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Neoglycoproteins: Preparation and Properties of Complexes of Biotinylated Asparagine–Oligosaccharides with Avidin and Streptavidin†

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ABSTRACT: Neoglycoproteins in which the oligosaccharide moieties are attached noncovalently to the protein through a high-affinity ligand have been prepared from biotinylated oligosaccharides and avidin or the nonglycosylated microbial analogue streptavidin. One of the asparagine–oligosaccharides purified from Pronase-digested ovalbumin (Man_6-$GlcNAc_2$-Asn) was reacted with an excess of the hydroxysuccinimide ester of biotin or, for the purpose of quantitation, [^{3}H]biotin. Derivatives were also prepared with an extension "arm", a 6-aminohexanoyl group, between biotin and asparagine. When the purified biotinyl-Asn–oligosaccharide was added to avidin or streptavidin, a complex was formed containing 3 mol of oligosaccharide/mol of protein. The complexes were stable at neutral pH in the absence of biotin and could be dialyzed for 2 weeks without any significant loss of ligand. In the presence of biotin, or under denaturing conditions, the oligosaccharide derivative was released and could be quantitatively recovered. To assess the influence of the protein matrix on the reactivity of the oligosaccharide units, free biotinyl-Asn–oligosaccharide and the corresponding avidin and streptavidin complexes were exposed to α-mannosidase in parallel experiments. The rate of hydrolysis of the free derivative was severalfold faster than that of the two protein complexes, and at the time when about 90% of the free derivative had all five α-mannosyl residues removed, the majority of the protein-bound derivatives contained two to four undigested α-mannosyl residues and also had a significant amount of undigested starting material. The ease of preparation and the properties of these neoglycoproteins suggest that they should be excellent models for the study of glycoprotein–receptor binding and glycoprotein processing.

A variety of biological recognition processes use glycoproteins as specificity determinants. Some examples of these processes include circulatory clearance of serum proteins (Neufeld & Ashwell, 1980; Ashwell & Morell, 1974), compartmentalization of lysosomal enzymes (Neufeld & Ashwell, 1980; Kaplan et al., 1977), developmental processes of slime mold and certain vertebrates (Barondes, 1981), and the specificity involved in legume–symbiont interaction (Schmidt, 1979). In order to fully evaluate how various oligosaccharide

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structures attached to a polypeptide may provide structural determinants in recognition processes, one needs glycoproteins homogeneous in composition of both amino acids and sugar residues. Since the majority of glycoproteins known today are heterogeneous with respect to the number and nature of the glycosyl moieties (Sharon & Lis, 1982), it appears that at this time studies must be carried out employing appropriate model glycoproteins prepared synthetically by conjugating well-characterized oligosaccharides to proteins. Such artificial conjugates, neoglycoproteins, have been used extensively in several areas of biochemistry and cell biology (Stowell & Lee, 1980).

In exploring different methods for the preparation of neoglycoproteins that contain naturally occurring oligosaccharides attached to proteins in defined location and at specific stoichiometry, we recently demonstrated the feasibility of preparing noncovalent oligosaccharide–protein complexes (Chen & Wold, 1984). The work proposed that ligands (such as coenzymes) exhibiting high affinity for specific sites on proteins (such as the corresponding apoprotein) can be exploited as cross-linking reagents in joining oligosaccharides to proteins. In this paper, we report results obtained from extending the application of this concept to the use of biotin as the cross-linking reagent for attaching an oligosaccharide to avidin and its microbial analogue streptavidin, both of which will be designated as biotin-binding protein (BBP)[1] in this paper. The avidin–biotin system has been employed in many areas of biochemical research (Bayer & Wilchek, 1980), and on the basis of the thorough characterization of the two BBPs (Green, 1975; Chaiet & Wolf, 1964), it was natural to select it for neoglycoprotein preparation. The previous studies have established the high affinity of biotin for avidin ($K_d = 10^{-15}$ M) over a wide pH range and the ability of BBP to bind a variety of biotinylated compounds including steroids (Manz et al., 1983), spin-labels (Chignell et al., 1975), and polypeptide hormones (Finn et al., 1984). Avidin and streptavidin behave alike in their reactions with ligands (Green, 1975); they are both tetrameric with one biotin site in each of the four identical 14000-Da subunits. The primary structure of avidin is known (DeLange & Huang, 1971); it is naturally glycosylated at Asn-17. However, streptavidin does not contain covalently linked sugar. Avidin has been crystallized (Green & Joynson, 1970) and studied by X-ray diffraction methods.

