Interactions of Concanavalin A with Asparagine-Linked Glycopeptides: Formation of Homogeneous Cross-Linked Lattices in Mixed Precipitation Systems†

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ABSTRACT: We have previously shown that certain oligomannose and bisected hybrid type glycopeptides are bivalent for binding to concanavalin A (Con A) [Bhattacharyya, L., Ceccarini, C., Lorenzoni, P., & Brewer, C. F. (1987) *J. Biol. Chem. 262*, 1288–1293]. Each glycopeptide gives a quantitative precipitation profile with the protein which consists of a single peak that corresponds to the binding stoichiometry of glycopeptide to protein monomer (1:2). We have shown that the affinities of the primary and secondary sites of the glycopeptides influence their extent of precipitation with the lectin [Bhattacharyya, L., & Brewer, C. F. (1988) *Eur. J. Biochem.* (in press)]. In the present study, we demonstrate that equimolar mixtures of any two of the glycopeptides result in a quantitative precipitation profile which shows two protein peaks. Using radiolabeled glycopeptides, the precipitation profiles of the individual glycopeptides were determined. The results show that each glycopeptide forms its own precipitation profile with the protein which is independent of the profile of the other glycopeptide. For mixtures containing an equimolar ratio of two glycopeptides, the glycopeptide with lower affinity shows a precipitation maximum at a lower concentration than the one with higher affinity. However, this can be reversed by increasing the ratio of the lower affinity glycopeptide in the mixture. Thus, the relative precipitation maxima of the glycopeptides are determined by mass-action equilibria involving competitive binding of the two carbohydrates to the protein. These equilibria, in turn, are sensitive to the relative amounts and affinities of the carbohydrates at both their primary and secondary sites. These findings indicate that each glycopeptide forms a unique homogeneous cross-linked lattice with the lectin which excludes the lattice of another glycopeptide in a mixture. The results are discussed in terms of the structure–function properties of asparagine-linked carbohydrates and lectins, as well as other multivalent binding systems.

The oligosaccharide chains of glycoproteins and glycolipids have been implicated as receptors in cellular recognition processes (Lennarz, 1980; Monsigny, 1984; Brandley & Schnaar, 1986). Asparagine-linked (N-linked) oligosaccharides constitute one class of oligosaccharide chains associated with glycoproteins (Kobata, 1984; Kornfeld & Kornfeld, 1985). Recently, we have shown that many of the N-linked oligosaccharides possess multivalent binding activities. For example, bi-, tri-, and tetraantennary complex type N-linked carbohydrates are bi-, tri-, and tetravalent for binding a variety of D-galactose- and L-fucose-specific lectins (Bhattacharyya et al., 1988a,b). We have also demonstrated that certain N-linked oligomannose and bisected hybrid type glycopeptides are bivalent for the glucose/mannose-specific lectin concanavalin A (Con A)[1] and that they can bind and precipitate the protein (Bhattacharyya et al., 1987a,b). The two Con A binding sites in the glycopeptides were located on the $\alpha(1-6)$ and $\alpha(1-3)$ arms of the core β-mannose residue, respectively. The site on the $\alpha(1-6)$ arm was identified as the high-affinity or primary site and the site on the $\alpha(1-3)$ arm as the low-affinity or secondary site (Bhattacharyya et al., 1987a).

Recently, we have shown that the affinities of the primary and secondary sites of certain oligomannose and bisected hybrid type glycopeptides influence their extent of precipitation of Con A (Bhattacharyya & Brewer, 1988). In the present paper, we show that in the presence of two such glycopeptides Con A forms homogeneous aggregates with each carbohydrate. These findings are discussed in terms of the structure–function properties of N-linked carbohydrates and lectins, as well as other multivalent systems.

Materials and Methods

Materials. Con A was purchased from Miles-Yeda. The concentration of Con A was determined spectrophotometrically at pH 5.6 with an absorbance $A^{1\%,1cm} = 12.4$ at 280 nm (Yariv et al., 1968) and expressed in terms of the monomer, M_r 26 000 (Wang et al., 1971). Glycopeptide **2** was obtained as a gift from Dr. Annette Herscovics, and **3**, **4**, and **5** were kindly provided by Dr. William Chaney. Soybean lectin was purified as described previously (Bhattacharyya et al., 1988a). Oligomannose-type glycopeptide **1** was prepared from soybean lectin by Pronase digestion of the lectin (Lis & Sharon, 1978). The purity of each glycopeptide was checked by high-resolution ^{1}H NMR at 500 MHz (Vliegenthart et al., 1983). Concentrations of glycopeptides were measured by the phenol–sulfuric acid method (Dubois et al., 1956) using D-mannose as standard. Monosaccharides were obtained from Sigma Chemical Co.

