Kinetic Analysis of Subunit Oligomerization of the Legume Lectin Soybean Agglutinin†

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ABSTRACT: The reconstitution of soybean agglutinin (SBA), a tetrameric GalNAc/Gal-specific legume lectin, after denaturation in urea has been studied using fluorescence, far-UV CD, a hemagglutination assay, and chemical cross-linking with glutaraldehyde as a bifunctional reagent. The reconstituted protein exhibits similar quaternary structure and activity as of native lectin. The kinetics of subunit oligomerization has been determined from the cross-linking reaction of the reconstituting protein followed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Monomers and tetramers could be quantitatively analyzed during reconstitution. Dimers are not detectable. The reassociation reaction follows second-order kinetics. The results are described by a kinetic mechanism in which the monomer-to-dimer association (characterized by a second-order rate constant (k_1) of 1.4×10^4 M^{-1} s^{-1} at 37 °C) is involved in the rate-determining step of the oligomerization reaction.

Legume lectins, a class of homologous oligomeric carbohydrate-binding proteins, exhibit a large variation in their quaternary structure arising from small alterations due to sequence variations in essentially the same tertiary structural fold of the molecule (*1*−*3*). Subunit oligomerization and the multivalency that develops as a consequence are of considerable importance to the structure and biological activity of these proteins (*4*−*6*). The tertiary structure of each subunit of legume lectin describes a jelly roll motif that comprises three antiparallel β-sheets: a nearly flat six-stranded back β-sheet, a curved seven-membered front β-sheet, and a five-membered top β-sheet that forms a roof-like structure above the other two (*5*). 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. For example, in concanavalin A (ConA) (*7*, *8*), the two back β-sheets interact side-by-side to form a 12-stranded contiguous sheet, which has been described as the canonical mode of legume lectin dimerization. Tetrameric assembly of ConA then results from back-to-back association of the two side-by-side dimers that are inclined in perpendicular manner. 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 (*9*−*11*). These observations reveal that legume lectins, which display various dimeric as well as tetrameric quaternary structures, can serve as excellent model systems for the investigation of folding and association reactions of oligomeric proteins addressing the effect of subunit oligomerization on their stability, structure, and function.

Soybean agglutinin (SBA)[1] is a GalNAc/Gal-specific plant lectin of the legume family and shows important biological properties, including its ability to induce mitogenicity in lymphocytes (*12*) and to localize carbohydrate receptors on the surface of normal and transformed cells (*13*). SBA is a tetrameric glycoprotein with one Man_9 oligomannose type chain per monomer (*14*). The quaternary structure of the protein involves the back-to-back association of two canonical dimers aligned nearly in a parallel fashion (Figure 1) (*15*, *16*).

Despite extensive studies of lectin−carbohydrate interactions, and elucidation of over 200 three-dimensional structures of lectins from plants, animals, bacteria, and viruses, relatively little information is available on their mechanism of folding and quaternary association. The theoretical and experimental aspects of unfolding and folding processes of legume lectins are currently under intensive investigation in a number of laboratories. A theoretical study of oligomerization of legume lectins with a view to characterizing and rationalizing the variability in their quaternary association has been reported by Prabu et al. (*17*). The relationship between protein stability and oligomerization in the legume lectin family has been highlighted in a recent review (*18*). Using solvent denaturation experiments, Mitra et al. (*19*) have shown that the differences in conformational stability of the dimeric ConA and winged bean acidic agglutinin appear to reflect their different modes of subunit association. Recently, we have demonstrated that the equilibrium unfolding reactions of tetrameric SBA and ConA

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[1] Abbreviations: SBA, soybean agglutinin, lectin from soybean (*Glycine max*); PBS, 0.01 M sodium phosphate buffered with 0.15 M sodium chloride, pH 7.2; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism.

