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Topological and Functional Characterization of the *N*-Glycans of Soybean (*Glycine max*) Agglutinin[1]

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Soybean agglutinin (SBA), is a noncovalently bound tetramer comprised of four identical subunits having a single *N*-glycan chain, $Man_9GlcNAc_2$, that is known to be essential for regeneration of the functional tetrameric structure from unfolded subunits. In this study, SBA was found to have strong affinity for concanavalin A, indicating that the *N*-glycans are extensively solvent-exposed. The susceptibilities of the *N*-glycans to α-mannosidase and endo-β-*N*-acetylglucosaminidase revealed that their distal areas have nonreducing ends embedded among the subunits, whereas their proximal regions are solvent-exposed. Endo-β-*N*-acetylglucosaminidase-digested SBA was unable to retain its conformation and gradually unfolded. Periodate-oxidized SBA, whose *N*-glycans closely correspond to the invariant pentasaccharide core, tended to dissociate into the subunits, but permitted to stay as folded monomers. This SBA species was capable of refolding from unfolded subunits but unable to form the functional tetramer. It seems probable that the proximal regions of the *N*-glycans function in the formation and stabilization of the subunit conformation, whereas the branches outside the invariant cores stabilize the tetrameric structure.

Key words: glycoprotein, *N*-glycan, *N*-glycan function, *N*-glycan topology, soybean agglutinin.

N-glycans have been implicated in many different biological functions (*1*). In addition to their direct participation in biological activities, they are known to promote the folding of glycosylated proteins and stabilize their functional conformations (*2-5*). There is, however, little direct evidence that the *N*-glycans of oligomeric glycoproteins perform such functions (*6*).

Soybean (*Glycine max*) agglutinin (SBA) is a noncovalently bound tetramer having a single *N*-glycan, $Man_9GlcNAc_2$, on each of its four identical subunits (*7*). Because of its homogeneous *N*-glycan structure and also because SBA is readily available in a sufficient quantity, studies on SBA are expected to provide clear information about the functional role of the *N*-glycans in the folding, assembly, and stabilization of this oligomeric protein. We have previously revealed that, when dissociated into its subunits and completely denatured in 6 M guanidine hydrochloride, SBA is quantitatively reconstituted to the active tetrameric structure by simple dilution and that the *N*-glycans of SBA play an essential role in the folding and assembly of the subunit polypeptides (*8, 9*). To obtain more detailed information on the function of the *N*-glycans of SBA, their relation to the protein conformation was investigated in connection with their topological features. The results indicate that the proximal regions of the *N*-glycans function in the formation and stabilization of the subunit conformation, whereas their distal areas stabilize the tetrameric structure.

MATERIALS AND METHODS

Materials—SBA was prepared from soybean as described previously (*8*). Concanavalin A (Con A) was extracted and purified from jack bean meal (Sigma) as described by Agrawal and Goldstein (*10*), then coupled to Cellulofine GLC-2000-m (Seikagaku Kogyo) by the method of Ito *et al.* (*11*). GalN-Sepharose CL-4B was prepared as reported by Matsumoto *et al.* (*12*). Bovine pancreatic RNase B (type XII-B) was obtained from Sigma and purified by gel-filtration on a Bio-gel P-60 column eluted with 0.1 M ammonium acetate (pH 7.8). 1-Anilino-8-naphthalenesulfonate (ANS) was also purchased from Sigma. Sodium periodate, trifluoroethanol, and guanidine hydrochloride were from Wako Pure Chem. α-Mannosidase (jack bean) and endo-β-*N*-acetylglucosaminidase (*Flavobacterium* sp.) (Endo F) were products of Seikagaku Kogyo. The high-mannose type pyridylamino (PA-) oligosaccharides, $Man_1GlcNAc_2$-PA (M1), $Man_3GlcNAc_2$-PA (M3), $Man_5GlcNAc_2$-PA (M5), $Man_6GlcNAc_2$-PA (M6), Man_7Glc-

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Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; Con A, concanavalin A; Endo F, *Flavobacterium* sp. endo-β-*N*-acetylglucosaminidase F; e-SBA, Endo F-digested SBA; m-SBA, α-mannosidase-digested SBA; M1-M9, $Man_1GlcNAc_2$-PA-$Man_3GlcNAc_2$-PA; PA-, pyridylamino; PB, 10 mM sodium phosphate buffer (pH 6.9); p-SBA, sodium periodate-oxidized SBA; SBA, soybean (*Glycine max*) agglutinin.

