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Specificity studies of the GDP-[L]-fucose: 2-acetamido-2-deoxy-β-[D]-glucoside (Fuc → Asn-linked GlcNAc) 6-α-[L]-fucosyltransferase from rat-liver Golgi membranes †

Ming-Chuan Shao, Charles W. Sokolik and Finn Wold *

The Department of Biochemistry and Molecular Biology, The University of Texas Medical School, P.O. Box 20708, Houston, TX 77225 (USA)

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ABSTRACT

The specificity of Golgi-membrane glycoprotein 6-α-[L]-fucosyltransferase [GDP-[L]-fucose: 2-acetamido-2-deoxy-β-[D]-glucoside (Fuc → Asn-linked GlcNAc) 6-α-[L]-fucosyltransferase; EC 2.4.1.68] has been assessed with regard to substrate covalent structures and the effect of a protein matrix on the conformational display of those covalent structures. Specificity was studied by direct comparison of the substrate quality of nine 6-biotinamidohexanoylAsn (= R) derivatives of intermediates and products in the pathway from $Man_5GlcNAc_2$-R to a fully sialylated biantennary complex-type glycan. The Man_5 derivative and the sialic acid-containing glycans were completely inactive as substrates. The other glycans were all fucosylated; the best substrate was $GlcNAcMan_3GlcNAc_2$-R. The protein-matrix effect was studied by comparing the substrate quality of the same 6-biotinamidohexanoylAsn derivatives as well as the corresponding biotinylAsn derivatives free in solution and bound to streptavidin. On the basis of a model derived from the known 3D structure of biotin (biocytin)-saturated streptavidin, it was predicted that the fucosylation site in the substrates would be completely masked in the biotin-binding pocket in the biotinyl derivatives (proximal display), and at least partially masked in the 6-biotinamidohexanoyl derivatives (distal display). The activity measurements were in agreement with these predictions; the glycan structures $GlcNAcMan_5GlcNAc_2$-, $GlcNAcMan_3GlcNAc_2$-, and $GlcNAc_2$-$Man_3GlcNAc_2$- were readily fucosylated as derivatives free in solution, but were totally inert in the proximal complex with streptavidin. In the distal complexes the latter two structures were found to be fucosylated very slowly while the former structure was inactive.

INTRODUCTION

The biosynthesis of glycoproteins appears to be under very tight control, yielding a large variety of protein-bound glycans in different cells and organisms. The regulatory mechanisms must as a minimum reflect the total enzymatic makeup in individual cells, the individual enzymes' specificities, and the substrate availabil-

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* Corresponding author.

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ity in each compartment[1]. We have attempted to explore both enzyme specificity and substrate availability using an artificial system in which pure, well characterized glycans can be evaluated as substrates for individual Golgi-membrane enzymes either free or bound to avidin through a covalently attached biotinyl group[2–5]. This system has provided a useful model, but since it has not been possible to describe the precise relationship of the glycan to the avidin-protein matrix, the model has been only a descriptive one. The 3D structure of the related biotin-binding protein, streptavidin, has recently became available[6], and it should now be possible to explore the substrate quality of these protein–glycan complexes more precisely using the streptavidin–biotinylglycan model system.

In this paper we report the results of the initial experiments with this model system. Because of the possible key role of the core α1,2-fucosyltransferase both in determining the conformation of *N*-linked glycans[1] and in reflecting unique biological activities[7,8], the present studies focus on this enzyme. We first make a systematic comparison of the relative substrate quality of all the intermediates involved in the conversion of the oligomannose derivative $Man_5GlcNAc_2$(6-biotinamidohexanoyl)Asn to the biantennary product $Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$(6-biotinamidohexanoyl)Asn, confirming and expanding the original observations of Longmore and Schachter[9] on the specificity of this enzyme. Then, using the best substrates, the effect of the protein matrix is evaluated by a direct comparison of the rate of fucosyl transfer from GDP-Fuc to the free and streptavidin-bound acceptor substrates. On the basis of information derived from the the 3D model of the glycan substrates bound to the streptavidin through the attached biotin moiety, the site of the fucosylation, i.e., the GlcNAc residue linked to asparagine, is extensively shielded within the binding pocket and should not be available for fucosyl transfer. The rate data are consistent with this picture.