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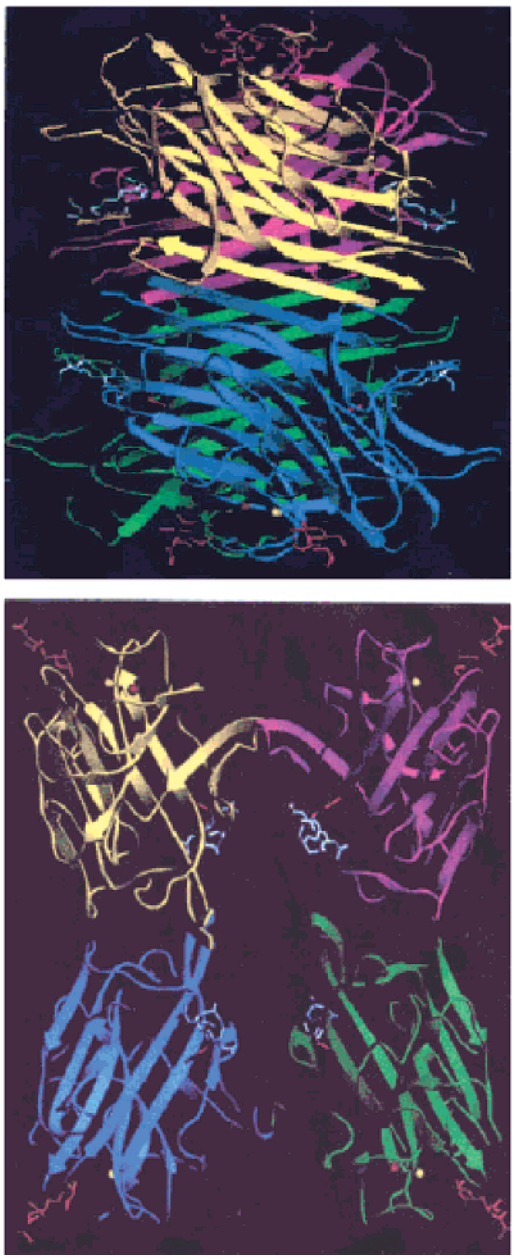

FIGURE 1: Structure of the SBA tetramer. (Top panel) Schematic diagram of two SBA dimers that compose the tetramer from the perspective of the *z* axis (first sheet: yellow and blue subunits; second sheet: green and pink subunits). The tetramerization involves back-to-back association of two side-by-side dimers that are nearly parallel to each other. (Bottom panel) The representation of the SBA tetramer from the perspective of the *x* axis of the unit cell (a 90° rotation from the top panel). (Reprinted with permission from ref *15*.)

involve the formation of a structured monomeric intermediate (*20*, *21*). These studies have shown that the dissociation process can be isolated from the gross unfolding of the polypeptide chains. Thus, dissociation−association phenomena may be studied without the added complication of monomer unfolding and refolding.

Reconstitution studies of oligomeric proteins, using kinetic approaches, give access to intermediates of association that, in turn, serve to delineate the pathway of reconstitution of native quaternary structure (*22*). In this paper, we present some aspects of kinetics of subunit oligomerization of SBA. To determine whether the rate-limiting step of the association reaction may be attributed to dimer or tetramer formation, we have used an approach based on a fast cross-linking reaction during reassociation (*23*). The present study reveals the sequence of events that are involved in the subunit association of SBA during its reconstitution from the denatured state.

MATERIALS AND METHODS

Materials. Soybean meal was purchased from Sigma. Cross-linked guar gum matrix was prepared as described (*20*). Sephadex G-100 was obtained from Pharmacia. Urea (AR, E. Merck, India) was further crystallized from hot ethanol to remove possible contamination by cyanate ions, and its stock solution was prepared on a dry weight basis. Glutaraldehyde [50% (m/v) aqueous solution] and sodium deoxycholate were purchased from Sigma. All other reagents used were of analytical grade. Double distilled deionized water was used throughout.

