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Interactions of concanavalin A with glycoproteins. A quantitative precipitation study of concanavalin A with the soybean agglutinin*,†

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ABSTRACT

Certain oligomannose-type glycopeptides have been previously shown to be bivalent for binding to concanavalin A and capable of precipitating the lectin by forming homogeneous cross-linked lattices [L. Bhattacharyya, M. I. Khan, and C. F. Brewer, *Biochemistry*, 27 (1988) 8762–8767]. In the present study, the effect of protein environment on the binding properties of an oligomannose-type oligosaccharide has been examined through quantitative precipitation analysis of the interactions of concanavalin A (Con A) with the soybean (*Glycine max*) agglutinin (SBA), which is a tetrameric glycoprotein possessing a single Man_9-oligomannose chain per monomer. The results showed that SBA forms two different types of cross-linked complexes with tetrameric Con A, depending on the relative ratio of the two molecules in solution. At a concentration of one equivalent or less, SBA forms a 1:1 complex with Con A. At concentrations exceeding one equivalent, SBA forms a 2:1 complex with Con A. However, SBA forms only 1:1 cross-linked complexes with dimeric forms of Con A, such as acetyl- and succinyl-Con A. The results demonstrated that the total valency of the carbohydrate of SBA is a function of both the quaternary structure of Con A, as well as the relative ratio of SBA to Con A. In addition, the individual Man_9-oligosaccharide, which as a glycopeptide is bivalent for binding to Con A, expresses univalency when present on the protein matrix of SBA.

INTRODUCTION

Oligosaccharide chains covalently linked to proteins and lipids have been implicated as receptors in a variety of biological processes[1–3]. Our investigations of the molecular recognition properties of asparagine-linked (*N*-linked) oligosaccharide and their interactions with lectins have revealed that many of these oligosaccharides are multivalent and can bind, cross-link, and precipitate with the proteins[4–7]. For example, certain *N*-linked oligomannose- and "bisected" hybrid-type glycopeptides were shown to be bivalent for the D-glucose and D-mannose-specific lectin, concanavalin A (Con A) and to form homogeneous cross-linked precipitates with the lectin[8,9].

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Generally, studies on the molecular recognition properties of *N*-linked oligosaccharides have been carried out with one amino acid residue (asparagine) or none attached to the carbohydrate chain. It is known that the protein on which *N*-linked oligosaccharide chains are located often influences processing of the oligosaccharides during post-translational modification[10–13]. Therefore, it is of interest to determine the effects of the protein matrix on the molecular recognition properties of the oligosaccharide chains attached to it. In order to address this question, we have carried out quantitative precipitation analysis of the interaction of soybean agglutinin (SBA), which is a glycoprotein possessing a single Man_9-oligomannose-type chain (**1**) on each of its four subunits, with Con A. The results are discussed in terms of the factors affecting the valency of oligosaccharide chains when present on a protein matrix.

EXPERIMENTAL

Materials and methods. — Con A was prepared according to the method of Agrawal and Goldstein[14]. The concentration of Con A was determined spectrophotometrically at pH 5.6 by use of $A_{280}^{1\%,1cm}$ 12.4 and is expressed in terms of monomer (M_r 27 000)[15,16]. SBA was prepared as described previously[17] and its concentration was determined by use[18] of $A_{280}^{1\%,1cm}$ 12.8, and is expressed in terms of monomer (M_r 30 000). A portion of SBA was digested with Pronase and the glycopeptide isolated was characterized by ^{1}H-n.m.r. spectroscopy at 500 MHz, which confirmed it to be the Man_9-glycopeptide[19,20]. The carbohydrate content of SBA was determined by the phenol–H_2SO_4 method[21] using D-mannose as the standard. 2,4,6-Trinitrobenzenesulfonic acid (TNBS), acetic anhydride, succinic anhydride, and the monosaccharides were purchased from Sigma Chemical Co (St. Louis, MO). [^{3}H]- and [^{14}C]-formaldehyde were purchased from New England Nuclear (0.99GBq/mmol) and Sigma Chemical Co. (0.37GBq/mmol), respectively. All other materials used were of analytical grade.