EXPERIMENTAL

The general procedures, analytical tools, and chemicals have been described in previous publications in which avidin was used as the protein matrix[3,10–12]. Pure glycans, still containing the asparagine residue, were obtained from pronase digests of glycoproteins[12,13], and additional derivatives were prepared from these by enzymatic modification[11]. The α-amino groups of the different glycan-Asn derivatives were reacted with the hydroxysuccinimide ester of biotin or 6-biotinamidohexanoic acid to yield, after purification, the final substrates. GDP-β-fucose was obtained from Sigma, GDP-β-[U-^{14}C]fucose (262 mCi/mmol) was from New England Nuclear. Fucosidases were obtained from Oxford Glycobiology Systems, Inc.; the conditions for the fucosidase experiments were those specified by the manufacturer in the enzyme kits. In order to conserve reagents, minor modifications of the established methods were introduced. Thus, all the fucosylations were carried out in a 50-μL volume in capped, conical polyethylene tubes in 10 mM MES buffer, pH 6.7, containing 10 mM $MnCl_2$ and 50 μL of 10% Nonidet P-40

per mL. The mixture contained, in addition to 8 mM GDP-β-fucose and 30 μL of rat-liver Golgi membranes (~30–60 μg of protein), 0.8 mM (40 nmol) glycan substrate, either free, in the presence of 1 mg of streptavidin saturated with biotin, or bound to 1 mg of streptavidin (1.25 mM in subunits). Where needed, the mannosidase II inhibitor swainsonine was added at a concentration of 40 μM. The reaction products were analyzed by the various procedures described before[2–5]. Exploratory assessment of enzyme activity and establishing proper reaction conditions was generally most readily done by determining the incorporation of [^{14}C]fucose into the acceptor substrates[2]. Characterization of the products was done by FABMS, using a sample matrix consisting of 1 μL of 1:1 thioglycerol–glycerol (instead of the 9:1 mixture of glycerol and a saturated aqueous oxalic acid solution employed previously) mixed with 1 μL of an aqueous solution of the sample, and by ion-exchange chromatography in the Dionex Model BioLC Carbohydrate Analyzer. Three different elution conditions were used in the chromatographic analyses. After fucosylations with Man_5-R(R') *, $GlcNAcMan_5$-R(R'), $GlcNAcMan_3$-R(R'), and $GlcNAc_2Man_3$-R(R') as substrates the elution was carried out isocratically with 50 mM sodium acetate in 275 mM NaOH, i.e., 45% A (water), 10% B (500 mM NaOH containing 500 mM NaOAc), and 45% C (500 mM NaOH) for 15 min. With $GalGlcNAcMan_5$-R(R') and $Gal_2GlcNAc_2Man_3$-R(R') as substrates elution was done isocratically with 40 mM sodium acetate in 270 mM NaOH, i.e. 46% A, 8% B, and 46% C for 15 min. For the sialylated glycans the eluting gradient was started at 50 mM NaOAc in 275 mM NaOH (45% A, 10% B, and 45% C) and ramped to 200 mM NaOAc in 275 mM NaOH (45% A, 40% B and 15% C) in 30 min, after which a 10-min reverse gradient returning to the starting condition was run, followed by a further 10-min equilibration with the starting eluent before the next sample was injected. The flow rate was 1 mL/min, and the detection system was the same as that used previously except that the applied potential was 0.65 V and the detector output range was set at 1 μA.

The relative rates of fucosylation were determined directly from the integrated substrate and product peaks observed in the ion exchange separations. Each result in the tables was obtained by adding the integration values of all the integrated peaks, and calculating the fractional contribution of each component to this total. In a few cases, peaks with the proper retention times were observed at levels too low to be integrated under standard assay conditions; these cases have been scored as containing a "trace" of the component in question. Since sufficient quantities of pure products were not available to permit the direct determination of the electrochemical signal for each individual glycan, these quantitative calculations are based on the assumption that the substrate, intermediate, and product signals are identical. Although observations made on a number of other glycans[11] and the close correspondence of the values for substrate disappearance and product

* All the structures in the text are abbreviated by substituting R for $GlcNAc_2$(biotinyl)Asn, R' for $GlcNAc_2$(biotinamidohexanoyl)Asn, and B for either (biotinyl)Asn or (biotinamidohexanoyl)Asn.

appearance tend to support this assumption, it needs to be stated that the values given in the tables have this uncertainty. In principle the quantitation could have been checked using the FABMS data, again assuming that the molecular ion signals are identical. Since the quantity of substrate and product was cut down to a minimum in this work, the MS data generally had high background noise, and so were unsuitable for proper quantitation and were used only qualitatively to establish the presence of fucosylated products.