Protein Purification. SBA was purified from the crude extract of soybean meal by affinity chromatography on cross-linked guar gum matrix (*24*). The integrity of the tetrameric structure of SBA was confirmed from gel filtration analysis on a 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) (*25*). Protein concentration was determined spectrophotometrically using $A^{1\%,1cm} = 12.8$ at 280 nm and expressed in terms of monomer ($M_r = 30\,000$) (*26*).

Activity Assay. The hemagglutinating activity of the lectin was assayed by the 2-fold serial dilution technique (*27*) in PBS (0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) containing 0.1 mM Mn^{2+} and 0.1 mM Ca^{2+}. The assay was done in microtiter plates using 25 μL of lectin solution and 25 μL of a 3% suspension of trypsin-treated rabbit erythrocytes. A unit of activity is defined as the lowest concentration of lectin giving visible agglutination (*28*).

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 37 °C in PBS containing 0.1 mM Mn^{2+} and 0.1 mM Ca^{2+}, pH 7.2 using a Sigma fluorimeter cuvette (volume: 2 mL; path length: 1 cm). The excitation wavelength was fixed at 280 nm, and the emission was scanned from 300 to 400 nm. The relative change (%) of the emission maximum was calculated on the basis of the difference of the wavelength maximum between the structured monomeric state and the native tetrameric state taken as 100%.

Far-UV CD measurements were made on a JASCO 600 spectropolarimeter at ambient temperature in a 1 mm path length cell using a scan speed of 50 nm/min and a response time of 2 s. The spectra were measured at a protein concentration of 0.33 μM and averaged over five scans to eliminate signal noise. The buffer used during measurement was PBS containing 0.1 mM Mn^{2+} and 0.1 mM Ca^{2+}, pH 7.2. The data are represented as molar ellipticity [θ], which is defined as $[\theta] = 100\theta_{obs}/(lc)$, where θ_{obs} is the observed ellipticity in degrees, l is the length of the light path in

centimeters, and *c* is the molar concentration of the protein. The values obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration of denaturant under similar conditions.

Denaturation and Reconstitution. The protein solution (20 μM) was denatured in 8 M urea in PBS for 30 min at 37 °C. Reconstitution of the protein was initiated by dilution with reconstitution buffer (PBS containing 0.1 mM Mn^{2+} and 0.1 mM Ca^{2+}, pH 7.2) to a residual denaturant concentration of 0.08−0.2 M urea. The final protein concentration during renaturation was 0.2−0.5 μM. Immediately after dilution, the mixtures were vigorously stirred in a vortex mixer, and a series of tubes containing the mixtures, in duplicate, was incubated at 37 °C for different periods of time up to a total time of 3 h. The kinetics of reassociation was examined, using aliquots taken at defined times during reconstitution, by fluorescence as mentioned previously and by a cross-linking reaction with glutaraldehyde described next.

Cross-Linking Reaction and SDS−PAGE. Cross-linking of native SBA and the reassociating protein at various times was achieved by a rapid reaction with glutaraldehyde as a bifunctional reagent as described (*23*). The cross-linking reaction was optimized with respect to the glutaraldehyde concentration and the time required for cross-linking. It was also found that residual denaturant (0.14 M urea) present in the solution during reconstitution did not interfere with the cross-linking reaction. Briefly, the procedure is as follows. To 3 mL aliquots of native or reassociating protein (30 μg) was added an aliquot of 50% (m/v) glutaraldehyde so as to make a final concentration of 1% glutaraldehyde. After 2 min, the cross-linking reaction was quenched by adding a ~10-fold molar excess of $NaBH_4$ (dissolved in 0.1 M NaOH). From this solution, the cross-linked protein was precipitated by a combined sodium deoxycholate/trichloroacetic acid precipitation. After $NaBH_4$ reduction (15 min), aqueous sodium deoxycholate (0.15%) was added so as to make a final concentration of 0.015% deoxycholate followed by a careful addition of trichloroacetic acid to its final concentration of 7% when excess $NaBH_4$ was destroyed and the cross-linked protein was precipitated. After centrifugation (4000*g*, 30 min), the obtained precipitate was redissolved in 0.1 M Tris-HCl buffer, pH 6.8 containing 1% SDS and heated at 100 °C for 5 min.