NAc_2-PA (M7), $Man_8GlcNAc_2$-PA (M8), and $Man_9GlcNAc_2$-PA (M9), were obtained as described previously (*13*).

Affinity Fractionation of Glycoproteins on a Con A-Cellulofine Column—A glycoprotein (0.36 mg of SBA or 0.30 mg of RNase B) was dissolved in 10 mM sodium phosphate buffer, pH 6.9 (PB) (0.2 ml) containing 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, and 0.15 M NaCl. The resulting solution was applied to a Con A-Cellulofine column (0.6× 7.0 cm) equilibrated with the same buffer. After being washed with the same buffer (7 ml), the column was eluted successively with 20 mM methyl α-D-mannoside (10 ml) and 0.2 M methyl α-D-mannoside (15 ml) in the same buffer. The effluent fractions were monitored by measuring the absorbance at 280 nm.

Chemical Deglycosylation of SBA—Chemical deglycosylation of SBA was carried out with trifluoromethanesulfonic acid as previously described (*8*).

Glycosidase Digestions of SBA—SBA (0.50 mg/ml) was incubated with α-mannosidase (6 U) in 0.1 M sodium acetate buffer (pH 5.0) in the presence of a small amount of toluene at 37°C. After 8 and 24 h, additional enzyme (1 U) was added, and the digestion was continued for 48 h. SBA was also digested with α-mannosidase as just described after being heated with 0.25% SDS in the same buffer at 100°C for 5 min. Endoglycosidase digestion of SBA (0.50 mg/1 ml) was done with Endo F (0.10 U) in the same buffer for 48 h at 37°C. The resulting precipitate was separated from the supernatant by centrifugation, washed repeatedly by dispersion in water followed by centrifugation, then lyophilized.

SDS-PAGE—SDS-PAGE was performed by the method of Laemmli (*14*) using 12% acrylamide slab gels. All samples for electrophoresis were heated at 100°C for 5 min in 1.7% SDS. Gels were stained for protein with Coomassie Brilliant R-250. Molecular weight standards were ovalbumin (M_r 43,000), bovine α-chymotrypsinogen (M_r 25,700), and horse skeletal muscle myoglobin (M_r 17,000).

Periodate Oxidation of SBA—SBA (7.2 mg/4.2 ml) was oxidized with 9.5 mM sodium periodate in 0.2 M sodium acetate buffer (pH 4.8) at 0°C for 10 h in the dark. Ethylene glycol (50 mg) was then added, and the mixture was allowed to stand at 0°C for 1 h, then dialyzed against 8 changes of 0.1 M sodium phosphate buffer (pH 8.0). The oxidized product was reduced by leaving the dialyzed solution with sodium borohydride (10.4 mg) at 0°C overnight. The excess sodium borohydride was decomposed by adding 2 M acetic acid in portions at 0°C, and the solution was dialyzed successively against 0.2 M sodium acetate buffer (pH 4.8) and PB for 2 days each. The dialyzed solution was applied to a GalN-Sepharose CL-4B column (0.6×7.0 cm) equilibrated and eluted with PB. The protein retained on the column was eluted with PB containing 0.2 M lactose, and the eluate was thoroughly dialyzed against water, then lyophilized.

Fluorescence Measurements—Fluorescence measurements were performed on a Shimadzu RF-1500 fluorescence spectrophotometer at 25°C. Intrinsic tryptophyl fluorescence spectra from 300 to 400 nm were recorded with excitation at 280 nm. To study the binding of ANS to SBA species, aliquots of 4 mM ANS were added to a stock solution of SBA in PB (0.89 μM final protein concentration), and the fluorescence spectra were recorded from 400 to 500 nm, with excitation at 380 nm.

Guanidine Hydrochloride-Induced Denaturation—Guanidine hydrochloride-induced unfolding was determined by following the change in intrinsic fluorescence. A stock solution (10 μl) of SBA (8.9 μM) in PB was mixed with the same buffer (0.99 ml) containing guanidine hydrochloride at appropriate concentrations, then incubated at 25°C for 24 h.