Radiolabelling of Con A and SBA. — Con A and SBA were radiolabelled with ^{3}H and ^{14}C, respectively, by reductive methylation by modification of a previously described procedure[22]. Con A (~ 150–200 mg) was dissolved in 0.1M HEPES buffer (pH 7.2) containing mM Ca^{2+} and mM Mn^{2+} (10 mL). Sodium cyanoborohydride (50 mg) was added to the protein solution, followed by 20-μL aliquots of [^{3}H]formaldehyde (2% v/v in water) at 10-min interval over a period of 1 h. The protein was desalted on a column (1.5 × 90 cm) of Sephadex G-25, equilibrated with 0.02M Tris·HCl buffer (pH 7.2) containing 0.15M NaCl, mM Ca^{2+}, and mM Mn^{2+}. The desalted ^{3}H-labelled Con A was thoroughly dialyzed against water and lyophilized. SBA was radiolabelled with [^{14}C]formaldehyde by use of the same procedure.

Preparation of acetyl- and succinyl-Con A. — Acetyl- and succinyl-Con A were prepared by modifications of published procedures[23,24]. Con A (100 mg) in saturated sodium acetate (pH 8.3; 10 mL) was stirred with 200-molar excess of either acetic anhydride or succinic anhydride in an ice bath for 1 h. The protein solution was then thoroughly dialyzed against water, lyophilized and subjected to a second cycle of the above described steps. The crude acetyl- or succinyl-Con A obtained after the second

cycle was dissolved in 0.02M Tris·HCl buffer (pH 7.2) containing 0.15M NaCl, mM Ca^{2+}, mM Mn^{2+}, and 0.2M D-glucose, and applied to a column (2 × 150 cm) of Sephacryl S-200 equilibrated with the same buffer. The column was eluted with the same buffer, and pure dimeric acetyl- or succinyl-Con A was separated from the tetrameric Con A, which eluted earlier than the dimeric Con A. The dimeric acetyl- or succinyl-Con A obtained was dialyzed against water and lyophilized.

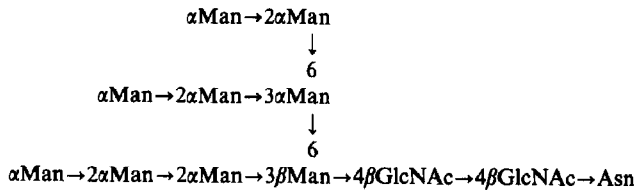
The free amino groups of dimeric acetyl- and succinyl-Con A were determined with the TNBS method[25] which showed that 85–90% of the amino groups were modified. Similar results were obtained for reductively methylated [^{3}H]Con A and [^{14}C]SBA. Both radiolabelled proteins remained tetramers after modification.

Quantitative precipitation assay. — The assay was performed on a solution containing 0.1M Tris·HCl buffer (pH 7.2), 0.9M NaCl, mM Ca^{2+}, and mM Mn^{2+}. Increasing amounts of [^{14}C]SBA were delivered into a series of tubes and buffer was added to make the final volume to 500 μL. [^{3}H]Con A solution (500 μL, 66μM) was added to each tube to make the final concentration of Con A 33μM. Precipitation was allowed to continue for ~20 h at room temperature. The supernatant solution was removed after centrifugation at 5000*g* and the precipitate washed three times with cold buffer (500 μL). After the final washing, the precipitate was dissolved in M methyl α-D-mannopyranoside to a final volume of 2 mL (M methyl α-D-mannopyranoside was used to speed up the dissolution process). The solutions were then analyzed for total protein content (absorbance) as well as radioactivity (LKB Rackbetta).