Radiolabeling of Glycopeptides. The glycopeptides were radiolabeled with ^{3}H or ^{14}C by reductive methylation by modification of a previously described procedure (Means & Feeney, 1968). About 3–4 mg of each glycopeptide was

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[1] Abbreviations: Con A, concanavalin A with unspecified metal ion content; NMR, nuclear magnetic resonance.

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Table I: Estimated Affinities (K_d) of the Primary and Secondary Binding Sites of the Glycopeptides[a]

glycopeptides	K_d values	
	primary sites	secondary sites
1[a]	50 nM	5 μM
2[a]	150 nM	5 μM
3[b]	1.5 μM	30 μM
4[b]	1.5 μM	150 μM
5[b]	1.5 μM	75 μM

[a] From Bhattacharyya and Brewer (1988). [b] From Bhattacharyya et al. (1987a).

dissolved in 200 μL of 0.1 M sodium borate (pH 9.3) containing sodium borohydride (5 mg/mL), and 20 μL of radiolabeled (^3H or ^{14}C) formaldehyde (2% v/v in water) was added at 10-min intervals over a total period of 1 h. The glycopeptides were desalted on a 0.9 × 90 cm Sephadex G-25 column equilibrated in 10 mM acetic acid (pH 3.2) and lyophilized before use. [^3H]- and [^{14}C]formaldehydes were obtained from New England Nuclear (25 mCi/mmol) and Sigma Chemical Co. (10 mCi/mmol), respectively, and were diluted 100-fold with cold formaldehyde solutions.

Quantitative Precipitation Assays. The assays were performed in 0.1 M Tris-HCl buffer, pH 7.2, containing 0.9 M KCl, 1 mM Mn^{2+}, and 1 mM Ca^{2+}. Increasing amounts of a mixture of two glycopeptides in the appropriate ratio were taken in a series of tubes, and buffer was added to make a final volume of 200 μL. Two hundred microliters of Con A solution (approximately 4 mg/mL) was added in each tube, and the precipitation was allowed to continue for about 20 h at 22 °C, after which time equilibrium was attained. The supernatant was centrifuged off. The precipitates were washed twice with 100 μL of cold buffer and dissolved in 0.1 M methyl α-D-mannopyranoside to a final volume of 1 mL. The resulting solutions were analyzed for protein concentration as well as for radioactivity.

Hemagglutination Inhibition Assays. The assays were done at room temperature by the 2-fold serial dilution technique (Osawa & Matsumoto, 1972) in 10 mM Tris-HCl buffer, pH 7.2, containing 0.15 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, using a 3% (v/v) suspension of rabbit erythrocytes.

RESULTS

Hemagglutination Inhibition Assays. The results of inhibition studies show that the N-dimethylated glycopeptides are essentially as inhibitory as the respective parent glycopeptides (Bhattacharyya et al., 1987a; Bhattacharyya & Brewer, 1988).

The inhibitory concentrations of the carbohydrates show a linear relationship with their affinity constant values (K_d) for Con A [cf. Loontiens et al. (1975)]. Thus, K_d values of the glycopeptides were calculated from the hemagglutination inhibition data and are listed in Table I (Bhattacharyya et al., 1987a; Bhattacharyya & Brewer, 1988).

Properties of Radiolabeled Glycopeptides. In order to follow the precipitation profiles of individual glycopeptides with Con A in a solution containing two glycopeptides, individual carbohydrates were radiolabeled with [^3H]- or [^{14}C]formaldehyde by reductive alkylation (Means & Feeney, 1968). ^1H NMR analysis at 500 MHz showed that the procedure leads to dimethylation of the free amino group of the asparagine residue of the glycopeptides.