Other Analytical Methods—Size-fractionation HPLC of PA-oligosaccharides was done on a TSK-GEL Amido-80 column (0.46×25 cm) (Tosoh) as described previously (*13*). Size-exclusion chromatography for estimation of the quaternary structure of proteins was also performed as described previously (*8*). Regeneration of SBA species from denatured subunits was performed essentially as reported previously (*8*) but with the following modifications as follows. A SBA species at 1.0 mg/ml was denatured in PB containing 6 M guanidine hydrochloride for 0.5 h at 37°C. For regeneration, the protein solution was diluted 100-fold with PB containing 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, and 0.15 M NaCl, with vigorous stirring in a vortex mixer, then allowed to stand for 24 h at 25°C. Other analytical procedures were described previously (*8, 9*).

RESULTS

Topological Features of the N-Glycans of SBA—To estimate the extent of solvent-exposure of the intramolecular *N*-glycans, the affinity of SBA for Con A was examined, using bovine pancreatic RNase B as a reference glycoprotein. RNase B has a single *N*-glycan of different structures ($Man_{5-9}GlcNAc_2$) (*15*), and the *N*-glycan chain is thought to be wholly solvent-exposed because it is susceptible both to α-mannosidase (*16*) and to Endo F (*17*) digestions under nondenaturing conditions. On the basis of the carbohydrate-binding specificity of Con A (*18*), therefore, it is assumed that a small fraction (8%) of RNase B, which has a relatively large *N*-glycan, $Man_8GlcNAc_2$ or

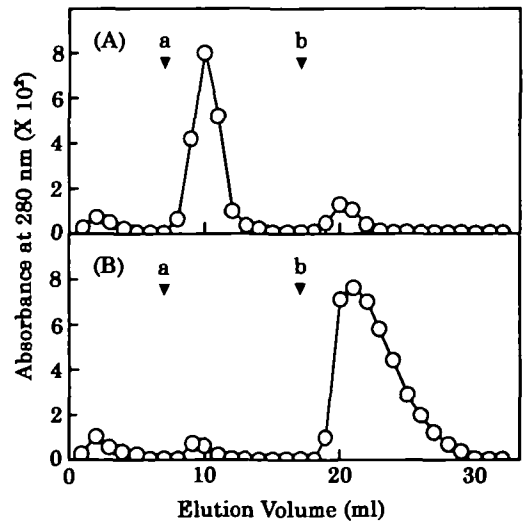

Fig. 1. **Comparison of SBA with RNase B in affinity for Con A.** RNase B (0.30 mg) (A) and SBA (0.36 mg) (B) were chromatographed on a Con A-Cellulofine column. The column was eluted stepwise with 20 mM (a) and 0.2 M (b) methyl α-D-mannoside. See the text for details.

$Man_9GlcNAc_2$ (*15*), shows a strong affinity for Con A. In fact, the greater part of RNase B was easily eluted from a Con A-Cellulofine column, whereas the elution of a small portion (*ca.* 9%) required a much higher concentration of methyl α-D-mannoside (Fig. 1A). The *N*-glycans of this minor fraction were analyzed for molecular size by size-fractionation HPLC and found to consist exclusively of $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ (data not shown). The elution feature of SBA from the Con A-Cellulofine column (Fig. 1B), in comparison with that of RNase B, strongly suggested that all four *N*-glycan chains of SBA are extensively solvent-exposed.

The susceptibilities of the intramolecular *N*-glycans of SBA to an exoglycosidase α-mannosidase and an endoglycosidase Endo F are expected to present reliable information about the solvent-exposure of their nonreducing and reducing ends, respectively. The *N*-glycans of SBA seemed to be resistant to α-mannosidase, though these of a denatured SBA were easily hydrolyzed (Fig. 2, lanes B and C). Further, α-mannosidase also appeared to be unable to hydrolyze the *N*-glycans of SBA in a weakly denaturing buffer containing 5-12% trifluoroethanol, in which α-mannosidase still retains about 70 to 25% of its activity (data not shown). These results suggest that the nonreducing ends of the *N*-glycans are sterically masked from interac-