Materials and Methods

Biotin, avidin, *p*-nitrophenyl α-D-mannopyranoside, jack bean α-mannosidase, ovalbumin (twice crystallized), methyl α-D-mannopyranoside, Con A–Sepharose 4B, and Sephadex G-25 (80 mesh) were obtained from Sigma Chemical Co. *N*-Hydroxysuccinimide was from Aldrich Chemical Co., and dicyclohexylcarbodiimide was from Pierce Chemical Co. Streptavidin was a generous gift from L. Chaiet of Merck Sharp & Dohme. Cation exchanger AG 50W (H^+ form) (200–400 mesh) was from Bio-Rad. Tritiated biotin was from New England Nuclear.

Asparagine–oligosaccharide from ovalbumin (fraction AC-D, Asn-$GlcNAc_2$-Man_6) was isolated by cation-exchange chromatography according to the methods of Huang et al. (1970). The identity and purity of the oligosaccharide was ascertained by proton nuclear magnetic resonance spectroscopy, chemical analysis, and fast atom bombardment mass spectrometry, $(M + 1)^+ = 1512.5$. The *N*-hydroxysuccinimide ester of [^{3}H]biotin was synthesized according to the methods of Bayer et al. (1979).

Buffers used were as follows: (1) cacodylate buffer, which contained 20 mM cacodylate and 1 mM zinc acetate, pH adjusted to 5.5 with NaOH; (2) Con A buffer, which contained 20 mM triethanolamine hydrochloride, 0.1 M NaCl, 1 mM each of $CaCl_2$ and $MgCl_2$, and 0.02% NaN_3, pH adjusted to 7.8 with NaOH.

Biotinylation of Asparagine–Oligosaccharide. Dried and desalted asparagine–oligosaccharide (2 μmol) and the *N*-hydroxysuccinimide ester of biotin (5 μmol) were separately dissolved in dimethyl sulfoxide (0.5 mL) previously dried over a 3-Å molecular sieve. The homogeneous solutions were mixed together with a drop (40 μL) of triethylamine, and the mixture was stoppered and left at room temperature overnight. Following removal of the solvent in vacuo, the unreacted biotin reagent was hydrolyzed with 1 M triethanolamine hydrochloride, pH 8 (1 mL), for 4 h at room temperature. Biotinylated asparagine–oligosaccharide (BAO) was purified by passage of the reaction mixture through a column (1.5 × 96 cm) packed with Sephadex G-25 (80 mesh) and eluted with water. The yield of biotinylated product was 60–80%, determined colorimetrically according to either the method of Green (1970) or that of McCormick and Roth (1970) with free biotin as standard. To incorporate a linker arm between biotin and Asn, N^{α}-(6-aminohexanoyl)asparagine–oligosaccharide was first prepared by the method of Yan and Wold (1984). This derivative was then biotinylated as described above with similar yields. Although the bulk of the data will refer only to BAO, both derivatives were used.

Reconstitution. A BAO–BBP complex was formed by mixing BBP (70–300 nmol of subunit) and BAO (1.0–1.2 equiv) in 0.5 M potassium phosphate, pH 6.9 (1.0 mL). The mixture was allowed to stand at room temperature overnight and was subsequently dialyzed against cacodylate buffer (200 mL) at 4 °C.

Stoichiometry and Stability. A single test sample was used for determining the following: (1) concentration of BBP, which was calculated from either the result of amino acid analysis with the published composition for BBP (Green, 1975) or absorbance at 282 nm with 24000 and 56000 for the values of molar absorptivity for avidin and streptavidin, respectively; (2) the total amount of BAO, which was accomplished by liquid scintillation counting; (3) the fraction of BAO dissociated, which was estimated by comparing the radioactivity in two identical aliquots of the sample in which one had been ultrafiltered through an Amicon YMT membrane in a Centrifree apparatus, to remove protein-bound BAO.