Quantitative precipitation assays with Con A and an equimolar mixture of any one of the glycopeptides in Figure 1 and the corresponding radiolabeled N-dimethylated derivative give a single peak in each case. The results for glycopeptide **5** and the corresponding radiolabeled ^{14}C-dimethylated derivative are shown in Figure 2. The profiles of percent Con A precipitated and cpm of the glycopeptide in the precipitates are similar in each case. The ratios of the total glycopeptide concentration to the concentration of Con A monomer at the maxima of the precipitation profiles [which gives the stoichiometry of the reaction (Kabat, 1976)] are 1:2.0, 1:1.8, 1:2.0, and 1:1.8 for **1**, **3**, **4**, and **5**, at protein concentrations of 74, 76, 176, and 76 μM, respectively, which agree with the results for the underivatized glycopeptides (Bhattacharyya et al., 1987a; Bhattacharyya & Brewer, 1988). Similar results were found for glycopeptide **2** (Bhattacharyya & Brewer, 1988). These results were also found for 70:30 ratios of the underivatized and radiolabeled glycopeptides. Under these conditions, the shapes of the profiles of percent Con A precipitated

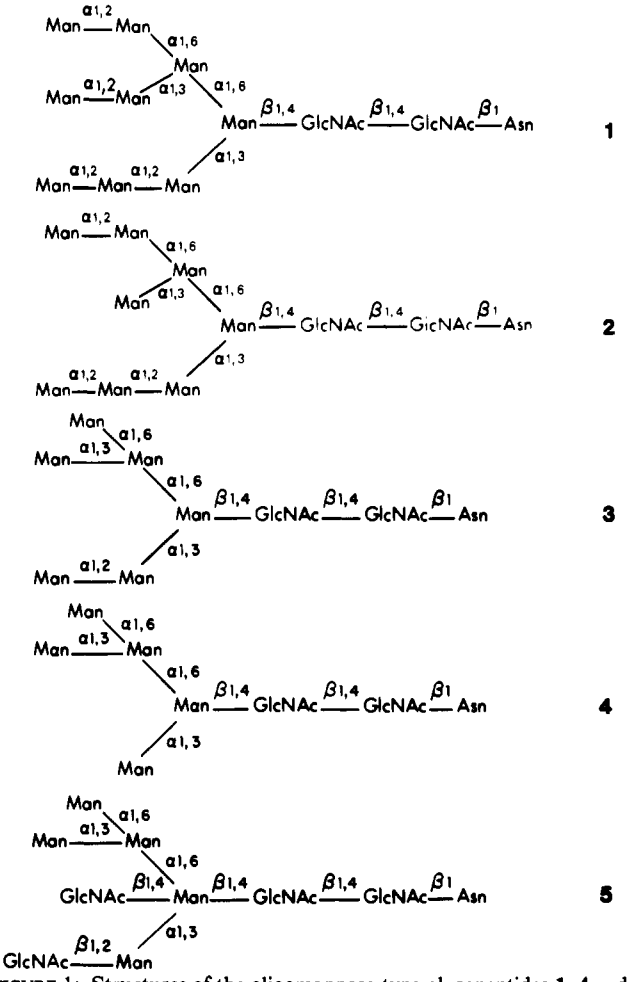

FIGURE 1: Structures of the oligomannose-type glycopeptides **1**–**4** and bisected hybrid type glycopeptide **5**.

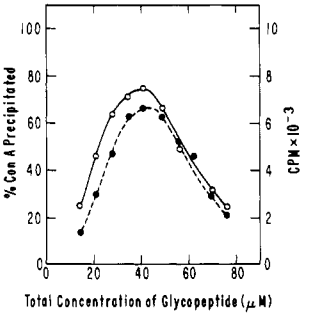

FIGURE 2: Precipitation profile of Con A (O) in the presence of a 50:50 mixture of glycopeptide **5** and the corresponding ^{14}C-dimethylated glycopeptide and cpm of the radiolabeled glycopeptide (●) in the precipitate. The protein concentration was 76 μM.

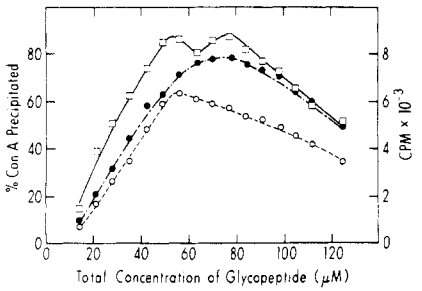

FIGURE 3: Precipitation profile of Con A (□) in the presence of a 50:50 mixture of ^{3}H-dimethylated glycopeptide **3** and ^{14}C-dimethylated glycopeptide **5**. The cpm of **3** (●) and **5** (○) in the precipitates are also shown. The protein concentration was 77 μM.

