The Regulation of Glycan Processing in Glycoproteins

THE EFFECT OF AVIDIN ON INDIVIDUAL STEPS IN THE PROCESSING OF BIOTINYLATED GLYCAN DERIVATIVES*

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The effect of the protein matrix on glycan processing by rat liver Golgi enzymes has been evaluated by a direct comparison of substrate→product conversion of a free glycan and of the same glycan linked to a protein. The glycan substrates had the general structure R-glycan where R represented either biotinyl-Asn-GlcNAc₂ or 6-(biotinamido)hexanoyl-Asn-GlcNAc₂ and the protein used was avidin; the extension arm in one of the glycan substrates permitted the additional comparison of two avidin-biotin-glycan complexes. By the use of different glycans as substrates, by the presence or absence of donor substrates (UDP-GlcNAc, UDP-Gal, and CMP-sialic acid (Sia)) and/or the inhibitor, swainsonine, it was possible to dissect the individual steps involved in the conversion of R-Man₆(6') (or R-Man₆) to a biantennary complex glycan R-Man₆,GlcNAc₆,Gal₄,Sia₂ or to the hybrid glycan R-Man₅,GlcNAc₆,Gal₄-Sia. Using fast atom bombardment-mass spectrometry to identify and quantify the substrates and products of each parallel incubation of free and avidin-bound substrates, the following observations were made. With the substrate without the extension arm, avidin-binding inhibited mannosidase I, GlcNAc transferase I, and the second step of the reaction catalyzed by mannosidase II (R-Man₅-GlcNAc→R-Man₅-GlcNAc); the second step of the reaction catalyzed by Gal-transferase was also inhibited to a lesser extent. This inhibition was greatly reduced or absent with the substrates with the extension arm and was consequently referred to as the short range effect. A long range effect of avidin binding expressed by both substrates with and without extension arm was observed for Gal-transferase acting in the hybrid glycan pathway (R-Man₅-GlcNAc→R-Man₅-GlcNAc-Gal) in the presence of swainsonine and also for Sia-transferase in the catalysis of the incorporation of the second Sia residue into the complex product (R-Man₅-GlcNAc₂-Gal₄-Sia→R-Man₅-GlcNAc₂-Gal₄-Sia₂) and to a lesser extent in the hybrid pathway (R-Man₅-GlcNAc→Gal→R-Man₅,GlcNAc-Gal→R-Man₅,GlcNAc-Gal-Sia). GlcNAc transferase II did not appear to be affected by avidin. Based on the information available on the biotin-binding site in avidin, it is proposed that the short range effect reflects the masking of the core chitobiose unit in the avidin-glycan complexes in the absence of the extension arm, but not in the presence of the arm, and that the early processing enzymes thus may require a fully exposed chitobiose for full activity. The long range effect is tentatively assumed to reflect conformational alterations of the glycan caused by protein-glycan interactions away from the biotin-binding site.

In the previous paper (1) we demonstrated that avidin-biotinyl glycan complexes represent reasonable models by which the effect of the protein matrix on glycan processing can be studied. Determining the processing efficiency of free biotinylated glycans in direct comparison to the same glycans bound to avidin, two separate types of inhibitory effects of the protein were observed. The first type was defined as a strong inhibition of processing of substrates with the glycan close to the biotin-binding site (biotinyl-Asn-glycans) which was partially or totally eliminated when the glycan was moved further away from the biotin-binding site and the protein surface (6-(biotinamido)hexanoylglycans). The second type of effect was expressed equally strongly for both kinds of substrates and these were interpreted to involve more subtle interactions between the glycan and the protein. The data were obtained entirely by following the incorporation of radioactive sugars into the glycan substrates and it was thus not possible to properly identify individual processing steps involved in the protein effects. The present work was undertaken to attempt to analyze each major enzyme of the processing sequence for its sensitivity to the effects of the protein matrix and to identify by the use of fast atom bombardment-mass spectrometry the major products of each reaction to establish whether the protein effects represent the specificity determinants that govern which type of glycan will be the final product at a given glycosylation site. A good deal of knowledge is available on the analysis, structure, and biosynthesis of the glycan units of glycoproteins (2, 3). Some of the information on the processing pathway is summarized in Fig. 1 below (3). Presumably the blockage of the early steps involving mannosidase I and GlcNAc transferase I would give rise to high-mannose type glycans, the specific blockage of mannosidase II would yield hybrid-type glycans, while full activity of all enzymes would yield the typical complex glycans.