Reaction with α-Mannosidase. Commercially obtained α-mannosidase was dialyzed into cacodylate buffer at 4 °C. The enzymatic activity was assayed with 10 mM *p*-nitrophenyl α-D-mannopyranoside as substrate. A unit of activity was taken as the amount of enzyme capable of hydrolyzing 1 μmol of substrate in 1 min. Digestion of neoglycoproteins and free oligosaccharide derivatives was carried out at 27 °C in a volume of 3 mL containing 15 units/mL α-mannosidase and 38–41 nmol of free BAO or complex in terms of subunit. At desired times during the course of the reaction, aliquots (0.2 mL) of the reaction mixture were removed and quenched by heating in boiling water with equal volumes of 1 M triethanolamine hydrochloride, pH 8, with or without 1 mM free biotin. The time course of the reaction was monitored by following the change in the ability of modified BAO to bind Con A–Sepharose. For this purpose, the reaction was

[1] Abbreviations: Con A, concanavalin A; BBP, biotin-binding proteins (used to collectively describe avidin and streptavidin); BAO, N^{α}-biotinylasparagine–oligosaccharide; BHAO, N^{α}-[6-(biotinylamino)hexanoyl]asparagine–oligosaccharide.

quenched in the presence of biotin. The released BAO was separated from the protein by ultrafiltration through the YMT membrane and allowed to pass through a small (1.4-mL) Con A–Sepharose column equilibrated in Con A buffer. The column was eluted with Con A buffer (5 mL) followed by an identical volume of the same buffer containing 0.1 M methyl α-D-mannopyranoside. All radioactivity eluted in Con A buffer was considered unbound while that eluted with methyl α-D-mannopyranoside was bound. For the BAO–streptavidin complex, a separate aliquot of the reaction mixture was quenched in the absence of biotin and similarly assayed for Con A binding without removal of streptavidin. At the end of the digestion by α-mannosidase, the remaining reaction mixture was quenched in the presence of biotin. After ultrafiltration across the YMT membrane, the released BAO was dried by lyophilization. The dried solid was redissolved in 0.5 mL of water containing 2 mg of maltotriose and methyl α-D-mannopyranoside as internal markers and allowed to pass through a column (1.5 × 166 cm) packed with Sephadex G-25 (80 mesh) eluted with 0.02 M triethanolamine hydrochloride containing 0.1 M NaCl, pH 8. Fractions collected (1.9 mL) were examined for radioactivity by liquid scintillation counting and for the location of markers by reaction with phenol and sulfuric acid (Dubois et al., 1956).

Results and Discussion

The experiments reported herein were performed with two purposes in mind. One was to establish that neoglycoproteins could be prepared with defined stoichiometry through the interaction of BAO and BBP. The second was to explore the effect of the protein matrix on the properties of the oligosaccharide moiety, by comparing the susceptibility to α-mannosidase of the free and BBP-bound biotinyl oligosaccharide.

Both avidin and streptavidin are known to bind 4 equiv of the dye 4-hydroxyazobenzene-2′-carboxylic acid per tetramer (K_d = 7 and 100 μM, respectively; Green, 1970) to give a complex exhibiting a distinct absorption band centered at 500 nm. The bound dye can be displaced by biotin with concomitant loss of absorption at that wavelength. Complete displacement of bound dye can also be accomplished with BAO, which is consistent with the conclusion that BAO binds avidin at the biotin site. The titration curves are given in Figure 1. Similar observations were made with streptavidin; however, the higher concentration of free dye needed to saturate streptavidin caused higher background absorption at 500 nm and led to substantial scattering of data near the end point (data not shown). Linearity is observed in both titration curves shown in Figure 1 up to 80% saturation. This portion of the titration curve was employed for the quantitative determination of BAO with biotin as standard. Despite probable differences in the affinity of biotin and BAO for avidin, the amount of BAO estimated by this dye displacement assay is within 5% the value obtained colorimetrically with its imino derivative of *p*-(dimethylamino)cinnamaldehyde according to the method of McCormick and Roth (1970).

