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Quaternary association and reactivation of dimeric concanavalin A

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Abstract

The reconstitution of dimeric concanavalin A (ConA) in terms of quaternary association and reactivation, after denaturation in urea, has been investigated using intrinsic fluorescence, 8-anilino-1-naphthalenesulfonate (ANS) binding, far-UV circular dichroism (CD), and an activity assay developed through a combination of affinity binding and the *o*-phthalaldehyde (OPA) procedure of protein estimation. The equilibrium denaturation of dimeric ConA in urea exhibits a biphasic unfolding pathway involving an intermediate with hydrophobic exposure, and the overall free energy of stabilization for the dimeric protein is obtained as 16.3 kcal mol^{-1}. The time course of reassociation and regain of activity during reconstitution reveals that the reactivation of ConA runs almost parallel to the process of subunit association. The reactivation reaction follows second-order kinetics, with a rate constant (k) of 2.6×10^2 M^{-1} s^{-1}. These results may provide insight into the relationship between quaternary association and function of legume lectins.

Keywords: Concanavalin A; Reassociation; Reactivation kinetics

1. Introduction

Lectins are oligomeric carbohydrate-binding proteins that are recognized as ubiquitous in virtually all living systems, ranging from viruses to bacteria and plants to animals [1,2]. Lectins form a highly diverse group of proteins consisting of many protein families [3]. The legume lectin family [4,5], and the families of animal lectins such as the galectins [6], the C-, I- and P-type lectins [7–9] are classic examples of such families. However, the richest source of lectins is the seeds of leguminous plants, and legume lectins are among the most intensively investigated families of proteins. Their exquisite specificity and high affinity for glycoconjugates have found many applications in biological and biomedical research [10].

Concanavalin A (ConA), the lectin from *Canavalia ensiformis* (jack bean) is the most widely used lectin in biology. Its uses include probing normal and tumor cell membrane structures and dynamics, studying glycosylation mutants of transformed cells, and yielding preparations of polysaccharides, glycopeptides and glycoproteins from ConA affinity columns [11]. ConA is a Glc/Man-specific lectin, and exists as a dimer around pH 5. Each monomer (M_r = 26,000) possesses one carbohydrate-binding site as well as a transition metal ion site (S1) (typically Mn^{2+}) and a Ca^{2+} site (S2) [12]. The lectin, however, exhibits high affinity for oligosaccharides of cell surface glycoproteins and glycolipids by interactions through extended binding site [13–15].

Structural studies on lectins started with the crystal structure of ConA [16,17]. The three-dimensional structure of ConA has presently been refined at 0.94 Å [18]. The lectin monomer describes an antiparallel β-sandwich comprising a nearly flat six-stranded back β-sheet, a curved seven-stranded front β-sheet, and a five-stranded top β-sheet that forms a roof-like structure above the other two. This β-sandwich architecture of ConA monomer is essentially conserved in the family of legume lectins, and the structures are nearly superimposable, irrespective of the specificity of the lectins [19]. The process of oligomerization in legume lectins involves the six-stranded back β-sheet in various ways, and the resulting mutual disposition of these β-sheets in the participating monomers leads to diverse quaternary structures. In ConA, the two back β-sheets interact side-by-side to form a

Abbreviations: ConA, concanavalin A, lectin from jack bean (*Canavalia ensiformis*); ANS, 8-anilino-1-naphthalenesulfonate; OPA, *o*-phthalaldehyde; CD, circular dichroism

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12-stranded contiguous sheet, which has been described as the 'canonical' mode of legume lectin dimerization. On the other hand, the dimeric structure of the lectin from *Erythrina corallodendron* (EcorL), the winged bean basic agglutinin (WBA I), and the winged bean acidic agglutinin (WBA II) involves a 'handshake' kind of quaternary structure with a relatively reduced buried intersubunit interface [20–22]. Thus legume lectins, which display various kinds of quaternary association, can serve as excellent model systems for the investigation of folding and association reactions of oligomeric proteins.