RESULTS AND DISCUSSION

The relative efficiencies of the fucosylation of nine different intermediates in the normal pathway for biantennary glycan biosynthesis are given in Table I. The experiment was conducted under predetermined conditions that gave 60–70% conversion of the best substrate ($GlcNAcMan_3$-R′) to the fucosylated product; this substrate was used as a standard to permit direct comparisons of the rates of fucosylation of all compounds. The results are completely consistent with the data reported by Longmore and Schachter[9] for the α1,6-fucosyltransferase, showing that the coupling of the first GlcNAc β1,2 to the α1,3-linked Man of the core is an essential requirement for biological activity of the fucosyltransferase. The best substrate is this derivative in which the α1,6-linked Man of the core is unsubstituted ($GlcNAcMan_3$-R′); if the 1,6 arm is substituted either with additional Man ($GlcNAcMan_5$-R′) or with GlcNAc ($GlcNAc_2Man_3$-R′), the rate is decreased 25–50%. If Gal is added to the required GlcNAc residue on the 1,3 arm ($GalGlcNAcMan_5$-R′, $Gal_2GlcNAc_2Man_3$-R′), the rate is decreased more than 80%; apparently additional substituents on the other arm do not affect substrate quality significantly. The incorporation of sialic acid eliminates the fucosyl transferase activity as completely as does the absence of the required GlcNAc residue in the case of Man_5-R′.

TABLE I

Substrate specificity of α1,6-fucosyltransferase from rat liver

Substrate	Swainsonine added	Relative rate of fucosylation
Man_5-R′ [a]	–	0
$GlcNAcMan_5$-R′	+	35
$GalGlcNAcMan_5$-R′	+	10
$SiaGalGlcNAcMan_5$-R′	+	0
$GlcNAcMan_5$-R′	–	51 [b]
$GlcNAcMan_3$-R′	–	63
$GlcNAc_2Man_3$-R′	–	39
$Gal_2GlcNAc_2Man_3$-R′	–	10
$SiaGal_2GlcNAc_2Man_3$-R′	–	0
$Sia_2Gal_2GlcNAc_2Man_3$-R′	–	0

[a] R′ = $GlcNAc_2$(6-biotinamidohexanoyl)Asn. [b] The product is exclusively fucosylated $GlcNAcMan_3$-R′.

It is well known that several fucosyltransferases are involved in the incorporation of fucose into different oligosaccharides in the cell[14], but the fucosylation of *N*-linked glycans in the Golgi membranes appears to involve only two enzymes: an α1,6-transferase that transfers Fuc from GDP-Fuc to the 6 position of the Asn-linked GlcNAc of the glycan core[9], and an α1,3-transferase that transfers fucose from GDP-Fuc to the 3 position of the GlcNAc residue in the sequence Gal(β1–4)GlcNAc(β1–2)Man(α1–3) on the 1,3 arm of the glycan[15,16]. Judging from the relative abundance of the two kinds of fucose-containing glycans, the former enzyme is likely to be the more common one; indeed, because of the requirement for Gal incorporation for the activity of the α-3 transferase, the α-6 transferase is the only enzyme that can act on the early intermediates in Table I. Nevertheless, the possible introduction of fucose into two positions could cause ambiguities in the interpretation of fucose incorporation, and it became necessary to establish to what extent the two transferases were involved. To this end we treated the products of each reaction in Table I with a specific α1,6-fucosidase and in separate incubations with a less specific α1,3(4)-fucosidase, monitoring for the conversion of fucosylated products back to the original substrates with FABMS or ion-exchange chromatography, in which case free fucose could also be determined. In all cases the α(1,6)-fucosidase gave complete removal of fucose, while the α1,3(4)-fucosidase had no effect (data not shown). We conclude from these results that our rat-liver Golgi membrane preparations express the activity of a single α1,6-fucosyltransferase with the substrates used in this work.