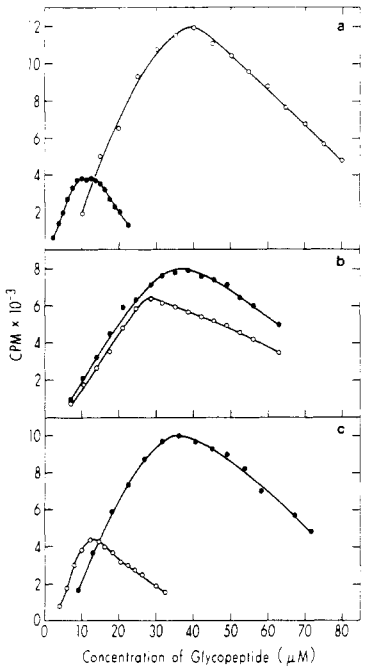

FIGURE 4: cpm in the precipitation profile of Con A with (a) 80:20, (b) 50:50, and (c) 30:70 mixtures of ^{14}C-dimethylated glycopeptide **5** (○) and ^{3}H-dimethylated glycopeptide **3** (●), respectively. The protein concentrations were 70, 77, and 73 μM, respectively.

were the same as those with the 50:50 ratios. In addition, the affinity of each N-dimethylated glycopeptide is similar to that of the native glycopeptide (Table I). Thus, the affinities and precipitation activities of the N-dimethylated glycopeptides are essentially the same as those of the parent glycopeptides.

Precipitation of Con A by Mixtures of Radiolabeled N-Dimethylated Glycopeptides. Figure 3 shows the quantitative precipitation profile of Con A in the presence of an equimolar mixture of radiolabeled glycopeptides **3** and **5** and the counts of each glycopeptide in the precipitate. The cpm values are corrected to reflect the relative amounts of both glycopeptides in the precipitate. Two protein peaks are observed, with ratios of the total concentration of the glycopeptides to protein of 1:1.4 and 1:1.0 at the first and second peaks, respectively. The radioactivity profiles show that the precipitation maximum for **5** corresponds with the first protein peak while that for **3** corresponds with the second protein peak. Figure 4 shows the profiles of the counts of the two glycopeptides in the precipitates with different ratios of the two carbohydrates. The concentrations of protein are similar in all three profiles. At a 50:50 ratio (Figure 4b), maximum precipitation of **5** occurs at a concentration of 28 μM and that for **3** at 37 μM, with the former precipitating less protein than the latter. At a 30:70

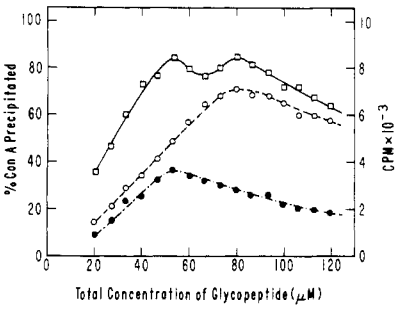

FIGURE 5: Precipitation profile of Con A (□) in the presence of a 50:50 mixture of ^{14}C-dimethylated glycopeptide **4** and ^{3}H-dimethylated glycopeptide **1**. The cpm of **4** (●) and **1** (○) in the precipitates are also shown. The protein concentration was 78 μM.

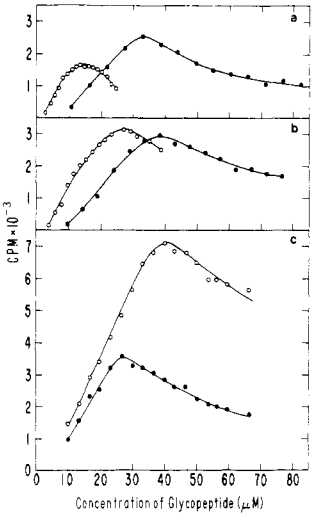

FIGURE 6: cpm in the precipitation profile of Con A with (a) 80:20, (b) 70:30, and (c) 50:50 mixtures of ^{14}C-dimethylated glycopeptide **4** (●) and ^{3}H-dimethylated glycopeptide **1** (○), respectively. The protein concentrations were 68, 75, and 78 μM, respectively.

ratio of **5** to **3** (Figure 4c), the precipitation maximum of **5** occurs at a glycopeptide concentration of 12 μM and that for **3** at 37 μM, with the amount of precipitate formed by **5** much less than that formed by **3**. However, at 80:20 ratio of **5** to **3** (Figure 4a), the precipitation maximum of **3** occurs at a glycopeptide concentration of 11 μM and that for **5** at 40 μM, with the former precipitating much less protein than the latter.