Quantitative precipitation assays with acetyl- and succinyl-Con A were carried out similarly, except that increasing amounts of SBA were made up to a final volume of 100 μL, and acetyl-Con A (100 μL, 122μM) or succinyl-Con A (100 μL, 176μM) was added to make the final concentration of acetyl-Con A and succinyl-Con A 61μM and 88μM, respectively.

RESULTS

The carbohydrate content of SBA, as determined by the phenol–sulfuric acid method in the present study (6.5% mannose per molecule), confirmed that there is one Man_9-oligomannose-type chain (1) per monomer, and a total of four chains per molecule[19]. Fig. 1 shows the profile for the quantitative precipitation of ^{3}H-labelled Con A by ^{14}C-labelled SBA. The curve for the total protein precipitated (A_{280}) consists of three different slopes with two break points. Titration of 33μM Con A with increasing amounts of SBA led to an increase in the total amount of protein precipitated with a constant slope until the concentration of SBA reached 33μM. At this point, a break occurred with a decrease in slope until the SBA concentration reached 66μM, at which point the curve became flat. The radioactivity profiles showed increasing amounts of [^{3}H]Con A precipitated up to 33μM of [^{14}C]SBA, at which point essentially 98% of Con A was precipitated. Further increase in the concentration of [^{14}C]SBA had no effect on the amount of Con A precipitated. The radioactivity profile of SBA is similar to that of the total protein precipitated. The radioactivity profiles of Con A and SBA showed that



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Man = D-Man*p*-1
GlcNAc = D-Glc*p*NAc-1

the increase in the total amount of protein precipitated after the first break point (33μM SBA) is due only to increasing amounts of SBA precipitated.

The molar ratio of SBA to Con A in the precipitates (Fig. 1B) showed that it remains constant at 1:1 up to an SBA concentration of 33μM. This ratio increased with further additions of SBA until it reached a value of 2:1 at a total SBA concentration of

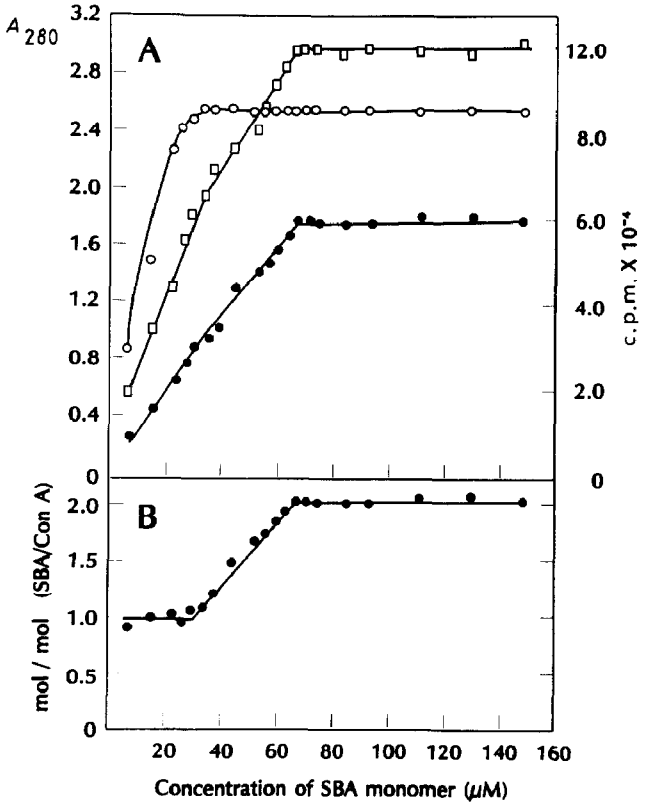

Fig. 1. Precipitin curves for the quantitative precipitation of Con A by SBA at 22°: (A) Profiles of the total protein precipitated (□) and radioactivity (c.p.m.) of [^{3}H] Con A (○) and [^{14}C]SBA (●) in the precipitate; and (B) ratio (●) of mol of SBA precipitated per mol of Con A. [^{3}H]Con A concentration was fixed at 33μM. For details see the Experimental section.

