryptophanamide; NBS, N-bromosuccinimide; FPLC, fast-protein liquid chromatography; REES, red-edge excitation shift.

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monomer [13], and exists as a tetramer in native state. The tetrameric structure of the protein involves back-to-back and nearly parallel association of two 'canonical' dimers that interact through contacts between their two outermost strands, creating a channel between them [14,15].

In recent years, the theoretical and experimental investigations of folding and association problem of legume lectins have been providing important insights into their structure, stability and function [16–18]. The role of glycosylation on the stability and unfolding of SBA has been reported [19]. For SBA, it has been shown that the quaternary dissociation of the tetramer to the tertiary monomers can occur under conditions of i) urea-induced equilibrium unfolding [20,21], and ii) relatively low pH [22]. Recently, we have demonstrated that structured SBA monomer develops and accumulates during renaturation from the completely unfolded state, and reforms the tetramer with a kinetic mechanism in which the monomer-to-dimer association is involved in the rate-determining step of the oligomerization reaction [23]. In this article, we have explored the distinguishing structural aspects of SBA in its native quaternary state vis-à-vis the intermediate tertiary and the unfolded states under aqueous conditions, the ubiquitous solvent condition for biological systems. SBA has six tryptophan residues per subunit. Utilizing these tryptophans as intrinsic probes, we have investigated the organization and dynamics of these probes in tetrameric, monomeric and denatured states of SBA by steady-state and time-resolved fluorescence, and by phosphorescence at 77 K. Further, the structural environments of tryptophan residues in the native, intermediate and unfolded states of SBA have also been studied by selective chemical modification (oxidation) of tryptophans [24] that become exposed in quaternary dissociation and unfolding.

2. Materials and methods

2.1. Materials

Soybean meal was purchased from Sigma. Cross-linked guar gum matrix was prepared as described [25]. 8-Anilino-1-naphthalenesulfonate (ANS), N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES), N-acetyl-L-tryptophanamide (NATA) and acrylamide were obtained from Sigma. The concentration of ANS was determined spectrophotometrically [26] using its molar extinction coefficient, $\varepsilon = 5000\ M^{-1}\ cm^{-1}$ at 350 nm. The purity of acrylamide was checked from its absorbance at 295 nm using $\varepsilon = 0.23\ M^{-1}\ cm^{-1}$ and optical transparency beyond 310 nm [27]. Urea (AR, E. Merck) was further crystallized from hot ethanol to remove possible contamination by cyanate ions. All other reagents used were of analytical grade. Double distilled water was used throughout.

2.2. Protein purification

SBA was purified from the crude extract of soybean meal by affinity chromatography on cross-linked guar gum matrix [28]. Since aggregation of SBA occurs on storage in the lyophilized state, affinity-purified SBA was precipitated by ammonium sulfate (80% saturation) and dialyzed against appropriate buffer before use in different experiments. The integrity of quaternary structure of SBA was confirmed from gel filtration analysis on Sephadex G-100 column when the protein was eluted as a single peak corresponding to its tetrameric molecular mass. The purity of the preparation was also checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [29]. Protein concentration was determined spectrophotometrically using $A^{1\%,\,1\ cm} = 12.8$ at 280 nm and expressed in terms of monomer ($M_r = 30\ 000$) [30].

2.3. Size-exclusion chromatography

The experiments on size-exclusion chromatography were carried out using a Superose-12 10/300 GL column attached to a Pharmacia FPLC system. The column was preequilibrated with 10 mM Glycine–HCl buffer, pH 2.0, containing 0.15 M NaCl, 1 mM Ca^{2+} and 1 mM Mn^{2+}. The protein solution (20 mM) was prepared at pH 2.0 and kept at room temperature for 15 min before injecting an aliquot of 200 μL into the column. The flow rate was 0.5 mL/min, and eluent was detected on-line by absorbance at 280 nm. The gel filtration of SBA by FPLC was also performed in 10 mM HEPES buffer pH 7.2, containing 0.15 M NaCl, 1 mM Ca^{2+} and 1 mM Mn^{2+}.

2.4. Chemical modification

Oxidation of tryptophan to oxindolealanine with NBS [24] was performed at ambient temperature using different protein samples of SBA. SBA tetramer was oxidized in 20 mM sodium acetate buffer, pH 5.0 containing 0.15 M NaCl, and the reaction with SBA monomer was carried out in 10 mM Glycine–HCl buffer, pH 2.0 containing 0.15 M NaCl. For oxidation of unfolded SBA, the sample was obtained after incubation of SBA for 18 h at 37 °C in acetate buffer, pH 5.0 containing 8 M urea. Samples (500 μg/mL) were treated with aliquots of NBS (2 M, freshly prepared) in a 1-cm path length cuvette. The reaction was monitored by the increased absorbance at 250 nm (oxindolealanine) and decreased absorbance at 280 nm (tryptophan). Following 5 min incubation, the absorbance at 280 nm was recorded and corrected for dilution. The number of tryptophans oxidized by NBS was calculated as described by Spande and Witkop [24] using a $-\Delta\varepsilon_{280}$ value of 4200.

2.5. 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 performed 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 SBA. The fluorescence quantum yield (Φ) was determined in each case by comparing the corrected emission spectrum of the samples obtained at excitation wavelength of 295 nm with that of L-tryptophan in water ($\Phi = 0.14$) [31] considering the total area under the emission curve.

ANS binding experiments were carried out with SBA at different pH values in the pH range 2–7.2, and also with denatured SBA in 8 M urea. The buffers used were 10 mM Glycine–HCl containing 0.15 M NaCl (pH 2–3.5), 20 mM sodium acetate containing 0.15 M NaCl (pH 4–5.5), 10 mM sodium phosphate containing 0.15 M NaCl (6–6.5), and10 mM HEPES containing 0.15 M NaCl (pH 7.2). In a typical experiment, protein sample (6.7 μM) was incubated with 100 μM ANS for 5–30 min at 25 °C. Excitation wavelength was fixed at 370 nm, and the emission scanned from 400 to 600 nm.

Anisotropy measurements were performed using Hitachi polarization accessories. Steady-state fluorescence anisotropy (A) was calculated as follows:

$$A = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively. G is the grating factor that corrects for