EXPERIMENTAL PROCEDURES AND RESULTS

* Portions of this paper (including "Materials and Methods," part of "Results," Footnote 2, Figs. 2 and 3, and Tables I-VIII) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-2532, cite the authors, and include a check or money order for $6.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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DISCUSSION

Before evaluating the results obtained in this work, it is important to assess both the advantages and disadvantages of the system and the experimental design. The main advantage of the noncovalent avidin-glycan neoglycoprotein is the unique feature of permitting a direct comparison of the processing of a given glycan or a glycan mixture free in solution and bound to a protein. As shown previously (1), the complex is stable during the processing and the bound glycan can be completely recovered for analysis at the completion of the processing reaction. By always comparing only the parallel incubations of free and bound glycan it appears that the results allow meaningful interpretations even in cases where the main substrate is only 50% pure (steps 5 and 6) and where a number of secondary reactions take place. By following strictly the established experimental protocol we have found the individual experiments to be remarkably reproducible in terms of the relative extent of processing of free and bound glycan, even if the absolute values may vary from experiment to experiment. This point is particularly important in view of the analytical technique used. The various uncertainties involved in using the molecular ion intensities from the mass spectra as a direct quantitative estimate of the different components in the reaction mixture have been considered under “Results.” The concerns for an unknown extent of fragmentation and for the linearity of ion intensities and concentration outside the narrow range tested (Table I) and for glycans not tested at all are all valid and impose serious restrictions on any interpretation based on strict quantitation. The way the experiments were designed, however, absolute quantitation is not essential; the important feature through-out is the relative extent of processing of free and bound substrate. The parallel analysis of each experimental pair of free and bound substrate includes the same set of substrates and products, whose fractional amounts were estimated in the same way from ion intensities. Even if there are significant errors in determining the absolute quantities involved, the direct comparison of the extent of processing should nevertheless be valid for each pair.

The main disadvantages of the system are related to the practical limitations imposed on the kinetic analysis of processing and to the inherent limitations of the system itself in making available only a single type of protein-glycan interaction in the avidin-biotinylglycan neoglycoprotein. The self-imposed experimental limitations are mostly based on availability of substrates and the investment of time for each analysis; to do a proper kinetic analysis of every processing step would be a monumental task. The decision to make each comparison of the extent of processing of free and protein-bound glycan at a single time point was thus a practical one, but the limitations it imposes on the analytical data need to be evaluated. Based on preliminary data, the single time point of 8 h selected for each incubation was one that gave about 90% conversion of R-Man to ["C]GlcNAc-containing products under the standard assay conditions (1); over a period of time we have found that the actual conversion does vary from experiment to experiment; in the experiment shown in Fig. 3, only 73% of the starting substrate was converted to products. Assuming that the experimental range of conversion in 8 h is 75–100%, it is clear that the sensitivity in detecting minor rate effects is minimal; in fact for single step reactions that may go to completion in even shorter times, fairly large rate effects might be missed by observing complete conversion for both free and bound substrate in 8 h. There is only one set of data for which that possibility may have to be considered, that of \(\alpha\)-mannosidase II in step 3, in which the extension arm substrate gave essentially complete conversion to product. In all the other steps, enough unreacted substrate remains to suggest that the one time point should permit detection of significant rate differences. The point here is that the processing rates observed in 8 h for free and bound substrate may underestimate the actual rate differences and, in fact totally fail to detect small differences for fast reactions; however, where differences are observed they can be considered to be significant. The other problem associated with the present kinetic analysis is the fact that the actual experimental substrate concentration is ambiguous. The amount of glycan was identical in the parallel samples of free and bound substrate, but while in the case of free glycan the substrate (["C]glycan]) concentration was \(2 \times 10^{-7}\) M in the case of bound glycan, the substrate with 2.5 mol of glycanc/mol of avidin tetramer (["C]avidin-glycan \(2\)) concentration was \(8 \times 10^{-4}\) M. In the absence of a complete kinetic analysis, it is impossible to properly evaluate this effect. It may be insignificant or it may reduce the rate with the protein-bound substrate by a factor of 2.5, and in that case it could lead to a wrong interpretation of the effect of the protein on processing. In the results presented in Tables II-VIII, most of the observed differences in processing are fortunately too large to reflect this substrate concentration effect, but some differences, notably those of free and bound 6-(biotinamido)-hexanoylglycans are small enough to simply reflect a substrate concentration effect. In the case of Gal incorporation (Table VII) and Sia \(^3\) incorporation (Table VIII) some of the differences are also small enough to give cause for concern; however, the fact that 2 residues are incorporated in both 5A and 6A and that apparently only the incorporation of the second residue is inhibited (the sum of mono- and diglycosylated product relative to substrate is very similar for free and bound substrate) lends support to the interpretation that these are protein effects on processing rather than substrate concentration effects. The fact that GlcNAc transferase II gives identical rates for free and bound substrate provides additional evidence that the effective substrate concentration may be identical in the absence and presence of avidin (Table VI).