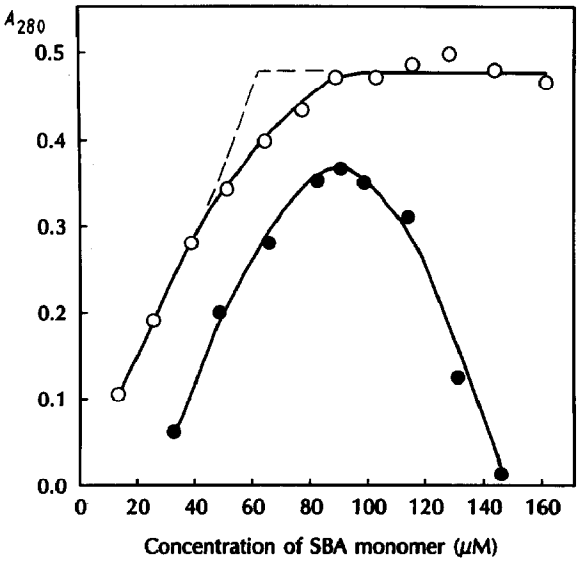

Fig. 2. Precipitin curves for the quantitative precipitation of dimeric acetyl-Con A (61μM) by SBA at 22° (○), and dimeric succinyl-Con A (88μM) by SBA at 4° (●).

66μM. A further increase in SBA concentration led to no further change in the ratio. These results were obtained over a Con A concentration range of 2.5 to 100μM and at 4 and 22°.

The quantitative precipitation profile of acetyl-Con A (61μM) in the presence of increasing amounts of SBA at 22° (Fig. 2) showed increasing total protein precipitated (A_{280}) until a broad plateau was reached. The point of intersection of the initial slope and final plateau region corresponds to 62μM SBA (monomer), which indicates formation of a 1:1 complex between the two proteins in the precipitates. Under the same conditions, succinyl-Con A failed to precipitate with SBA. However, at 4°, succinyl-Con A precipitated with SBA. The quantitative precipitation profile of succinyl-Con A (88μM) with SBA at 4° in Fig. 2 shows a bell shaped profile with an equivalence point (maximum precipitation) near 90μM SBA, indicating a 1:1 ratio of mol (monomer) of succinyl-Con A and SBA in the precipitates.

Inhibition (50%) of the formation of the precipitates of Con A and its dimeric forms with SBA occurred in the presence of a 0.2M solution of the specific competing monosaccharide, methyl α-D-mannopyranoside, but not by nonspecific monosaccharides such as D-galactose or L-fucose. Dissolution (50%) of the precipitates also occurred with methyl α-D-mannopyranoside at a concentration of 0.2M.

DISCUSSION

Complex formation between Con A and various glycoproteins is well documented and has been the basis of purification of glycoproteins by affinity chromatography[26,27]. Glycoproteins such as ribonuclease B[28], immunoglobulins[28,29], ovalbumin[30], and SBA[28] have been shown to bind and precipitate Con A. These earlier precipitation studies with Con A were qualitative in that the glycoproteins were not well characterized in terms of structure and number of their oligosaccharide chains.

Con A consists of monomeric subunits of M_r 27 000 that possess one D-glucose/D-mannose binding site[14,15]. The lectin is a tetramer at pH 7.2, and is, therefore, tetravalent in its carbohydrate-binding activity[16]. SBA is also a tetramer with four similar subunits[18]. The present results confirm that each subunit has one Man_9-oligosaccharide (**1**) attached to it and, therefore, SBA contains four Man_9-oligosaccharide chains per molecule[19].