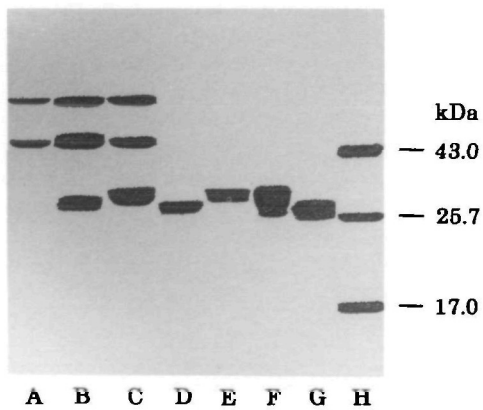

Fig. 2. **Susceptibilities of the intramolecular *N*-glycans of SBA to glycosidase digestion as estimated by SDS-PAGE.** SBA, after being heated with 0.25% SDS (lane B), or in its native state (lane C), was digested with α-mannosidase. The supernatant (lane F) and precipitate (lane G) separated from the Endo F-digest of SBA were also analyzed. Lane A, α-mannosidase; lane D, SBA deglycosylated with trifluoromethanesulfonic acid; lane E, native SBA; lane H, molecular weight standards.

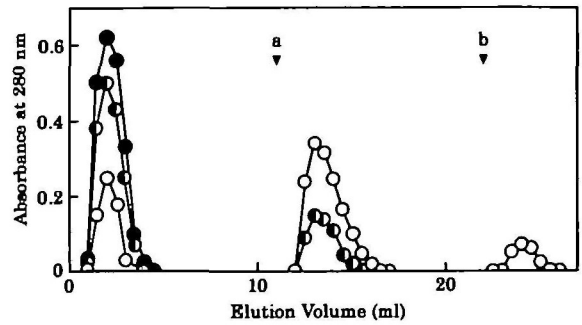

Fig. 3. **Periodate oxidation of the *N*-glycans of SBA monitored by the change in affinity for Con A.** SBA (1.0 mg) was oxidized for 2 h (○), 4 h (◐), or 6 h (●) as described under "MATERIALS AND METHODS," and then analyzed for the oxidative degradation of the *N*-glycans by affinity chromatography on a Con A-Cellulofine column (0.6×7.0 cm) equilibrated with PB containing 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, and 0.15 M NaCl. After being washed with the same buffer (11 ml), the column was eluted stepwise with the same buffer containing 20 mM (a) and 0.2 M (b) methyl α-D-mannoside.

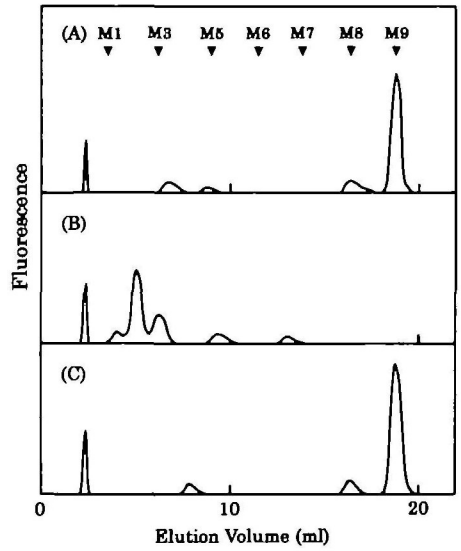

Fig. 4. **Size-analysis of the *N*-glycans of m-SBA and p-SBA.** PA-oligosaccharides from m-SBA (A) and p-SBA (B) were submitted to size-fractionation HPLC. PA-oligosaccharides from the native SBA were also analyzed (C). Arrowheads indicate the elution positions of standard PA-oligosaccharides.

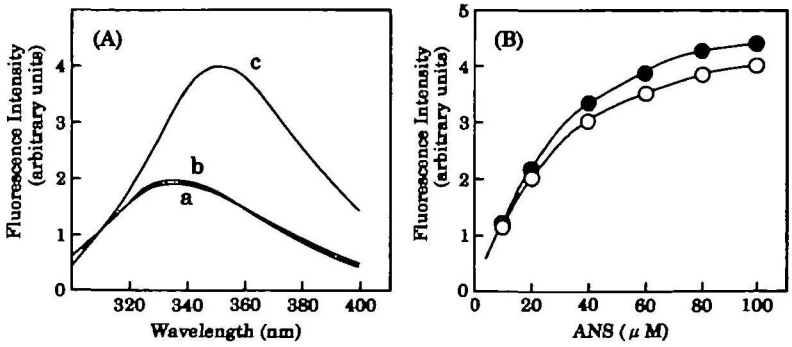

Fig. 5. **Comparison of the conformation of p-SBA with that of the native SBA.** (A) Tryptophyl fluorescence spectra of the native SBA (a), p-SBA (b), and fully unfolded SBA (c). (B) ANS binding of the native SBA (○) and p-SBA (●).