The stoichiometry of ligand binding was estimated with radiolabeled BAO. The specific radioactivity of BAO was deduced from combined results from dye displacement and liquid scintillation counting, and the amount of protein was arrived at by amino acid analysis. It was found, after correction for unbound ligands, that both avidin and streptavidin bound 2.8 (±0.4 confidence limit) BAO per tetramer. This value and the results in Figure 1 are unexpectedly low, and we have no explanation for this apparent discrepancy. On the basis of the fact that both proteins give identical dye titrations

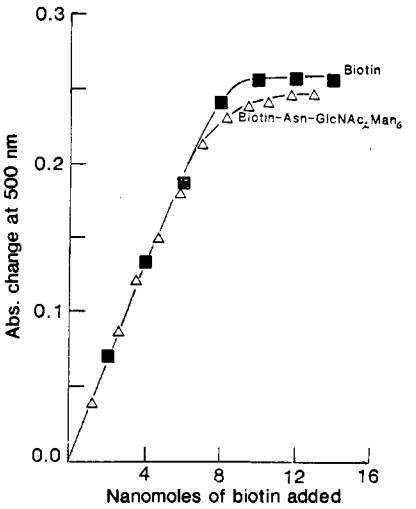

FIGURE 1: Titration of avidin–dye complex with biotin and biotinylasparagine–oligosaccharide. The protein (0.2 mg in 1 mL of buffer, containing a calculated total of 14 nmol of subunits) was treated with 2-μL aliquots of 1 mM biotin or biotinylasparagine–oligosaccharide. The end points correspond to approximately 0.7 biotin equiv per subunit.

for biotin and biotinyl oligosaccharides, we had expected to find 4 mol/mol of the oligosaccharide bound to the two BBPs, but the analytical data clearly gave 3 mol/mol. Other workers have reported similar anomalous behavior for the binding of biotin derivatives to avidin, suggesting that negative cooperativity may be involved with bulky ligands. Thus, biphasic kinetics were observed in binding of both biotinylated peptides to avidin derivatives (Hofmann et al., 1982) and biotinylated spin-labels to avidin (Chignell et al., 1975); an electron microscopic examination of avidin containing four molecules of 1-biotinamido-12-[(dinitrophenyl)amino]dodecane revealed that while the majority of the avidin complex reacted with two Fab fragments of anti-dinitrophenyl antibody, most interestingly, an occasional complex reacted with three (Green, 1975).

In assessing the stability of the noncovalent neoglycoproteins, it was found that the fraction of unbound BAO in two samples of complexes (8% for avidin and 12% for streptavidin) remained constant for a period of 22 h at pH 5.5. It appears, therefore, that the unbound BAO at zero time was the result of incomplete dialysis and that no dissociation took place. In a separate experiment using other preparations of the two BAO–BBP complexes, dialysis after reconstitution was carried out against 0.1 M potassium phosphate, pH 7, for 2 weeks. In this case, the amount of free BAO in both complexes was reduced to less than 5% while the BAO bound remained three per tetramer. In the presence of 1 mM biotin, however, BAO was released from the complexes (Figure 2). A faster rate of BAO dissociation from the streptavidin complex than from the avidin complex suggests that the latter is more tightly associated.

It was pointed out previously (Chen & Wold, 1984) that a noncovalent neoglycoprotein was a suitable model substrate for studying enzymatic processing of a maturing glycoprotein. To further test this prediction and to assess the effect of the protein matrix on the substrate quality of the oligosaccharides, the two BAO–BBP complexes and free BAO were subjected to a processing-like reaction under identical conditions with jack bean α-mannosidase as the model processing enzyme. The assay designed to follow the mannosidase action was based on the binding of high-mannose oligosaccharides to Con A and the loss of this binding when most of the α-mannosyl residues are removed. Intermediates in the reactions thus cannot be