In our previous, quantitative-precipitation studies of oligomannose-type glycopeptides with Con A, we demonstrated that the Man_9-glycopeptide can bind and precipitate the lectin[4,5]. The quantitative-precipitation profile showed a sharp bell-shaped curve, indicating high-affinity binding of the glycopeptide in both the soluble and cross-linked complexes with the lectin. The point of maximum precipitation in the profile, the equivalence point, provided the stoichiometry of the precipitation reaction which demonstrated that the glycopeptide was bivalent. Detailed structure-activity studies showed that the two Con-A-binding sites are located on the α-D-(1→6) and α-D-(1→3) arms of the core β-D-mannosyl residue of the glycopeptide. The site on the α-D-(1→6) arm was identified as the high-affinity or primary site, and the site on the α-D-(1→3) arm as the low-affinity or secondary site[4]. The α-D-(1→6) arm of the glycopeptide was found to bind with a 3000-fold higher affinity than methyl α-D-mannopyranoside to Con A, while the α-D-(1→3) arm of the glycopeptide bound with a ~20-fold higher affinity[6]. The predominant high-affinity-binding determinant on the α-D-(1→6) arm was identified as the tri-D-mannosyl component, 3,6-di-*O*-(α-D-mannopyranosyl)-D-mannose, which binds by extended binding interactions with Con A[31]. The outer two (1→2)-linked α-D-mannopyranosyl residues of the trimannosyl component on the α-D-(1→6) arm of the glycopeptide provide smaller increases in binding affinity *via* statistical-enhancement mechanisms[6]. Recent precipitation studies have demonstrated that the glycopeptide forms a homogeneous, cross-linked lattice with Con A, even in the presence of binary mixtures of the Man_9-glycopeptide and other oligomannose- and "bisected" hybrid-type glycopeptides[8,9].

These results contrast with the quantitative precipitation profiles of acetyl- and succinyl-Con A with SBA (Fig. 2) in which both dimeric forms of Con A show only 1:1 cross-linked complexes with SBA, and their profiles differ in shape from that of Con A. Interestingly, the precipitating activities of the two dimeric Con A derivatives differ from each other. Acetyl-Con A precipitates with SBA at 22°, whereas succinyl-Con A precipitates with SBA only at low temperature (4°). This indicated that succinyl-Con A forms a weaker cross-linked lattice with SBA than that of acetyl-Con A. In any case, the

geometry of the 1:1 cross-linked complexes between the dimeric forms of Con A with SBA resembles the lattice shown in Fig. 3B, but with the SBA and Con A molecules reversed.

The present findings provided new observations on the specificity of binding and cross-linking interactions of the carbohydrate chains of glycoproteins with lectins. The valency of the carbohydrate chains of a glycoprotein are determined by several factors, including the quaternary structure of the glycoprotein and the total number of carbohydrate chains on the protein. For example, even though the Man_9-glycopeptide is bivalent for Con A binding[4], the individual Man_9-oligosaccharide chains on SBA appear to be univalent in the 1:1 SBA–Con A cross-linked complex. The overall carbohydrate valency of SBA in the complex is tetravalent. Thus, in the 1:1 complex, the valency of the oligosaccharide chain appears to be determined by the quaternary structure of the glycoprotein (or accessibility of the oligosaccharide), and not by the valency of the individual carbohydrate chains. Furthermore, the α-D-(1→6) arm of the Man_9 chain on SBA appears to preferentially binds to Con A in the cross-linked complexes. This is supported by the 2000-fold higher concentration of methyl α-D-mannopyranoside, relative to SBA, that is required to dissolve or inhibit the formation of the precipitates. This concentration difference corresponds to the difference in affinities between the monosaccharide and the pentasaccharide component of the α-D-(1→6) arm of the Man_9-oligosaccharide[6]. Other possible binding geometries involving the secondary binding site on the Man_9 chain cannot be ruled out; however, they would appear to be less likely based on thermodynamic and steric arguments.

Another finding was the observation of a change in the stoichiometry of the SBA–Con A cross-linked complexes from 1:1 to 2:1 upon addition of increasing amounts of SBA. This resulted in a change in the total carbohydrate valency of SBA from four to two, and a required change in the geometry of the cross-linked complexes.