Figure 5 shows the quantitative precipitation profile of Con A in the presence of an equimolar mixture of radiolabeled glycopeptides **1** and **4** and the counts of each glycopeptide in the precipitate. The cpm values are corrected to reflect the relative amounts of both glycopeptides in the precipitate. Two protein peaks are observed, with ratios of the total concentrations of the glycopeptides to protein of 1:1.5 and 1:1 at the first and second peaks, respectively. The radioactivity profiles show that the precipitation maximum of **4** corresponds with the first peak of Con A and that of **1** corresponds with the second protein peak. Figure 6 shows the precipitation profiles of the two glycopeptides with different ratios of the two carbohydrates. The concentrations of protein are similar in all three profiles. At a 50:50 ratio (Figure 6c), maximum precipitation of **4** occurs at a concentration of the glycopeptide of 27 μM, while maximum precipitation of **1** occurs at 40 μM. Under these conditions, **4** precipitates less protein than **1**. However, at a 70:30 ratio of **4** to **1** (Figure 6b), glycopeptide **1** exhibits maximum precipitation at 27 μM and **4** shows maximum precipitation at 38 μM, with both glycopeptides precipitating about the same amount of protein. At an 80:20 ratio of **4** to **1** (Figure 6a), the point of maximum precipitation

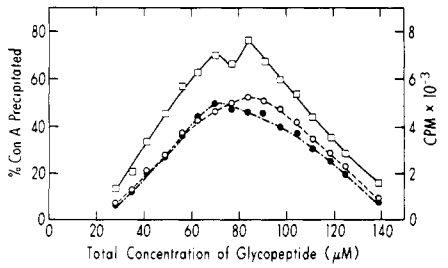

FIGURE 7: Precipitation profile of Con A (□) in the presence of a 50:50 mixture of ^{14}C-dimethylated glycopeptide **4** and ^{3}H-dimethylated glycopeptide **5**. The cpm of **4** (●) and **5** (○) in the precipitates are also shown. The protein concentration was 127 μM.

of **1** occurs at a glycopeptide concentration of 15 μM and that for **4** occurs at 33 μM, with **1** precipitating much less protein than **4**.

Figure 7 shows the quantitative precipitation profile of Con A in the presence of an equimolar mixture of radiolabeled glycopeptides **4** and **5** and the counts of each glycopeptide in the precipitate. The cpm values are corrected to reflect the relative amounts of both glycopeptides in the precipitate. Two closely spaced protein peaks are observed, with ratios of the total concentration of the two glycopeptides to protein of 1:1.8 and 1:1.5 at the first and second peaks, respectively. The radioactivity profiles show that the precipitation maximum of **4** corresponds to the first protein peak while that for **5** corresponds to the second protein peak.

The quantitative precipitation profile of Con A (71 μM) in the presence of an equimolar mixture of glycopeptides **1** and **2** shows two closely spaced protein peaks (similar to that in Figure 7) with the ratios of the total concentration of the two glycopeptides to protein of 1:2.1 and 1:1.5 at the first and second peaks, respectively (profiles not shown). The radioactivity profiles show that the precipitation maximum for **2** corresponds to the first protein peak, while that for **1** corresponds to the second protein peak.

Quantitative precipitation studies with 80:20, 50:50, and 30:70 mixtures of **4** and **5** and of **2** and **1** show radioactivity precipitation profiles similar to those in panels a–c of Figure 4, respectively (data not shown).

The quantitative precipitation profile of Con A (77 μM) in the presence of an equimolar mixture of glycopeptides **1** and **5** shows two well-separated protein peaks (as in Figure 5). The radioactivity profiles show that the precipitation maximum for **5** occurs at a total glycopeptide concentration of 56 μM which corresponds with the first protein peak and that the precipitation maximum for **1** occurs at 90 μM, which corresponds with the second protein peak (profiles not shown).

Discussion

Glycopeptides **1–5** have previously been shown to be bivalent for Con A binding, and each gives a quantitative precipitation profile with the protein that consists of a single peak (equivalence point) corresponding to the binding stoichiometry of glycopeptide to protein monomer (1:2) (Bhattacharyya et al., 1987a; Bhattacharyya & Brewer, 1988). (In each case, the profile is similar to the protein profile in Figure 2.) The present results provide evidence that each glycopeptide forms a unique homogeneous cross-linked lattice with the protein in the precipitates.