Samples were analyzed by 10% SDS−PAGE (*25*), and the electrophoretic run was calibrated with marker proteins of known molecular mass. Densitometric scanning of the gels was performed in a Thermal Imaging System (Image Master VDSFTI-500), and the relative amounts of the cross-linked species involved in the reconstitution pathway were determined from the densitometric plots.

RESULTS

We have studied kinetically the process of quaternary association of SBA from its structured monomer monitored by fluorescence and far-UV CD and by a cross-linking reaction with glutaraldehyde as a bifunctional reagent that permits the detection and quantitative evaluation of intermediate(s) of association. We have previously shown that the equilibrium unfolding of SBA involves a structured monomeric intermediate having characteristic fluorescence properties (*20*). The restoration of structure at the monomeric

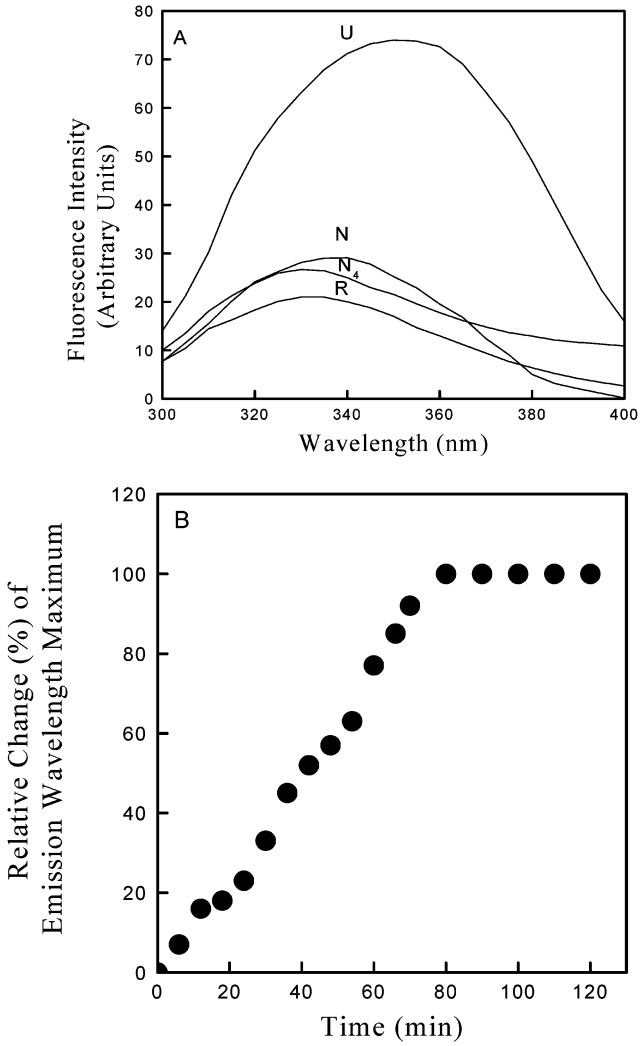

FIGURE 2: (A) Fluorescence spectra at 37 °C of native SBA (N_4), unfolded monomer (U), reconstituting monomer (N) within 10 s of reconstitution, and renatured SBA (R). The protein concentration was 0.33 μM. The spectra were corrected for the buffers containing requisite concentrations of urea. Excitation wavelength, 280 nm; excitation and emission band-pass, 5 nm each; scan rate, 60 nm/min. (B) A plot of relative change (%) of the emission wavelength maximum as a function of time during reconstitution. The percent change was calculated on the basis of the difference of the wavelength maximum between the structured monomeric state (within 10 s of reconstitution) and the native tetrameric state taken as 100%. Each data point represents an average of three determinations.

level, that is, the formation of structured monomer during reconstitution from the completely denatured state, takes place in a rapid reaction within the dead time of measurement (<10 s). Thus, the process of subunit association that occurs in a much longer time scale (hours) can be investigated after manual mixing of the denatured protein in 8 M urea with the reconstitution buffer (PBS containing 0.1 mM Mn^{2+} and 0.1 mM Ca^{2+}, pH 7.2).