tion with α-mannosidase, *i.e.*, they are embedded among the subunits. On the other hand, Endo F gradually cleaved the *N,N'*-diacetylchitobiosyl linkages at the reducing ends of the *N*-glycans of the native SBA (Fig. 2, lanes F and G), and Endo F-digested SBA settled as a precipitate giving a negative orcinol-sulfuric acid test. It is known that Endo F hydrolyzes high-mannose type *N*-glycans, but not complex-type ones (*17*), *i.e.*, this enzyme recognizes the structure of the invariant pentasaccharide core and its vicinity of a substrate *N*-glycan. This finding, therefore, indicates that the invariant core and its vicinity of the *N*-glycans of SBA are solvent-exposed. Another finding worth further consideration is that Endo F cleavage of the *N*-glycans of SBA resulted in denaturation of SBA protein.

To corroborate the extensive solvent-exposure of the *N*-glycans and to determine mild conditions under which the greater part of every *N*-glycan chain can be degraded, SBA was subjected to periodate oxidation as described under "MATERIALS AND METHODS," and the change in its affinity for Con A was followed. After 6 h oxidation, SBA completely lost its affinity for Con A, indicating that the greater part of the individual *N*-glycans is solvent-exposed and that the exposed areas of the *N*-glycans are easily degraded under the mild periodate-oxidation conditions (Fig. 3).

The *N*-glycans of α-mannosidase-digested SBA (m-SBA) and periodate-oxidized SBA (p-SBA) were analyzed for molecular size by size-fractionation HPLC. The size of the *N*-glycans of SBA remained unchanged during the treatment with α-mannosidase (Fig. 4, A and C), confirming the above result that the nonreducing ends of the three branches of every *N*-glycan chain are hindered from being hydrolyzed by α-mannosidase. It is also probable that periodate degraded the *N*-glycans of SBA to core structures consisting of two GlcNAc and two or three Man residues (Fig. 4B). Since the inner tetrasaccharide core Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc of the *N*-glycans has no vicinal glycols susceptible to periodate oxidation, periodate seems to have almost fully acted on the *N*-glycans.

Stabilizing Effect of N-Glycans on SBA Conformation—As described above, Endo F-digested SBA (e-SBA) is unstable and readily aggregates. It was of interest, therefore, to examine the stability of p-SBA, which has short *N*-glycans closely corresponding to the invariant pentasaccharide core.

Before dealing with this subject, p-SBA was characterized with regard to the amino acid composition, protein conformation, and lectin activity. There was no significant difference in amino acid composition between p-SBA and the native SBA, in agreement with the result previously reported (*19*). p-SBA proved to be tetrameric by size-exclusion chromatography performed as described under "MATERIALS AND METHODS" (data not shown) and to have a specific lectin activity a little higher than that of the native SBA. The higher activity of p-SBA may arise from the periodate degradation of the *N*-glycans: this leads to a decrease in steric hindrance of the lectin function. Although there was no appreciable difference in tryptophyl fluorescence between p-SBA and the native SBA (Fig. 5A), a slight difference in ANS-binding features was observed between them (Fig. 5B). p-SBA bound a few more ANS molecules per molecule of protein than the native SBA, indicating that

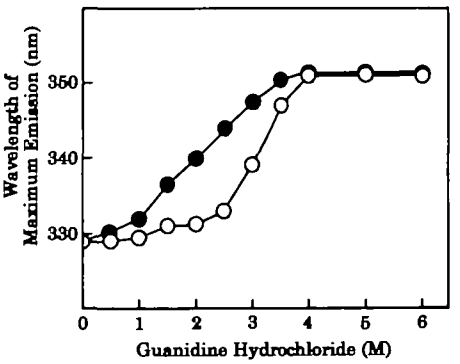

Fig. 6. **Guanidine hydrochloride-induced denaturation of SBA species monitored by tryptophyl fluorescence.** The effect of guanidine hydrochloride on the conformation of the native SBA (○) and p-SBA (●) was followed as described under "MATERIALS AND METHODS." Individual points represent the mean of at least three independent measurements.