Quantitative Precipitation Profiles of Con A in the Presence of Equimolar Concentrations of Two Glycopeptides. The quantiative precipitation profile of Con A in the presence of an equimolar mixture of any two of the glycopeptides in Figure 1 shows two peaks for the percent protein precipitated. The presence of two distinct protein peaks indicates that each glycopeptide forms a homogeneous cross-linked lattice with the lectin. However, in order to gain further insight into the precipitation profiles, radiolabeled glycopeptides were used in order to monitor the precipitation activities of the individual glycopeptides.

Radiolabeled glycopeptides **3** (^{3}H) and **5** (^{14}C) bind nearly equally well to the protein (Table I) due to their common primary binding sites [the trimannosyl moiety on the core α(1–6) arm (Brewer & Bhattacharyya, 1986)]. However, the precipitation profile of the protein in the presence of an equimolar mixture of the two radiolabeled glycopeptides shows two peaks (Figure 3), as observed with the unlabeled glycopeptides (data not shown). In addition, the ratio of total glycopeptide (obtained from their respective specific activities) to protein monomer in the precipitates across the entire profile is close to 1:2, as found in the individual precipitation profiles of the glycopeptides (Bhattacharyya et al., 1987a; Bhattacharyya & Brewer, 1988). The presence of different precipitation maxima for the two glycopeptides, which correspond to the two protein peaks, and different precipitation profiles is consistent with the formation of an independent homogeneous lattice for each carbohydrate. Similar results were obtained with equimolar mixtures of glycopeptides **1** and **4**, **4** and **5**, **1** and **5**, and **1** and **2**. The results also show that, in every case, the weaker binding glycopeptide displays a precipitation maximum at a lower glycopeptide concentration than that of the higher affinity glycopeptide. In order to determine the factors that influence the concentration dependence of the precipitation maxima of the glycopeptides, the precipitation reactions were performed with altered ratios of the carbohydrates.

Effects of Altered Ratios of the Glycopeptides on Their Precipitation Profiles. When an equimolar ratio of glycopeptides **3** and **5** is used in a precipitation profile (Figure 4b), the precipitation maximum of **5** occurs at a lower concentration than that of **3**. However, when the ratio of **5** to **3** is 80:20, the precipitation maximum of **3** shifts to a lower glycopeptide concentration, while that for **5** shifts to higher concentration (Figure 4a). When the ratio of **5** to **3** is 30:70, the precipitation maximum of glycopeptide **5** shifts to a lower concentration, while that for **3** shifts to a higher relative concentration (Figure 4c). The amount of the glycopeptides precipitated at their respective precipitation maxima also changes in relation to the relative ratios of the two carbohydrates. Thus, although the two exhibit separate precipitation profiles, the precipitation maximum of each glycopeptide is sensitive to the relative ratio of the two carbohydrates.

These results suggest that the relative positions of the precipitation maxima of the glycopeptides are determined by mass-action equilibria which arise from competitive binding of the two carbohydrates to the protein. Competitive binding of one glycopeptide effectively lowers the concentration of protein available to bind to the other glycopeptide. Since the *intrinsic* ratio of carbohydrate to protein in the precipitate of an individual glycopeptide is always 1:2 (or close) (Bhattacharyya et al., 1987a; Bhattacharyya & Brewer, 1988), lowering the available protein concentration shifts the position of the precipitation maximum of a glycopeptide to lower concentration. Thus, by raising the concentration of glycopeptide **5** relative to **3** in each sample, **5** binds to more protein than **3**. Hence, the precipitation maximum of **3** shifts to a lower concentration (Figure 4a), and the apparent equivalence ratio of **3** to total protein concentration (1:6.4) reflects the loss of protein available to bind to the glycopeptide. (If the available protein concentration drops, then the carbohydrate

concentration must drop to maintain the fixed 1:2 ratio in the precipitate.) When the ratio of the two glycopeptides is reversed, the opposite occurs, as is shown in Figure 4c. Similar results are observed for mixtures of **1** and **4**, **4** and **5**, and **1** and **2**.