Figure 1 summarizes the results presented in Table II-VIII in the Miniprint Supplement. At each processing step the letter S (for short range proximity effect) signifies that a substantial decrease in processing rate was observed for the avidin complex of biotinylglycan but not for the avidin complex of 6-(biotinamido)hexanoylglycan; the letter L (for long range proximity effect) signifies that an inhibitory effect of avidin was observed for the complexes of both glycan derivatives. An asterisk signifies that for a particular two-step reaction, only one step appeared to be affected. Based on the quantitative limitations discussed above, the results can only be reported in an all or none fashion; but it is clear that the magnitude of the protein effect on processing varies considerably. Only one enzyme, GlcNAc transferase II, appeared not to be affected by avidin. The short range proximity effect may be visualized as a simple steric masking of the critical residues and bonds from access to processing enzymes in the cavity of the biotin binding pocket. These effects are similar to those observed previously with this and similar neoglyco-proteins when they are exposed to isolated jackbean \(\alpha\)-mannosidase or endoglycosidase H (4, 16, 17). There is one disturbing feature of this model, namely the size of the cavity that could accommodate the majority of the glycan. Green (18) has estimated that the carbohydrate of biotin is only about

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The abbreviations used are: Sia, sialic acid; FAB-MS, fast atom bombardment-mass spectrometry (Miniprint); MES, P-(N-morpholine)ethanesulfonic acid (Miniprint).
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The glycan processing steps (3) studied in this work (C, Man; ■, GlcNAc; o, Gal; ▼, Sia; the hybrid pathway and the GlcNAc transferase I step were isolated by the addition of the mannosidase II inhibitor, swainsosine). The observed effects of the protein matrix (avidin) on each processing step are indicated by the letters S (short range effect observed only for the avidin complex of biotinylglycan) and L (long range effect observed for the avidin complex of both biotinylglycan and 6-(biotinamido)hexanoylglycan). (S) and (L) indicate moderate effects; S and L substantial effects. The asterisk in the three two-step reactions signifies that only one step was significantly altered by the protein matrix.

0.8-0.9 nm below the van der Waal's surface of avidine. With Aan and a fully extended chitobiose attached to the biotin the nonreducing half of the second GlcNAc of the chitobiose should be well clear of the van der Waal's surface of the protein; with the aminohexanoyl arm inserted between biotin and Aan, the entire chitobiose unit should be fully exposed.