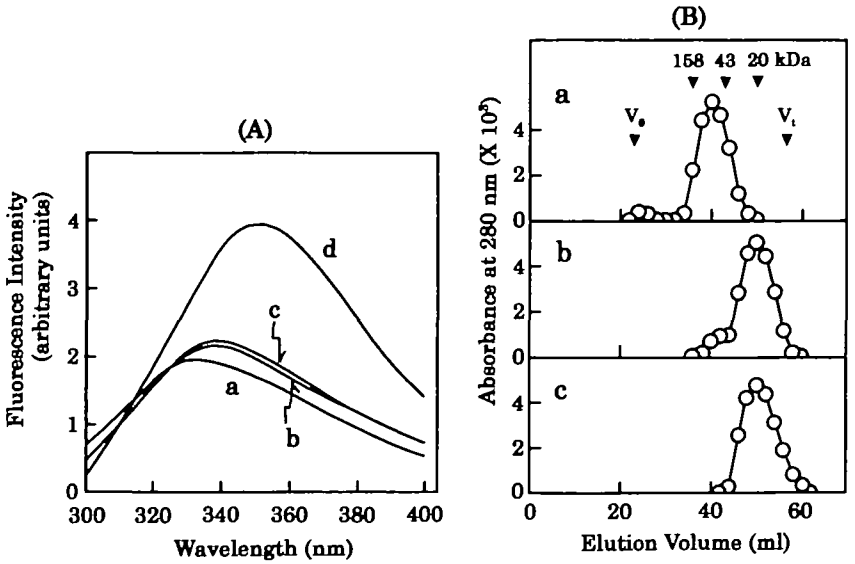

Fig. 7. **Stability and refolding of p-SBA monitored by tryptophyl fluorescence (A) and size-exclusion chromatography (B).** p-SBA was incubated in PB at 40°C for 24 h, then the tryptophyl fluorescence emission (A, b) and molecular size (B, b) were examined. p-SBA was also fully denatured in 6 M guanidine hydrochloride, then allowed to regenerate by dilution, and the tryptophyl fluorescence emission (A, c) and molecular size (B, c) were examined. The spectra of the native SBA (A, a) and fully denatured SBA (A, d), and the molecular size (B, a) of the native SBA are also shown. The elution positions of molecular weight standards are indicated by arrowheads.

there are more binding sites exposed on p-SBA molecules. It seems likely that the attachment of the *N*-glycan chains to the protein covers hydrophobic regions of SBA, making them unavailable to the ANS. These findings together indicate that p-SBA probably retains the overall native protein conformation, though the *N*-glycan structures are severely modified.

The responsibility of the *N*-glycans for the stability of the protein conformation was examined by comparison of guanidine hydrochloride-induced denaturation of p-SBA and native SBA. Unfolding of the protein was monitored by the change in wavelength of maximum fluorescence emission (Fig. 6). The midpoint of denaturation was 3.0 M for the native SBA, but 2.1 M for p-SBA. This distinct difference between the stabilities of the two SBA species appeared to derive from the difference between their *N*-glycan structures, and hence it is probable that the *N*-glycan chains of SBA stabilize the protein conformation to guanidine hydrochloride-induced denaturation. A decreased stability of p-SBA was also observed when it was incubated in PB at 40°C. The tryptophyl fluorescence emission peak of p-SBA, in contrast with that of the native SBA, was gradually red-shifted and reached a steady wavelength of 339 nm (Fig. 7A, b). This metastable transition product of p-SBA was found to be monomeric by size-exclusion chromatography (Fig. 7B, b). Judging from its tryptophyl fluorescence spectra, this monomeric SBA seemed to retain the native protein conformation. Interestingly, this monomeric SBA quantitatively regained the tetrameric structure in PB containing 1 mM GalNAc, a binding sugar for SBA. The resulting tetrameric p-SBA was retained on a GalN-Sepharose CL-4B column and eluted with PB containing 0.2 M lactose, suggesting the restoration of lectin activity (data not shown).