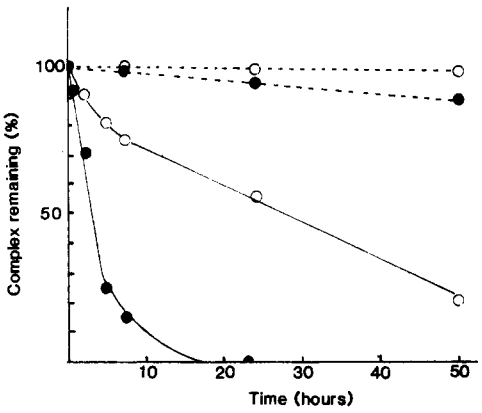

FIGURE 2: Stability of complexes of biotinylasparagine–oligosaccharide with avidin (O) and streptavidin (●) in the absence (– –) and presence (—) of 1 mM biotin. The complexes (2×10^{-5} M) had been dialyzed against 0.1 M potassium phosphate buffer, pH 7.0, for 2 weeks prior to the start of the experiment. At zero time, they were diluted 10-fold (final concentration 2×10^{-6} M) with 0.1 M triethanolamine hydrochloride, pH 7.5, with or without biotin. At the indicated times, samples were assayed for free biotinylasparagine–oligosaccharide as described in the text.

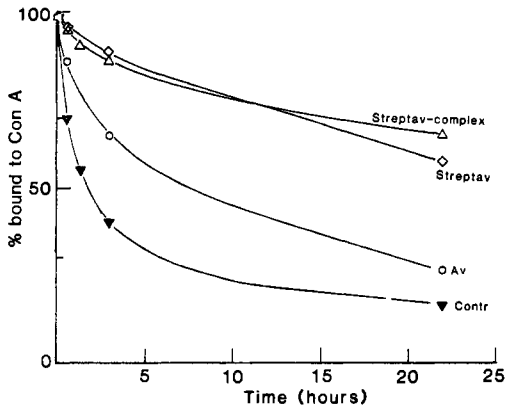

FIGURE 3: Effect of α-mannosidase digestion on the concanavalin A binding quality of free biotinylasparagine–oligosaccharide (▼) and the same oligosaccharide derivative associated with avidin (O) or with streptavidin (◇, △). One samaple of the streptavidin complex (△) was subjected to affinity chromatography without prior dissociation of the streptavidin–oligosaccharide complex (streptavidin is not a glycoprotein); for the other two protein complexes, the oligosaccharide was liberated from the complex before the affinity chromatography assay.

detected; only the binding starting material and intermediates and the nonbinding intermediates and products can be distinguished. There is still a good deal of uncertainty about the specificity of Con A (Carver & Brisson, 1984); in general, oligomannosidic structures containing more α-mannosyl residues than the typical trimannosylchitobiose core of N-linked oligosaccharides bind tightly to this lectin (Baenziger & Fiete, 1979). However, it is noteworthy that recently a tetramannosylchitobiose oligosaccharide was found to have low affinity for the lectin as well (Harpez & Schachter, 1980). Even if we allow for this ambiguity of interpretation, the time course of the reactions of free BAO and of the two BAO–BBP with α-mannosidase still indicates that free BAO was digested faster than the protein-bound BAO (Figure 3). The reaction was allowed to proceed until free BAO had lost about 90% of its Con A binding ability. At this point, all protein-bound BAO was released by heating in the presence of free biotin, and the total BAO in each sample was analyzed by gel permeation chromatography on Sephadex G-25 (80 mesh). The elution profiles (Figure 4) show that the majority of the digested material from the avidin complex was larger in size