The roles of multiple enzymes competing for a single substrate and of enzyme specificity in regulating the pathway of glycan processing in glycoproteins are well established[1,9,15,16], and fucosyltransferases represent excellent examples of this kind of regulation both in the all-or-none selection of specific pathways[1,15] and in the determination of the obligatory sequence of steps in a given pathway[1,16]. The data in Table I along with our knowledge of which structures have been found in nature put the α1,6-transferase primarily in the second category, obligatory order of reactions. The enzyme is inactive until GlcNAc transferase I has incorporated the first GlcNAc residue into the acceptor substrate. Furthermore, since substrates containing sialic acid are not acted upon by the enzyme, and since products containing both sialic acid and fucose are well known, it seems clear that fucosylation must precede the incorporation of terminal sialic acid into complex glycoproteins. It is in fact quite likely that the substrate preferences suggested by the data in Table I reflect the actual sites of α1,6 fucosylation in vivo.

It is also well established that another key regulatory mechanism involved in directing the pathways of glycan processing is the environment in which each individual glycan is displayed in glycoproteins. One purpose of the present work is to explore the use of the complexes of the biotinylated glycans with streptavidin to assess the question. To what extent does the protein environment of an individual glycan affect its availability and quality as a substrate for the various processing enzymes encountered during the biosynthesis of glycoproteins? With the availabil-