Role of the N-Glycans in Folding and Assembly of SBA Subunit Polypeptides—The *N*-glycans of SBA are known to be essential for the folding and assembly of the SBA subunit polypeptides (*8, 9*). It is of interest, therefore, to see whether p-SBA, which has short *N*-glycans closely corresponding to the invariant core, is able to regenerate from its unfolded subunits. Although p-SBA seemed to fold properly as judged by the change of the tryptophyl fluorescence spectrum (Fig. 7A, c), it failed to regain the tetrameric structure, remaining monomeric (Fig. 7B, c). This monomeric p-SBA, however, was completely retained on a GalN-Sepharose CL-4B column and quantitatively eluted with PB containing 0.2 M lactose, similarly to p-SBA dissociated into the monomers. The p-SBA thus eluted was found to be entirely tetrameric by size-exclusion chromatography (data not shown). These findings, together with the ready subunit dissociation of p-SBA, suggest that the invariant core *N*-glycans enable the subunits to fold properly but are insufficient to form and stabilize the tetrameric structure.

DISCUSSION

The *N*-glycans attached to single-chain proteins, to our knowledge, are invariably solvent-exposed and susceptible to exoglycosidase digestion. Those *N*-glycans projecting into the solvent, although they look foreign to the conformation of the glycosylated proteins, are known to function not only in direct promotion of the protein folding (*2-4*) but also in stabilization of the protein conformation (*5, 16*). On the other hand, the high-mannose type *N*-glycans on oligomeric proteins, whose functions with regard to protein conformation have received little attention, are more or less buried among the subunits (*20, 21*). SBA, therefore, is not unique among glycoproteins in having *N*-glycans embedded among the subunits. The *N*-glycans of some oligomeric glycoproteins from a kidney bean (*Phaseolus vulgaris*) are only partially accessible to α-mannosidase and are not hydrolyzed to the extent that they fail to bind Con A (*21*). The tetrameric α-mannosidase from jack bean has *N*-glycans almost completely masked from interaction with either Con A or endo-β-*N*-acetylglucosaminidase H (*20*). These high-mannose type *N*-glycans seem to be required for formation of oligomeric protein structures in the endoplasmic reticulum and to be inaccessible to the Golgi processing enzymes.

There seems no doubt that the proximal regions of the *N*-glycans function in the formation and stabilization of the tertiary structure of the subunits, for the following reasons. First, Endo F digestion causes aggregation of SBA, and the resulting deglycosylated SBA is, in contrast with the native SBA, unable to refold from unfolded subunits. Second, p-SBA, which carries short *N*-glycans closely corresponding to the invariant core, is permitted to stay as folded monomers, though it is easily dissociated into the subunits. Third, p-SBA is able to refold from its unfolded subunits. It also seems probable that the distal areas of the *N*-glycans are required for formation and stabilization of the tetrameric structure, because periodate degradation of the branches outside the invariant cores remarkably reduces the stability of the oligomeric structure and renders the subunits unable to assemble. It appears that the subunits are tethered tightly together by the *N*-glycans.

Recently, in attempt to refold the inclusion bodies of recombinant SBA expressed in *E. coli*, an SBA identical with native SBA in its activity and oligomeric structure has been isolated in small yield (0.2 to 0.4%) (*22*). This fact is contrary to our results of the functional roles of the *N*-glycans in the folding and assembly of SBA. It should be noted, however, that the recombinant SBA was expressed by growing the bacteria in the presence of isopropyl β-D-galactopyranoside inducer. As described above, p-SBA is able to recover the native tetrameric structure with the aid of GalNAc, a binding sugar for SBA. In addition, SBA is known to bind Gal as well as GalNAc. Although it is uncertain whether e-SBA is able to recover the native structure with the aid of Gal, the small amount of the tetrameric SBA from the inclusion bodies might be attributed to the action of isopropyl β-D-galactopyranoside used to induce the bacterial growth.

The present study provided direct evidence of two different roles of the intramolecular high-mannose *N*-glycans of SBA in the folding and assembly of the subunit glycopolypeptides. It seems likely that some oligomeric proteins require high-mannose type *N*-glycans of large size for the subunit assembly and stabilization of the oligomeric structures. It is still unknown, however, what kind of interaction exists between *N*-glycans and proteins.

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