Effect of the Affinities of the Glycopeptides on Their Relative Precipitation Maxima. The observation of mass-action effects on the relative positions of the precipitation maxima of the glycopeptides suggest that the relative affinities of the glycopeptides are important in this regard. The order of the precipitation maxima of the glycopeptides in equimolar mixtures can thus be explained by their relative affinities for the protein. For example, in the equimolar mixture of **1** and **4**, the precipitation maximum of **4** corresponds to the first protein peak while that for **1** corresponds to the second peak (Figure 5). Bhattacharyya and Brewer (1988) have shown that the primary site of **1**, the pentamannosyl moiety on the core $\alpha(1-6)$ arm, has higher affinity for the protein by a factor of 30 compared to the primary site of **4**, the trimannosyl moiety (Table I). Glycopeptide **1** also has higher affinity at its secondary site [the trimannosyl moiety on the core $\alpha(1-3)$ arm] by a factor of approximately 20 compared to the α-mannosyl moiety at the same position of **4** (Table I) (Bhattacharyya & Brewer, 1988). Thus, glycopeptide **1** binds to more protein in solution than **4**, which reduces the effective concentration of Con A available to bind to the latter. Hence, the precipitation maximum of **4** occurs at a lower carbohydrate concentration than that of **1** (details discussed below). The large difference in affinities of the two glycopeptides results in a large separation of the precipitation maxima of the two carbohydrates (Figure 5). This is also true for **1** and **5**. However, when differences in affinities of the primary and secondary sites are smaller, such as for **1** and **2** [a factor of 3 higher affinity of the primary site of **1** (Table I)], the separation of the precipitation maxima of the glycopeptides is smaller.

These findings also suggest why the precipitation maximum of **5** occurs at a lower concentration than that of **3** in the presence of an equimolar mixture of the glycopeptides (Figure 4b). Although the primary high-affinity sites of the two glycopeptides are the same [the trimannosyl moiety on the $\alpha(1-6)$ arms], the secondary binding site of **3** [the mannobiosyl moiety on the $\alpha(1-3)$ arm] possesses a factor of approximately 3 higher affinity than the secondary site of **5** [the *N*-acetylglucosaminylmannosyl moiety on the $\alpha(1-3)$ arm] (Bhattacharyya et al., 1987a; Bhattacharyya & Brewer, 1988). Thus, the precipitation maximum of **5** occurs at a lower concentration than that of **3**, because of the lower affinity of the secondary site of the former. The lower affinity of the secondary site of **4** [the α-mannosyl residue on the $\alpha(1-3)$ arm] compared to **5** (Bhattacharyya et al., 1987a; Bhattacharyya & Brewer, 1988) also explains the occurrence of the precipitation maximum of **4** at a lower concentration in an equimolar mixture (Figure 7).

Differences in the affinities of the glycopeptides also influence their relative precipitation maxima in altered ratio experiments. For example, in the precipitation profiles of mixtures of glycopeptides **1** and **4**, which have large differences in the affinities of their primary and secondary sites (factors of 30 and 20, respectively) (Bhattacharyya & Brewer, 1988), increasing the ratio of **4** to **1** to 70:30 (Figure 6b) and 80:20 (Figure 6a) reverses the order of the precipitation maxima of the two glycopeptides from that in the 50:50 mixture (Figure 6c). However, the extent of separation is not as dramatic as similar changes between pairs of glycopeptides such as **3** and **5** (Figure 4) and **4** and **5**, which have close affinities for the lectin (Table I) (Bhattacharyya et al., 1987a). The difference in the behavior of mixtures of **1** and **4** is due to the relatively weak binding of **4** compared to **1**, which requires a much greater proportion of **4** in the mixture to reverse the order of the precipitation maxima of the two glycopeptides.

These results demonstrate that differences in the affinities of the glycopeptides at their primary and secondary binding sites, as well as differences in the ratios of the glycopeptides, determine the order of the precipitation maxima in the precipitation profiles.