To allow for these features, it is proposed that the regulatory signal to the processing enzymes in distinguishing between the substrates with and without extension arm is based entirely on the exposure of the chitobiose unit. This model has attractive features in terms of in vivo regulation; if the activity (i.e. a low $K_a$) for a given enzyme is determined by the chitobiose exposure, very dramatic effects on processing could perhaps result from quite minor alterations in the protein matrix involving the chitobiose environment only. It is interesting to note in this connection that the branch specificity reported for the bovine cholesterol $\alpha$-2-6-sialyltransferase is expressed only if at least one of the core GlcNAc residues is present in the substrate (14), and thus that the proposed regulatory role of the chitobiose unit is not without precedent.

Unfortunately, we do not have sufficiently detailed structural information on the biotin-binding site in avidin to select one model over the other.

The long range proximity effect was identified on the basis of its apparent independence of the presence of the extension arm in the substrate, the processing of both substrates was inhibited to the same extent in the protein complex as compared to their free forms. It is natural in this case to consider protein-glycan interactions which may affect glycan conformation, but it is difficult on the basis of current knowledge to present a very precise model. The long range effect was observed primarily for late steps and involving only one branch of the biantennary structure, and it is important to note the increasing body of evidence showing that the two branches indeed behave differently. Based on the knowledge that the rotation around the C5-C6 bond in the 1-6 branch adds considerable conformational flexibility to the common $\Psi$ and $\Phi$ rotation of the glycosidic bond and by examination of models (19) it was realized early that while the 1-3 branch is fairly rigidly fixed relative to the chitobiose core, the 1-6 branch may assume any one of several favorable orientations. These general features have been confirmed by a number of experimental approaches including NMR (20-23) and x-ray diffraction analyses (24) and have been evoked to explain branch specificity of several processing enzymes (12, 14, 25).

In the production of the hybrid glycan in this work only the 1-3 branch is involved and the observation that the incorporation of both Gal and Sia is inhibited in the avidin complex (Tables VII and VIII) consequently suggests that it is the 1-3 branch that is masked in the complex. This in turn could imply that the strong long range proximity effect observed for the incorporation of the second Sia residue in the biantennary product also involves the 1-3 branch and that the 1-6 branch was fully exposed as substrate. Such an extrapolation does not seem valid, however. The two substrates are clearly different and the presence of the 1-6 mannosyl residue in the hybrid pathway could obviously have unique effects on the transferase.

It appears that the avidin-glycan system provides a valid model system for glycoprotein processing. The proximity effects observed here confirm the general observation that the protein matrix has a significant effect on in vivo processing (26-28) and are consistent with the general mechanisms proposed to explain the effect. Evidence that the proximity effects such as those observed here must be involved in in vivo processing has been obtained in experiments that demonstrate that high-mannose structures at glycosylation sites that are resistant to endoglycosidase H in the native protein structure are not processed to complex glycans, while those that are susceptible to endoglycosidase H (from a mutant unable to make complex oligosaccharides) correspond to the ones that are processed in the wild type (29, 30). Thus, exposure of the core chitobiose unit by the criterion of endoglycosidase H digestion appears to be required for processing, just as was concluded from our observations for the short range proximity effect. It should be noted that the avidin-biotinylglycan complexes have been found to be resistant to endoglycosidase H digestion under conditions that gave complete hydrolysis of the corresponding free glycan (4) and intermediate rates of hydrolysis with 6-(biotinamido)hexanoylglycan. The new feature of the present work may be the strong indication that the proximity effects reflect different types of protein-glycan interaction, the short range ones which may involve the exposure of the core chitobiose and the long range ones, which...
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presumably reflect conformational alterations of the outer chains of the glycan. In glycoprotein biosynthesis the former could presumably represent one mechanism through which the commitment to produce either a high-mannose, hybrid, or complex-type glycan is made, and the latter one mechanism through which specific variations in the nonreducing termini of multiantennary glycans can be achieved.