Reassociation Monitored by Fluorescence and Circular Dichroism. The fluorescence spectra of native SBA, unfolded monomer, structured monomer, and the renatured protein are shown in Figure 2A. The unfolded protein in 8 M urea exhibits an emission maximum at 351 ± 1 nm with a relative intensity of ~2.5-fold higher than that of native SBA having an emission maximum at 330 ± 1 nm. When the denatured protein is subjected to reconstitution by dilution with

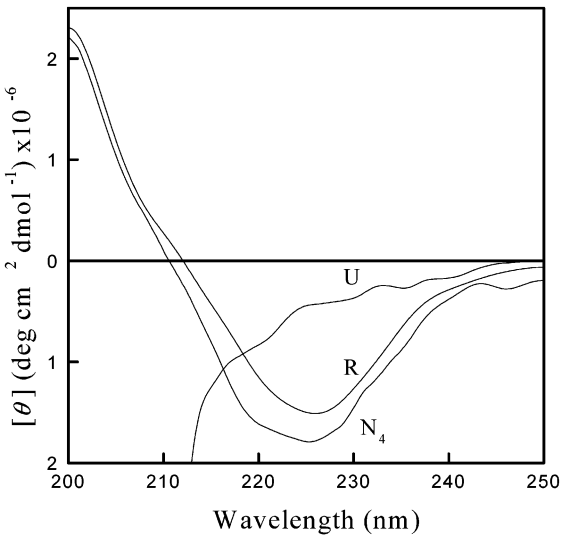

FIGURE 3: Far-UV CD spectra of SBA (0.33 μM) in native (N_4), unfolded (U), and renatured (R) states. The spectra were measured in 1 mm path length cell using a scan speed of 50 nm/min and averaged over five scans. The data are represented in molar ellipticities. The spectra were corrected for the buffers containing requisite concentrations of urea.

renaturation buffer, the emission maximum shifts immediately (<10 s) to 340 ± 1 nm with an emission intensity similar to that of native SBA, which is the characteristic property of the structured monomer (*20*). As there was no appreciable difference in the relative fluorescence intensities of native tetramer and the structured monomer in the entire emission envelope, the fluorescence intensity parameter could not serve as a suitable probe to monitor reassociation. However, the emission wavelength maximum showed a gradual blue shift to that of native protein during the process of reassociation; thus, the relative change (%) of the wavelength maximum was used to examine reassociation with time (Figure 2B). The reconstitution was found to be complete in about 80 min. It may be mentioned that the emission wavelength parameter can give a qualitative indication of the oligomerization reaction, and the use of emission maximum data for quantitative analysis of reassociation kinetics would be inappropriate (*29*, *30*).

The far-UV CD spectra of SBA in native, unfolded, and renatured states are shown in Figure 3. 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 SBA and that of renatured protein are similar. It may be mentioned that the far-UV CD spectrum of the reconstituting protein, at the initial stage of reconstitution, closely resembles that of native protein (data not shown), implying that the secondary conformation was restored immediately. 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 (data not shown).