Analysis of the Precipitation Profile of the Protein: Evidence for the Formation of Homogeneous Precipitates. The precipitation profile of Con A with a glycopeptide mixture is a result of titrating the binding sites of the protein with increasing concentrations of two glycopeptides. Consider, for example, the precipitation profile of Con A (78 μM) in the presence of an equimolar mixture of **1** and **4** (Figure 5). At low concentrations of the two carbohydrates, an excess of protein binding sites exists which essentially allows an equal number of complexes to be formed between both glycopeptides and the lectin. This continues until all of the protein sites are occupied by the primary and secondary binding sites of the carbohydrates (54 μM), with both glycopeptides showing nearly equal amounts of precipitate due to the formation of 2:4 glycopeptide/protein (monomer) complexes (the protein is a tetramer under these conditions). Furthermore, under these conditions, most of the protein is precipitated (85%). The presence of homogeneous or heterogeneous lattices between the glycopeptides and protein cannot be distinguished up to this point in the profile. However, beyond this point, the two possibilities can be distinguished. Further increase in the concentration of both glycopeptides leads to their competitive binding, with the higher affinity carbohydrate displacing the lower affinity one, which may be represented by the equilibrium

$$L_2P_4 \rightleftharpoons HLP_4 \rightleftharpoons H_2P_4$$

where H, L, and P represent high- and low-affinity glycopeptides and the lectin monomer, respectively. Previous results indicate that both L_2P_4 and H_2P_4 (2:4 homogeneous complexes between the glycopeptides and the lectin) form precipitates (Bhattacharyya et al., 1987a; Bhattacharyya & Brewer, 1988). Thus, with increasing concentration of total glycopeptide beyond 54 μM, the proportion of heterogeneous complexes of **1** and **4** (HLP_4) increases. If HLP_4 also precipitates, then its formation would not decrease the total amount of precipitated protein. However, as observed, the amount of precipitated protein declines at this point in the profile. This indicates that the formation of heterogeneous complexes in a 2:4 ratio of the two glycopeptides and the protein (HLP_4) leads to soluble complexes. The precipitates themselves, therefore, must be exclusively composed of homogeneous complexes with the glycopeptides.

The decrease in percent protein precipitated above 54 μM total glycopeptide in Figure 5 is thus due, in part, to conversion of the homogeneous precipitates of **4** to soluble heterogeneous complexes of **1** and **4**. A second mechanism involving formation of soluble 3:4 and 4:4 complexes between **4** and the protein also contributes to the decrease in the precipitation of **4**. A combination of these two pathways, which decreases the amount of protein precipitated by **4**, offsets the increase by **1**. At a total glycopeptide concentration of approximately 67 μM, however, the percent protein precipitated increases as the extent of precipitate formed by **1** offsets the decrease due to **4**. Precipitation by glycopeptide **1** rises until it forms a maximum of 2:4 complexes with the protein, after which

soluble complexes of **1** are formed in 3:4 and 4:4 ratios.

CONCLUSIONS

The present results demonstrate that a variety of bivalent oligomannose and bisected hybrid type glycopeptides form homogeneous aggregates with the lectin. The homogeneous nature of the precipitates appears to be due to the unique cross-linked lattice of each complex, which can be compared to selective crystallization of solutes from solutions. In any case, in the presence of mixtures of the glycopeptides, the specificity of binding of the oligomannose and the bisected hybrid type glycopeptides to Con A is far greater in their cross-linked complexes than in the corresponding soluble complexes. Since we have recently demonstrated that D-galactose- (Bhattacharyya et al., 1988a) and L-fucose-specific lectins (Bhattacharyya et al., 1988b) also undergo cross-linking with multivalent N-linked glycopeptides and related oligosaccharides containing terminal D-galactose and L-fucose residues, respectively, the present results suggest that homogeneous aggregates may occur in these systems as well.

The implications of this study are far reaching. If these interactions occur with multivalent glycoproteins, whether via the multivalency of an individual carbohydrate chain or the presence of multiple carbohydrate chains on a protein, then the above observations may explain one possibility for the specificity of N-linked-type carbohydrates as receptors on the surface of cells. The ability of such multivalent molecules to form homogeneous cross-linked complexes with multivalent proteins may be required for biological signal transduction effects. Furthermore, by varying the concentration of competing carbohydrate structures in the mileau, the precipitation (cross-linking) maximum of a given multivalent carbohydrate (glycoprotein) can be altered. Thus, microheterogeneity in glycosylation could serve as a regulatory mechanism controlling signal transduction events.

The results of the present study may also serve as a general model for a variety of multivalent ligand–receptor systems. These would include antibody–antigen, glycolipid–protein, and hormone–protein interactions. Evidence, in fact, has been obtained in many biological receptor systems that aggregation of specific receptors following ligand binding is required for signal transduction effects (DeLisi & Blumenthal, 1979). The present finding may provide insight into the physical mechanism involved in these aggregation processes.

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