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10 µl of a reaction containing 10 µM 4-Hydroxy-L-amino-2-carboxylic acid and 0.3 µM of avidin in 0.2 µl phosphate buffer, pH 7.0. Fractions containing terminal derivatives were identified by their ability to form the brown reagent yellow (5). The samples were then transferred from the titration plate to nitrocellulose and counted in the presence of 2 µl of hydrofluor as before to identify the fractions containing radioactive sugars. The positive fractions were pooled, hydrolyzed and finally desalted on a (0.5 x 30 cm) Sephadex G-25 column packed with water. Fractions of 14 drops were collected, and the fractions lasing positive for both UV and radioactivity (as assayed by above) were again pooled and lyophilized in preparation for mass spectrometry. The resulting gel filtration was found to be important for the production of reproducible mass spectra, and consequently was included as a standard step in the procedure. The dry product was dissolved in 10 µl of water, and 1 µl aliquots, containing 5-15 ng of glycan, were used for MS analysis.

The mass spectrometric analysis - 1 µl of glycan product was mixed with 1 µl of a 10:1 solution of glycerol and a saturated aqueous oxalic acid solution, and the entire sample was applied to the mass spectrometer probe. A Varian MAT 11200 high resolution mass spectrometer equipped with an ion trap saddle field atom gun and a Q250 data system was used. The mass-spectrometer was operated at a 3.5 keV accelerating and potential and a resolution of approximately 2500. The FAB gun was operated at 8 keV and gave 50 µamps current using xenon gas. Mass spectra were obtained using a wide-window multichannel analyzer program on the data system with magnetic steering and the line were calibrated with calcium oxide.

For each scan the sum of the intensities of the (M + H) and (M + Na) peaks for each species of N was taken as the relative amount of N in the mixture (with the established procedure of two gel filtration steps prior to MS analysis the (M + H) and (M + Na) were generally a minor component compared to (M + H) and the relative value was finally normalized to percent of the sum of all intensities observed in each run. It should be emphasized that starting glycan (acceptor substrate) is a component of the product fraction and thus is included in each analysis.

RESULTS

Assessment of Quantitative and Qualitative Aspects of FAB-MS. Since mass spectrometry will be used as a tool in the identification of all the components of the postnascent product peaks from gel filtration, and for the estimation of the relative amount of different products, it was essential to assert that limitations this experimental technique might impose on the spectra and their interpretation. Figure 2 shows a typical mass spectrum of the starting substrate, and Fig. 1 typical spectra of processing products obtained in the absence and presence of avidin. Fragmentation of the glycan derivatives during mass spectrometry could give rise to molecular ions with M+ values readily assignable to unprocessed processing intermediates; in fact, we felt that extensive fragmentation of the anticipated mixtures of processing products would seriously hamper this particular experimental approach. Other authors have reported fragmentation of oligosaccharides (6,7); the fragmentation patterns have been important sources of information in assigning specific structural features to glycans. Under the conditions used in this work we have observed some evidence for significant fragmentation of any of the pure compounds we have used (Fig. 1), and we have thus concluded that all components observed in a given reaction mixture are...
true products of glycan processing and not fragmentation products. Since it has so far been impractical to verify this conclusion by direct evaluation of every intermediate observed, we recognize that a potential limitation exists in this area, and that interpretations regarding minor components should be made with caution.