Hemagglutinating Activity. The minimal hemagglutinating concentration of the reconstituted lectin was the same as that of the native SBA (1.2 μg/mL). These results show that the activity of the protein was restored completely upon reassociation. It may be mentioned that the usual hemagglutination assay for the measurement of lectin activity requires small amounts of protein and can thus be carried out under

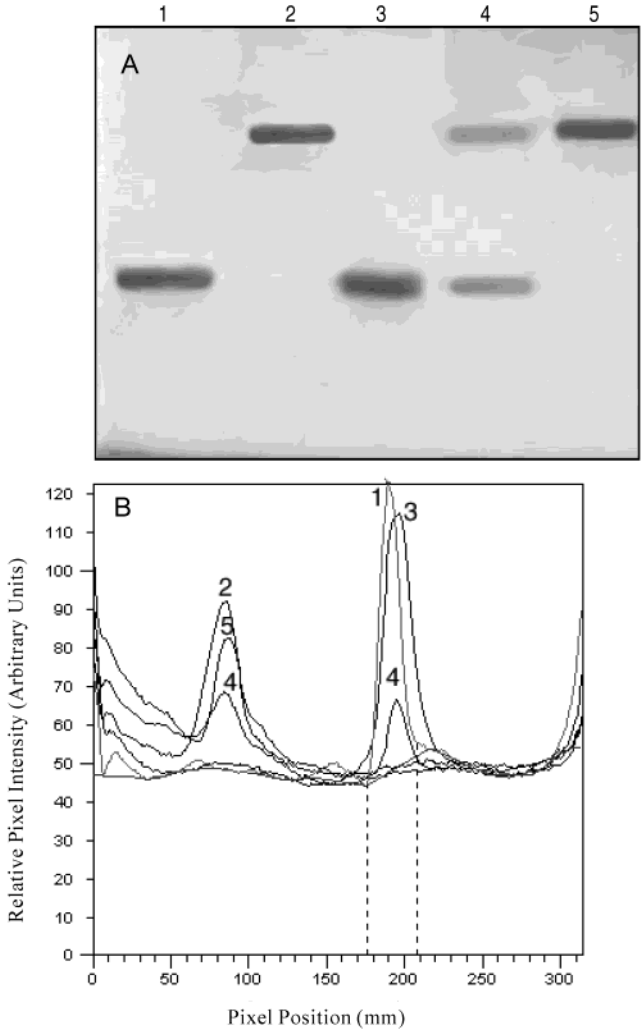

FIGURE 4: (A) SDS−PAGE profile of glutaraldehyde cross-linked SBA at various times of reconstitution. Lanes 1 and 2 represent native and glutaraldehyde cross-linked native SBA samples, respectively. Lanes 3−5 show, respectively, glutaraldehyde cross-linked samples of SBA at 30 s, 32 min, and 90 min of the reassociation reaction. (B) Densitometric plots of SDS−PAGE gel patterns as shown in panel A. The relative amounts of the cross-linked species were quantitated from the respective peak areas.

the conditions of low protein concentrations during reconstitution; however, the low accuracy of this method (*28*) does not entail quantitative analysis of reactivation kinetics.

Cross-Linking Studies. Snapshot fixation of reassociating oligomer by cross-linking with glutaraldehyde and subsequent analysis by SDS−PAGE is a powerful kinetic tool for the analysis of the reassociation of oligomeric proteins (*23*). Figure 4A shows some of the SDS−PAGE patterns for the cross-linking of native SBA and the reconstituting protein at various times. Lanes 1 and 2 show the patterns for native SBA and the cross-liked native SBA samples. As shown, quantitative cross-linking of native SBA was achieved under the optimized conditions of the experiment, and the cross-linked protein gave a single band showing the migration characteristics of a protein of M_r ~120 000. In the absence of a cross-linking reaction, native SBA gave the band corresponding to subunit molecular mass under denaturing conditions of SDS−PAGE. These results provide a control system for the analysis of the assembly process of SBA. Figure 4A also shows the gel patterns of the reconstituting