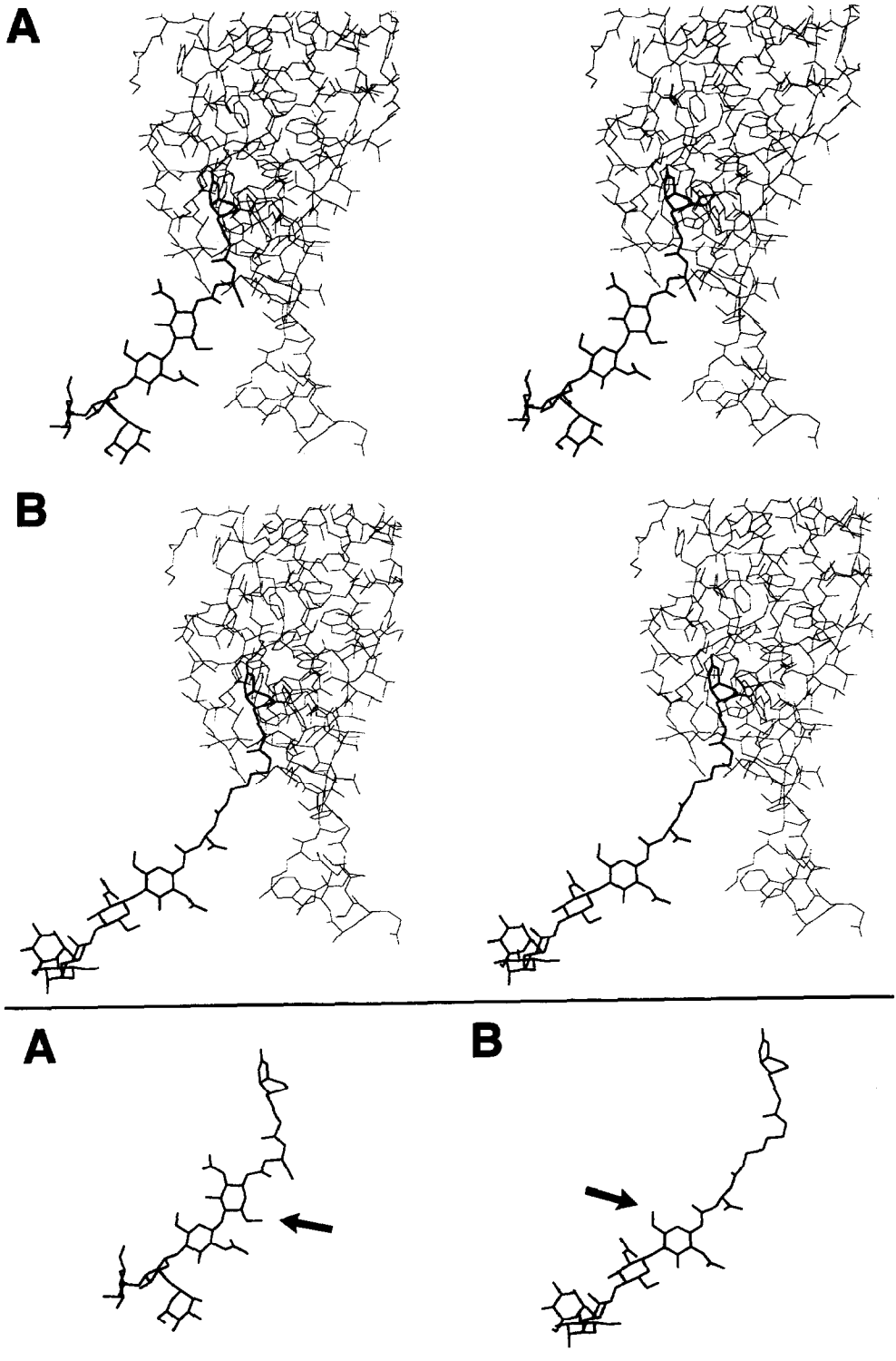
A
B
A
B

TABLE II

Effect of streptavidin on α1,6-fucosyltransferase acting on $GlcNAcMan_5GlcNAcGlcNAc$-B (GnM_5GnGn-B) in the presence of swainsonine

B	Biotin added	Substrate status	Fractional amount of glycan in reaction mixture	
			GnM_5GnGn-B	$GnM_5Gn(Fuc)Gn$-B
BiotinylAsn (proximal)	+	Free	38	62
	−	Bound	100	0
6-Biotinamido-hexanoylAsn (distal)	+	Free	54	46
	−	Bound	100	trace

ity of the structure of the streptavidin–biocytin complex it has become possible to approach this question with specific models in which the relationship of the glycan to the protein is varied by the introduction of a hydrocarbon arm between the biotin and the glycan. Fig. 1 illustrates the relationship of a glycan substrate proximally displayed on the streptavidin surface in the absence of any extension arm [glycan(biotinyl)Asn] and distally displayed in the presence of a 6-carbon extension arm [glycan(6-biotinamidohexanoyl)Asn]. From Fig. 1 it appears that the fucosylation site in the proximal orientation is inside the binding pocket and thus probably is shielded from the fucosyl transferase; even in the distal orientation, the site appears to be sterically hindered. The experimental test of the model is to compare the substrate quality of the free substrate (in the presence of biotin-saturated streptavidin) to that of the same substrate bound to streptavidin. This was done, and the results for the four best substrates (Table I) are given in Tables II–V, showing that as predicted by the model, the fucosylation is completely eliminated in the proximal and strongly inhibited in the distal complexes of glycans with streptavidin. As indicated under experimental, the qualitative identification of fucosylated products was based on mass spectrometry, and the quantitative analysis of the fucosylation was based on the integrated electrochemical signals obtained from ion exchange chromatography.

Typical data from both processes are illustrated in Figs. 2 and 3. Table II shows the results obtained with the earliest possible acceptor substrate, $GlcNAcMan_5$-R(R′) with the mannosidase II inhibitor swainsonine added to conserve the

Fig. 1. Stereoviews of the models of possible structures of (A) $Man_3GlcNAc_2$(biotinyl)Asn and (B) $Man_3GlcNAc_2$(6-biotinamidohexanoyl)Asn associated with the biotin-binding protein streptavidin. The protein component of the model is derived from the coordinates for the biocytin (N^{ϵ}-biotinylLys)–streptavidin complex[6]. The glycan complex was then produced by retaining the biotinamido group of biocytin in the binding site and replacing the Lys moiety with the glycosylAsn or the glycosyl(hexanoyl)Asn moiety of the glycan derivatives. Only part of the biotin-binding segment of the protein subunit is included in the figure, and the particular glycan conformation used was arbitrarily chosen. To help identifying the fucosylation site in the two views the structures of the two glycan derivatives have been reproduced below the dividing line, with an arrow indicating the 6-OH of the Asn-bound GlcNAc residue.

TABLE III

Effect of streptavidin on α1,6-fucosyltransferase acting on $GlcNAcMan_3GlcNAcGlcNAc$-B (GnM_3GnGn-B)

B	Biotin added	Substrate status	Fractional amount of glycan in reaction mixture	
			GnM_3GnGn-B	$GnM_3Gn(Fuc)/Gn$-B
BiotinylAsn (proximal)	+	Free	66	34
	−	Bound	100	0
6-Biotinamido-hexanoylAsn (distal)	+	Free	77	23
	−	Bound	97	3

integrity of the substrate; under conditions in which the free substrates were 50–60% fucosylated, no fucosylated product was observed for either the proximal or distal complexes with streptavidin. Table III shows the results obtained with what according to the data in Table I is the best substrate for the fucosyl transferase, $GlcNAcMan_3$-R(R'). Under conditions where the free substrates were 25–35% fucosylated, no fucosylation was observed for the proximal complex; in this case, however, the distal complex consistently showed a low level (~ 15% of that of the free substrate) of activity.