Figure 2. Typical mass spectra of substrates used in this work. A and B are primary substrates isolated from ovomucin and chemically modified. C and D are secondary substrates prepared from the primary ones by enzymatic conversion. A (R=H) is avidin-[α-D-GlcNAc]2-[ε-Bis (6-azido)hexanoyl]-[ε-Biotinyl]-[ε-Bis (6-azido)hexanoyl]-[ε-Avidin]-[α-D-GlcNAc]2 (Wm 1686.6). B (R=H) is avidin-[α-D-GlcNAc]2-[ε-Bis (6-azido)hexanoyl]-[ε-Biotinyl]-[ε-Bis (6-azido)hexanoyl]-[ε-Avidin]-[α-D-GlcNAc]2 (Wm 1686.6). C (R=MGN) is biotinyl-[α-D-GlcNAc]2-[ε-Bis (6-azido)hexanoyl]-[ε-Biotinyl]-[ε-Bis (6-azido)hexanoyl]-[ε-Avidin]-[α-D-GlcNAc]2 (Wm 1730.7). D (R=MGN) is biotinyl-[α-D-GlcNAc]2-[ε-Bis (6-azido)hexanoyl]-[ε-Biotinyl]-[ε-Bis (6-azido)hexanoyl]-[ε-Avidin]-[α-D-GlcNAc]2 (Wm 1744.8). An additional spectrum is also shown for Avidin (R=H) to illustrate the changes in the intact substrate. The figure shows the effect of the gel filtration step (see methods) in eliminating the multiple Na+ salt adduct peaks. C was not subjected to a second gel filtration step for Na+ removal. It was prepared according to the procedures given under methods.

Figure 3. Comparison of the mass spectra of ovomucin enzyme processing products obtained in parallel experiments in the presence and absence of avidin. The substrates (R=H) are underlined. A and B are identical. C, D, and E are identical. F is identical except that the biotinyl moiety is replaced by an avidin moiety. The quantitative estimates (from the peak intensities) of the relative amounts of substrate and products observed in the spectra are given in Table II.

It would be highly desirable to be able to estimate the amount of each component in a mixture of products directly from the molecular ion intensities observed in the mass spectrum. It appears, however, that FAB-MS inherently has a number of potential hazards to such quantitation, and that some empirical evaluation of the validity of such data should be made. The major unknowns in making direct quantitative extrapolation from the relative abundance of molecular ions observed in the spectrum are the relative tendency of different components to form ions, their relative distribution on the surface or in the interior of the sample drop (solubility), the relative stability toward fragmentation, and possibly the relative concentration. Townsend et al. have shown that with a family of oligosaccharides differing only in the number and nature of neutral sugar building blocks, most of the potential differences considered above do not appear to be significant, and that the quantification that is valid. They showed, however, that for derivatives containing bis, the positive ion intensity (presumably the ion formation) decreased significantly. They derived empirical constants to correct for the bis-effect; the peak intensity for the bis derivative relative to the corresponding neutral glycan was about 0.4, and similarly, that of the bis-derivative relative to the bis-derivative about 0.4. With these corrections, they were able to obtain good quantitative data. Table I shows data for the quantitative estimation of the ratio of different mixtures of glycans used in this work. We conclude from this data that relative ion intensity gives a valid qualitative measure of the relative abundance of the components in a mixture, this is in agreement with the conclusions arrived at by Townsend et al. (8). About much more extensive evaluation of the variables involved we could derive correction factors based on a correlation of 14C incorporation with molecular ion intensity, but we have decided not to try to correct the observed ion intensities in this manner. The main quantitation to be made in this work is the direct comparison between a set of products obtained in the absence and presence of avidin. The comparison illustrated by the data in Fig. 3 should be valid for each pair without a correction even if the absolute values are incorrect. Table II shows the quantitative estimate of molecular ions in Fig. 3 along with the relative intensity of the 14C-substrate with the extension arm. These reactions involve the combined effect of avidin on GlcNAc transferase I and II and mannosidase I. Also included in Table II is the quantitative data confirming that swine mucins bind mannosidase I but not mannosidase II (9).

| Sample no. | Compound | Quantities | Height of m/z (ratio) | m/z (ppm) | Ratio found |
|------------|----------|------------|----------------------|-----------|-------------|
| 1          | A        | 1          | 150                  | 1.0       |             |
| 2          | A        | 1          | 146                  | 1.0       |             |
| 3          | C        | 150        | 150                  | 1.0       |             |
| 4          | D        | 100        | 100                  | 1.0       |             |
| 5          | E        | 400        | 400                  | 1.0       |             |

Table I

Effect of Avidin on Glycan Processing. The results will be reported step by step according to Fig. 1, reporting the normalized (% state intensities of identified molecular ions ([M + H]+) ([M + Na]+)). The values for the starting substrate for each reaction will be reported and listed first in the tables whenever possible. In the previous paper, the two distinct protein-enzyme interactions were designated short-range and long-range proximity effects, and this nomenclature with all its implications and implied mechanistic knowledge will be used here.