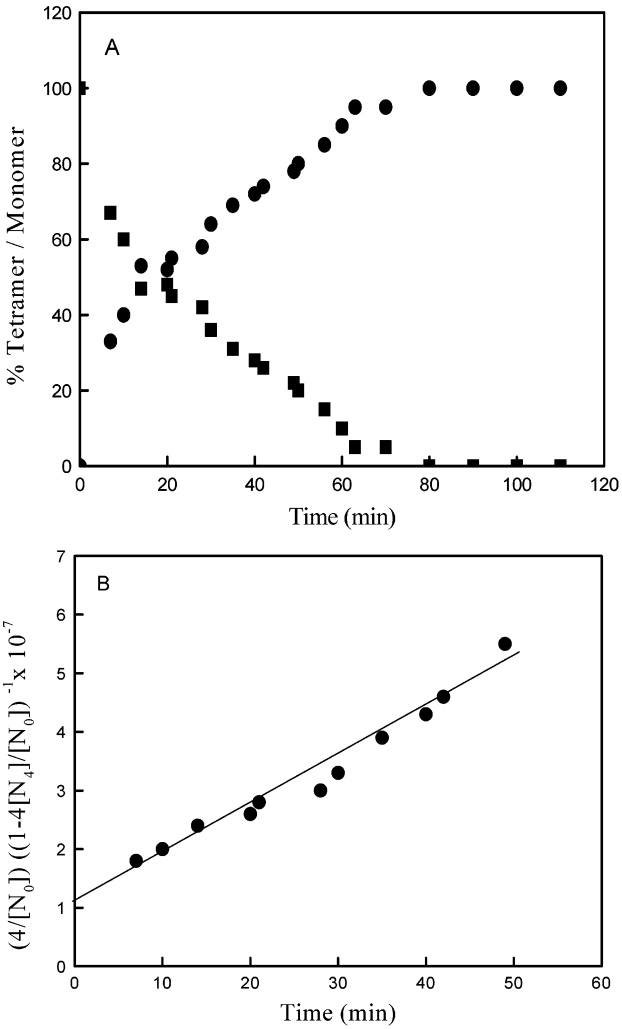

FIGURE 5: (A) Plot of percent tetramer (●) or monomer (■) vs time for the reassociation of SBA as determined by cross-linking with glutaraldehyde and subsequent SDS−PAGE. The data are shown for a final protein concentration of 0.33 μM. See the text for more details. (B) Second-order kinetic plot for the reassociation reaction (tetramer data) according to eq 6. The line has been drawn using the least-squares analysis of the data.

protein at some specified times: 30 s (lane 3: one band), 32 min (lane 4: two bands), and 90 min (lane 5: one band). The results show that the cross-linking of reconstituting SBA during the period of reconstitution reveals two bands on SDS−PAGE gels that are identified as monomers and tetramers. No dimeric intermediate is detectable. The densitometric scanning of the gels yielded the relative amounts of monomeric and tetrameric cross-linked species. A typical densitometric plot for the quantitative analysis of the gel (Figure 4A) is shown in Figure 4B. The relative amounts of monomers and tetramers were obtained from the respective peak areas. A plot of percent tetramer/monomer versus time is shown in Figure 5A. The data have been fitted to second-order kinetics (see Discussion).

DISCUSSION

The regain of tetrameric quaternary structure of SBA by subunit oligomerization after denaturation in urea has been characterized using fluorescence (Figure 2A,B), far-UV CD (Figure 3), and chemical cross-linking with glutaraldehyde (Figure 4A,B). The hemagglutinating activity of the fully reconstituted protein was also similar to that of native lectin. Thus, the reconstitution of SBA has been achieved in terms of its structure and activity. The kinetics of reassociation of SBA has been determined by the cross-linking technique (Figure 5A) and can be analyzed as follows.