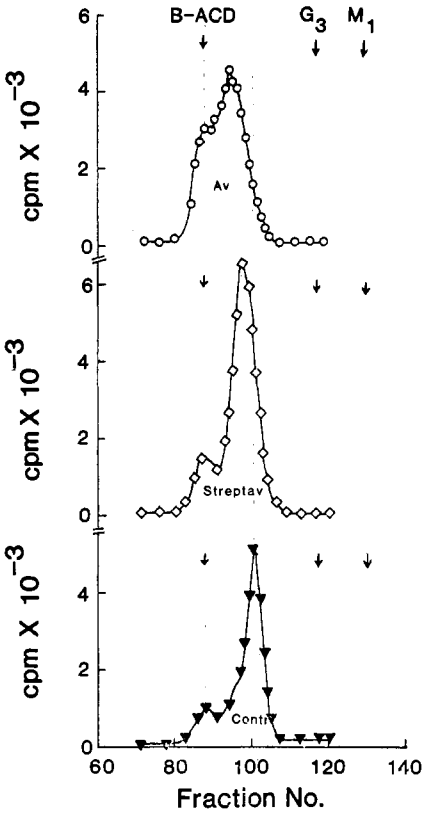

FIGURE 4: Gel permeation chromatography of the 24-h α-mannosidase digestion mixture from (from top to bottom) the avidin complex, the streptavidin complex, and free biotinylasparagine–oligosaccharide, after liberation of the oligosaccharide derivatives from their protein complexes. The Sephadex G-25 column was calibrated with methyl α-D-mannopyranoside (M_1), maltotriose (G_3), and the substrate biotinyl-Asn–$GlcNAc_2$-Man_6 (BACD; biotinylated fraction ACD from Pronase digestion of ovalbumin), the elution positions of which are indicated on the figure. Vertical dotted lines have been drawn at the elution position of the substrate and the main product from the α-mannosidase action on free substrate. The conditions are described in the text.

distribution than that from streptavidin, which, in turn, was larger than that from free BAO. This fact reflects differential protection from the action of α-mannosidase afforded by the polypeptides.

Comparison of the time courses and gel permeation profiles from the two complexes revealed a puzzling finding: it was anticipated that a faster rate of digestion would correlate with a smaller size distribution in the product, but the reverse was observed for the two BBP complexes. At the moment, there appears to be no good explanation other than to note once more the report of Harpez and Schachter (1980) mentioned above. It is possible that a low Con A affinity tetramannosylchitobiosyl oligosaccharide accumulated in the sample containing avidin while a high Con A affinity trimannosylchitobiosyl oligosaccharide accumulated in the one containing streptavidin. Unfortunately, the low resolving capacity of Sephadex G-25 (80 mesh) did not yield fractions of sufficient purity to permit structural characterization. However, regardless of the precise nature of the modified BAO, data from both Con A–Sepharose and gel permeation individually indicated that the polypeptide chain provided the bound BAO differential protection against α-mannosidase.

In the collection of data for the time courses, Con A binding was not evaluated with intact BAO–avidin because this protein is naturally N-glycosylated with a heterogeneous population of oligosaccharide of oligomannosidic and hybrid structural types (Bruch & White, 1982). Streptavidin is not glycosylated,

and the reaction of BAO–streptavidin with α-mannosidase could thus be monitored by Con A binding with intact complex as well as with BAO released from the protein. Almost identical time courses were seen (Figure 3), a fact that suggests that unlike the glycosidase the lectin's interaction with the oligosaccharide was not affected by the presence of the polypeptide. A similar observation was made previously in the study of the neoglycoprotein created from apo-aspartate aminotransferase and a phosphopyridoxylated derivative of the same asparagine–oligosaccharide (Chen & Wold, 1984).

Neoglycoproteins obtained by attaching coenzyme derivatives of oligosaccharides to protein have several unique features. First, since the oligosaccharide is bound only at the coenzyme site(s), the relative location and the number of oligosaccharide units per mole of a given protein should be constant for any oligosaccharide–coenzyme complex of that protein. This in turn permits the preparation of neoglycoproteins in which each protein matrix can be labeled with a series of oligosaccharides in specific positions. Second, inasmuch as the binding pockets for the coenzyme generally are located in a crevice, the coenzyme moiety is sequestered from reactions that may create artifactual results. Third, because of the noncovalent nature of the neoglycoproteins, the ready displacement of the oligosaccharide derivatives by native coenzyme greatly facilitates the isolation of the oligosaccharide for analysis. As a final, quite obvious point, it should perhaps be stated that the chemistry involved in preparing the biotinylated oligosaccharides would not in any way restrict the use of different types of Asn-linked oligosaccharides (complex, hybrid) in preparation of the biotin–avidin neoglycoproteins. The high-mannose oligosaccharides used here were simply selected on the basis of availability and well-characterized properties.