Step 1: Mannosidase I: α-Man-β-Man-β-Man. The results in Table I show that the processing of the substrate without the extension arm is strongly retarded in the avidin complex, and that the extension arm almost completely eliminates this retardation. The effect of avidin on mannosidase I is thus a typical short-range proximity effect of the protein matrix, it can be visualized as a typical static hindrance effect, with the substrate close to the protein surface or indeed partly buried in a binding cavity, certain residues become inaccessible to the processing enzymes, and when the substrate is pushed further out from the surface of binding pocket by the presence of the extension arm, the exposure of the α-mannosyl bond approaches that observed with the free oligosaccharide. The consistent appearance in the mass spectrum of a species corresponding to α-Man, appears to be real and probably reflects the ability of α-mannosidase I to also slowly (2-3 hours) cleave α1-3 and α1-6 bonds (5).

Table II

The Effect of Avidin and Swine Mucin on Glycan Processing by Rat Liver Genti Enzyme

| Reaction | Swollen Starch Products | Fractional Amount of Glycan in Reaction Mixture |
|----------|------------------------|-----------------------------------------------|
| A: R = H, B: R = H | BSA 0.4 ± 0.01 | α-Man-β-Man-α-Man-β-Man |
| B: R = H, C: R = H | BSA 0.3 ± 0.01 | α-Man-β-Man-α-Man-β-Man |
| C: R = H, D: R = H | BSA 0.4 ± 0.01 | α-Man-β-Man-α-Man-β-Man |
| D: R = H, E: R = H | BSA 0.3 ± 0.01 | α-Man-β-Man-α-Man-β-Man |
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| Substrates: R-MagGln | Fractional Amount of Glycans in Reaction Mixture |
|---------------------|-----------------------------------------------|
| R-Biotinyl-Ash-Gln-ΔX | BSA 0.07 0.16 0.08 0.03 0.06 |
| R-Biotinyl-Ash-Gln-ΔX | Avdin 0.12 0.15 0.03 0.03 0.03 |
| R-D-Biotinyl-Ash-Gln-ΔX | BSA 0.02 0.13 0.02 0.03 0.04 |
| R-D-Biotinyl-Ash-Gln-ΔX | Avdin 0.02 0.13 0.02 0.03 0.04 |

The data in Table V show that the step apparently is not at all affected by the protein matrix. Since the new linkage is B1-3 to the Man from which Man-Glucosyl is removed, the biotinylglycans that are formed with the Man residue intact are still biotinylated. The 2:1 ratio of Man-Glucosyl to B1-3 Man is consistent with this interpretation. The presence of partially degraded products R-MagGln is also evident in this experiment.

Table VI

| Substrates: R-MagGln | Fractional Amount of Glycans in Reaction Mixture |
|---------------------|-----------------------------------------------|
| R-Biotinyl-Ash-Gln-ΔX | BSA 0.32 0.30 0.06 |
| R-Biotinyl-Ash-Gln-ΔX | Avdin 0.32 0.30 0.06 |
| R-Biotinyl-Ash-Gln-ΔX | Gal 0.32 0.30 0.06 |

The data in Table VI again show that the step apparently is not at all affected by the protein matrix. Since the new linkage is B1-3 to the Man from which Man-Glucosyl is removed, the biotinylglycans that are formed with the Man residue intact are still biotinylated. The 2:1 ratio of Man-Glucosyl to B1-3 Man is consistent with this interpretation. The presence of partially degraded products R-MagGln is also evident in this experiment.