SBA is a dimer of dimers (Figure 1). Thus, the most plausible mechanism to fit the observed association data can be described by the following kinetic model involving monomers (N), dimeric intermediates (N_2), and native tetramers (N_4):

$$4N \xrightarrow{k_1} 2N_2 \xrightarrow{k_2} N_4 \quad (1)$$

Two limiting cases can give rise to simple second-order kinetics: $k_2 \gg k_1$ or $k_1 \gg k_2$. Since dimers are not detectable in the cross-linking experiments (Figure 4A,B), it appears that no significant amounts of N_2 accumulate during the course of the reaction, which implies $k_2 \gg k_1$. Therefore, dimerization of monomers (k_1) is probably the rate-determining step in the reassembly of SBA. This situation may be analyzed mathematically as follows. The formation of the tetramer is given by

$$d[N_4]/dt = k_2[N_2]^2 \quad (2)$$

Since $k_2 \gg k_1$, N_2 is present in a steady state so that

$$[N_2]^2 = (k_1/k_2)[N]^2 \quad (3)$$

Substituting eq 3 into eq 2 gives

$$d[N_4]/dt = k_1[N]^2 \quad (4)$$

If $[N_0]$ is the total initial concentration of monomeric protein, then from mass balance $[N_0]$ can be expressed as

$$[N_0] = [N] + 2[N_2] + 4[N_4] \quad (5)$$

Taking $[N_2]$ to be approximately equal to zero, eq 4 can be integrated to

$$-4/[N_0] + 4/([N_0] - 4[N_4]) = k_1 t \quad (6)$$

Hence, a plot of $(4/[N_0])(1-4[N_4]/[N_o])^{-1}$ versus t should yield a straight line whose slope is directly related to the rate constant (k_1). Such a second-order plot for the reassociation reaction (using tetramer data in Figure 5A) is shown in Figure 5B. The value of k_1 is obtained as 1.4×10^4 M^{-1} s^{-1}.

It may be mentioned that the rate of tetramer formation (eq 4) relates directly to the rate of disappearance of the monomers to form the dimer, which indicates that the overall association process is determined by this dimerization reaction. The second-order rate constant calculated in terms of the reactant monomer (using monomer data in Figure 5A) is also found to be consistent with the k_1 value given previously.

The cross-linking studies thus reveal the kinetic pathway of reassociation of SBA characterized by second-order kinetics, with the monomer-to-dimer association as the rate-limiting step of the oligomerization reaction. X-ray crystallographic analysis has shown (*15*) that the two monomers of the SBA dimer are joined such that their β-sheets align

to form a 12-stranded sheet spanning one face of the dimer (Figure 1, top panel). At the dimer interface, the first β-strand of the two subunits interacts through six main chain hydrogen bonds involving the uncharged Ser and Thr residues. Further intersubunit contacts occur through side chain−side chain and side chain−main chain interactions from Ala, Ser, and Asn residues within the interface. In the SBA tetramer, the 12-stranded β-sheets of each dimer face each other, and the two dimers interact with their two outermost strands, creating a large channel in the middle of the tetramer (Figure 1, bottom panel). The interface formed by these two outermost strands consists mainly of a number of relatively short side chains that intercalate in a zipper-like fashion. This type of dimeric structure of SBA resembles the canonical dimer of concanavalin A (ConA) and other legume lectins (*5*); however, its tetrameric assemblage is different from that in ConA, and unlike ConA, the SBA tetramer does not dissociate into two dimeric species at more acidic pH. The present study provides the kinetic mechanism for the tetrameric association of SBA with the dimerization of SBA dimers occurring faster than the dimerization of SBA monomers, which may arise from the requirement of different architecture of the β-sheet packing in the dimeric and tetrameric interface of the lectin. These results are also in agreement with the mechanism proposed previously for the equilibrium denaturation of SBA (*20*).

The reconstitution kinetics of oligomeric proteins including some tetrameric enzymes such as lactate dehydrogenase and phosphofructokinase offer several kinetic mechanisms in respect to the rate-limiting step involved in the kinetic pathway of reassociation (*22*, *31*−*33*). SBA seems to be the first multimeric protein in the legume lectin family for which such a kinetic analysis is being reported.

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