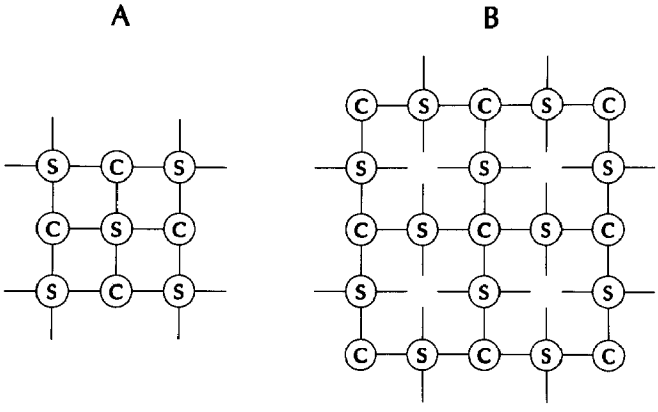

Fig. 3. Schematic representation of the 1:1 (A) and 2:1 (B) stoichiometric cross-linked complex of SBA and Con A. The circles with C represent Con A tetramers, and those with S SBA tetramers. The connecting lines between SBA and Con A molecules are the Man_9-oligosaccharide chains of SBA.

Thus, depending on the relative ratio of SBA to Con A in solution, the total carbohydrate valency of SBA may be two or four.

The quaternary structure of the lectin also influences the expressed valency of the carbohydrates on the glycoprotein. Dimeric acetyl- and succinyl-Con A showed exclusive formation of 1:1 cross-linked complexes with SBA. This contrasts with the change in total valency of the carbohydrates of SBA with tetrameric Con A. The cross-linked lattice geometry of SBA with acetyl- or succinyl-Con A must also be different from those observed with native Con A. In addition, the shape of the precipitation profiles of the SBA with the two dimeric forms of Con A differed from the profile observed with native Con A. Similar bell-shaped profiles have been obtained for the precipitation of tetrameric Con A with bivalent, oligomannose-type glycopeptides, including the Man_9-glycopeptide[4]. Interestingly, although acetyl- and succinyl-Con A are reported to possess similar carbohydrate-binding affinities[32,33], their precipitation activities differ with SBA, with the former possessing higher activity. This suggested that the charge state on the dimeric Con A derivatives influences the stability of the cross-linked complexes with SBA.

Although each Man_9 carbohydrate chain of SBA expresses only univalent interactions with Con A, single oligomannose-type chains on certain glycoproteins can express bivalency when present as the only oligosaccharide chain on the protein. An example is hen ovalbumin which possesses only one oligosaccharide chain per molecule and which was reported[30] to precipitate with Con A. However, it appears from the present study that the valency of an individual carbohydrate chain on a glycoprotein possessing multiple copies of the oligosaccharide is determined by the quaternary structures of the glycoprotein and the lectin with which it interacts.

A recent report described the precipitation of Con A by lysozyme, a non-glycosylated protein, and the dissolution of the precipitates by specific monosaccharides[34]. We have observed that this process is dependent on the type of buffer used and does not occur under our experimental conditions. Further experiments suggested that sequestration of the metal ions of Con A by specific buffers (phosphate) and other solution components may account for these observations, since the apoCon A that is formed is insoluble at physiological pH[35]. We emphasize that all of the cross-linked complexes observed between SBA and Con A were inhibited from forming, or dissolved by competing monosaccharide, and are thus due to specific carbohydrate–protein interactions and not to nonspecific protein–protein interactions.

In summary the present results demonstrated that glycoproteins with multiple oligosaccharide chains form different types of cross-linked aggregates with multivalent lectins depending on several factors. Each type of aggregated complex appears to have its own unique lattice which is dependent on the multivalency of the glycoprotein as well as the lectin. In view of the correlation, with a variety of cellular events[36–38], of the aggregation of cell-surface carbohydrate receptors upon addition of lectins the implications of these findings are intriguing. The results suggest that specific aggregation events, which could control signal transduction processes on the surface of a cell, could be regulated by the number and type of carbohydrate chains on a glycoprotein as well as the quaternary structures of both the glycoprotein and lectin.

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