Step 2. GalNAc-transferase I: R-MagGln + UDP-GalNAc = R-MagGln-GalNAc. This step was isolated by including the mannosidase-1 inhibitor swainsonine in the reaction mixture containing R-MagGln and UDP-GalNAc. The results in Table IV again show that the intermolecular product with substantial inhibition of the processing in the light and dark complex with release of the interaction or the complex with the extension arm. Since the GalNAc is added to the same Man residue from which Man was removed in step 3, this effect should perhaps be expected. The presence of partially degraded products R-MagGln is also evident in this experiment.
In Vitro Processing of Avidin-Biotinylglycans

TABLE VII
The Effect of Avidin on Glycan Processing by Galactosyl Transferase
(A: R-Man₃-GlcNAc₂-Gal + UDP-Gal; B: R-Man₃-GlcNAc-Gal + UDP-Gal, Glucosaminyl R-Man₃-GlcNAc-Gal + UDP-Gal)

| Substrates | Protein     | B-Man₃-GlcNAc | B-Man₃-GlcNAc | B-Man₃-GlcNAc | B-Man₃-GlcNAc | B-Man₃-GlcNAc | B-Man₃-GlcNAc | B-Man₃-GlcNAc | B-Man₃-GlcNAc | B-Man₃-GlcNAc |
|------------|-------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| A: R-Man₃-GlcNAc | BSA 0.03 | 0.15 | 0.37 | 0.09 | 0.06 | 0.13 | 0.12 | 0.02 | 0.02 |
|            | Avidin 0.32 | 0.14 | 0.20 | 0.09 | 0.05 | 0.07 | 0.04 | 0.04 | 0.05 |
|            | R-(Biotinamido) | BSA 0.32 | 0.15 | 0.36 | 0.13 | 0.14 | 0.09 | 0.08 | 0.10 |
|            | Hexamyl-Man-GlcNAc | Avidin 0.32 | 0.13 | 0.36 | 0.13 | 0.10 | 0.05 | 0.03 | 0.05 |
| B: R-Man₃-GlcNAc | BSA 0.06 | 0.05 | 0.06 | 0.20 | 0.51 | 0.05 | 0.03 | 0.02 | 0.07 |
|            | Avidin 0.04 | 0.04 | 0.05 | 0.22 | 0.53 | 0.04 | 0.03 | 0.02 | 0.07 |
|            | R-(Biotinamido) | BSA 0.02 | 0.02 | 0.02 | 0.22 | 0.52 | 0.06 | 0.03 | 0.02 |
|            | Hexamyl-Man-GlcNAc | Avidin 0.02 | 0.02 | 0.02 | 0.22 | 0.52 | 0.06 | 0.03 | 0.02 |

a Some of the molecular ions cannot be unambiguously assigned to a specific structure; unfortunately, the substrate in reaction B is one of them.

TABLE VIII
The Effect of Avidin on Glycan Processing by Sialyl Transferase
(A: R-Man₃-GlcNAc-Gal + CMP-Sia; B: R-Man₃-GlcNAc-Gal + CMP-Sia, Glucosaminyl R-Man₃-GlcNAc-Gal + CMP-Sia)

| Substrates | Protein | R-Man₃-GlcNAc | R-Man₃-GlcNAc | R-Man₃-GlcNAc | R-Man₃-GlcNAc | R-Man₃-GlcNAc | R-Man₃-GlcNAc | R-Man₃-GlcNAc | R-Man₃-GlcNAc | R-Man₃-GlcNAc |
|------------|---------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| A: R-Man₃-GlcNAc | BSA 0.37 | 0.14 | 0.08 | 0.07 | 0.10 | 0.07 | 0.04 | 0.03 | 0.02 | 0.01 | 0.04 |
|            | Avidin 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
|            | R-(Biotinamido) | BSA 0.11 | 0.06 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 |
|            | Hexamyl-Man-GlcNAc | Avidin 0.11 | 0.06 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 |

b Molecular ions cannot be assigned to a specific structure.