Notwithstanding numerous and extensive studies on lectin structure and lectin–carbohydrate interactions, relatively little information is available on the folding and association reactions of lectins. In the past few years, reports on reversible unfolding of several legume lectins have appeared in the literature [23,24]. Recently, we have demonstrated that the equilibrium unfolding reactions of soybean agglutinin and tetrameric ConA involve the formation of a structured monomeric intermediate [25,26]. These studies have shown that the dissociation process can be isolated from the subsequent unfolding of the polypeptide chains. Thus dissociation–association phenomena may be investigated without the added complication of monomer unfolding and refolding.

The oligomerization of legume lectins has important functional implications. The quaternary structure endows the lectin with a potential for multivalent binding to cells, and the requisite topology of the binding sites in the specific quaternary structure imparts the fine specificity necessary for its biological activity. In this context, studies on quaternary association and reactivation during reconstitution of the lectins from their denatured state would be of much interest. Reconstitution studies of oligomeric proteins, using kinetic approaches, serve to delineate the pathway of renaturation to native quaternary structure [27]. We have previously reported a kinetic study of the reassociation of tetrameric soybean agglutinin that follows second-order kinetics, with the monomer-to-dimer association as the rate-determining step of the oligomerization reaction [28]. In this paper, we present the characteristics of urea-induced equilibrium denaturation of dimeric ConA, and the kinetics of reconstitution of the protein from the denatured state in terms of quaternary association and reactivation. The present study reveals a biphasic denaturation pathway of dimeric ConA, and its reactivation occurring almost parallel to the process of quaternary association.

2. Materials and methods

2.1. Materials

Jack bean seeds were purchased from Sigma. Sephadex G-100 and Bio-Gel P-100 were obtained from Amersham Biosciences and Bio-Rad Laboratories, respectively. 8-Anilino-1-naphthalenesulfonate (ANS) and *o*-phthalaldehyde (OPA) were purchased from Sigma. Urea (AR, E. Merck, India) was further crystallized from hot ethanol to remove possible contamination by cyanate ions [29], and its stock solution was prepared on a dry weight basis [30]. All other reagents used were of analytical grade. Double distilled deionized water was used throughout.

2.2. Protein purification

Native ConA was purified from jack bean seeds according to the published procedure [31] using Sephadex G-100 as an affinity matrix. The purity of the preparation was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [32], and the assay of activity was done by hemagglutination assay [33] using 3% suspension of trypsin-treated rabbit erythrocytes. Native ConA exhibits dimer–tetramer equilibrium in aqueous solution, and exists as a dimer around pH 5. The integrity of dimeric structure of ConA at pH 5.2 (20 mM sodium acetate buffer containing 0.15 M NaCl, 1 mM Mn^{2+} and 1 mM Ca^{2+}) was confirmed by size-exclusion chromatography on a Bio-Gel P-100 column (1×75 cm) when the protein was eluted as a single peak corresponding to its dimeric molecular mass. The column was precalibrated with the following marker proteins: bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), and soybean trypsin inhibitor (20.1 kDa). The concentration of ConA was determined spectrophotometrically at 280 nm using $A^{1\%}$, 1 cm = 12.4 at pH 5.2, and expressed in terms of the monomer (M_r = 26000) [12].

2.3. Spectroscopic measurements

Absorption spectra were recorded on a Hitachi U 3210 UV–vis spectrophotometer using a Sigma cuvette (volume: 2 mL; path length: 1 cm).

Fluorescence spectroscopy was performed on a Hitachi 4010 spectrofluorimeter equipped with a constant temperature cell holder. The spectra were measured at 25 °C using a Sigma fluorimeter cuvette (volume: 2 mL; path length: 1 cm). For intrinsic fluorescence measurements, the excitation wavelength was fixed at 280 nm, and the emission scanned from 300 to 400 nm. The fluorescence intensity at 336 nm (which was the wavelength maximum for the native state) gave a greater measurable change and was used as the preferred signal for following the denaturation. Relative change of the fluorescence intensity at 336 nm in urea was determined as percent change relative to the intensity of the native protein at 336 nm. In case of ANS binding experiments, the excitation wavelength was fixed at 370 nm, and the emission scanned from 450 to 550 nm. The excitation and emission band pass was 5 nm each, and the scan rate was 60 nm min^{-1}.

Far-UV CD spectra were measured on a JASCO J-720 spectropolarimeter purged with N_2, and equipped with a constant temperature cell holder. The spectra were mea-