All these properties make the noncovalent neoglycoproteins excellent models for studying the specificity determinants involved in glycoprotein interaction with receptors (lectins) and processing enzymes. Although it is clear that the oligosaccharide moieties on glycoproteins are essential structural elements, several observations show that the protein matrix is involved as well. For example, in enzymatic modification of a maturing glycoprotein, the polypeptide has significant influence on the outcome of the process (Swiedler et al., 1985; Williams & Lennarz, 1984). This is not surprising since van der Waal's contact between the peptide and sugar residues has been observed in glycoproteins (Deisenhofer, 1981; Sutton & Phillips, 1983). On the one hand, either the polypeptide can simply sterically reduce the accessibility of the oligosaccharide (Hsieh et al., 1983; Trimble et al., 1983), or special bonding arrangement between the two may stabilize certain conformations in the oligosaccharide, thereby limiting the spectrum of possible interactions (Savvidou et al., 1984). On the other hand, since protein–protein interaction has been suggested to involve extensive surface areas of complementary properties in the participating partners (Salemme, 1977), it is not unreasonable to visualize the specificity determinants in glycoproteins as mosaic composites of the oligosaccharide and the surrounding side chains of the polypeptide.

The polypeptide–oligosaccharide interactions affecting the chemistry of glycoproteins can be studied with the BAO–BBP complexes described in this paper as model systems. The binding site of biotin has been estimated to be buried 9 Å below the van der Waals surface of avidin (Green, 1975). A biotinylated oligosaccharide bound to avidin should consequently be expected to have a portion of the reducing end masked by the polypeptide and show at least a partial resistance to α-mannosidase, consistent with the results reported here. As a further test of the extent of shielding afforded by the polypeptide chains, we have assessed the susceptibility of the chitobiose linkage of the oligosaccharide to endoglycosidase H, specifically evaluating the effect of inserting the six-carbon "arm" between biotin and asparagine (BHAO). Under conditions where both BAO and BHAO gave endoglycosidase H digestion half-lives of 70 min, the two BBP complexes of BHAO gave half-lives of about 10 h, while the two BBP complexes of BAO were completely resistant (Chen et al., 1985). These results are consistent with the simple model depicting the chitobiose part of the oligosaccharide buried in the biotin binding site in the case of BAO but being at least partially available to the enzyme through the six-carbon extension in BHAO. In exploring more specific uses of these models in glycoprotein processing reactions, we have recently found that BAO and BHAO, and to a lesser extent their BBP complexes, are substrates for glycoprotein processing enzymes in Golgi membrane preparations from rat liver, accepting first GlcNAc and then Gal from the appropriate nucleoside diphosphate sugar donors (M.-C. Shao, personal communication).

One other interesting aspect of the reaction of glycoproteins with cellular carbohydrate receptors that is still not well understood is the possible requirement of specific topological arrangements of glycosyl units for endocytosis (Ashwell & Morell, 1974; Connelly et al., 1982). Extensive studies have been done with BSA derivatives containing covalently linked carbohydrate in a range of stoichiometry and with several other glycoconjugates containing sugar clusters (Lee & Lee, 1982; Lee et al., 1984), establishing the fact that "sugar density" is an important feature in the process. In view of the fact that the number and location of noncovalently bound oligosaccharide in noncovalent neoglycoproteins are known, the BBP complexes should provide excellent complementary model systems to the clustered compounds in studies of both receptor specificity and oligosaccharide processing.

Registry No. Asn-GlcNAc$_2$-Man$_6$, 39114-02-8; biotin (*N*-hydroxysuccinimidyl ester), 35013-72-0; N^{α}-(6-aminohexanoyl)-asparagine–oligosaccharide, 99764-89-3; α-mannosidase, 9025-42-7.

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