The data in Table IV illustrate the situation in which the fucosyl transferase acts while $GlcNAcMan_5$-R(R') is converted to $GlcNAcMan_3$-R(R') through the action of mannosidase II in the absence of swainsonine, and in which the protein matrix effect on both fucosyl transferase and mannosidase II must be considered. We have recently established that streptavidin, like avidin[2,3,10], strongly inhibits mannosidase II action on the proximal complex, but has relatively little effect on the distal one (M.-C. Shao and F. Wold, unpublished results); this information along with the data in Tables II and III makes it possible to give a rational interpretation of the data in Table IV. Under conditions where all of the free substrate was converted to products, 60% of which was the fucosylated derivative $GlcNAcMan_3GlcNAc(Fuc)GlcNAc$-B, no fucosylation was observed for the strep-

TABLE IV

Effect of streptavidin on α1,6-fucosyltransferase acting on $GlcNAcMan_5GlcNAcGlcNAc$-B (GnM_5GnGn-B) in the absence of swainsonine

B [a]	Biotin added	Substrate status	Fractional amount of glycan in reaction mixture			
			GnM_5GnGn-B	GnM_4GnGn-B	GnM_3GnGn-B	$GnM_3Gn(Fuc)Gn$-B
BiotinylAsn (proximal)	+	Free	trace	trace	39	61
	−	Bound	67	27	6	0
6-Biotinamido-hexanoylAsn (distal)	+	Free	trace	8	29	63
	−	Bound	12	5	71	12

[a] The biotinylAsn samples and the 6-biotinamidohexanoylAsn samples were run on different dates.

TABLE V

Effect of streptavidin on α1,6-fucosyltransferase acting on $GlcNAc_2Man_3GlcNAcGlcNAc$-B ($Gn_2M_3GnGn$-B)

B	Biotin added	Substrate status	Fractional amount of glycan in reaction mixture	
			Gn_2M_3GnGn-B	$Gn_2M_3Gn(Fuc)Gn$-B
BiotinylAsn (proximal)	+	Free	70	30
	−	Bound	100	0
6-Biotinamido-hexanoylAsn (distal)	+	Free	73	27
	−	Bound	98	2

tavidin-bound proximal substrate. The strong inhibition of mannosidase II, which leaves 67% of the substrate unchanged, 27% as the Man_4-containing intermediate, and only 6% as the Man_3-product should be noted. For the distal complex, the mannosidase II activity is much higher, giving more than 80% of the activity for

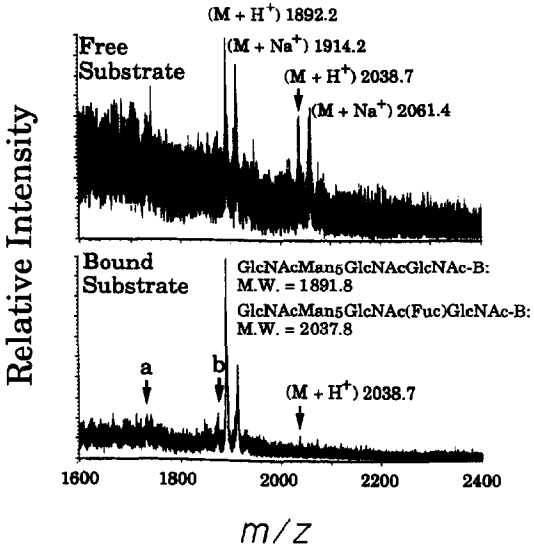

Fig. 2. Characteristic FABMS data, including the observed m/z values, illustrating the fucosylation of glycan substrates. The specific example shown is the conversion of $GlcNAcMan_5GlcNAcGlcNAc$(6-biotinamidohexanoyl)Asn to $GlcNAcMan_5GlcNAc(Fuc)GlcNAc$(6-biotinamidohexanoyl)Asn in the presence of the mannosidase II inhibitor, swainsonine, and in the presence of steptavidin presaturated with biotin (free substrate) or streptavidin alone (bound substrate). Although the samples were desalted by gel filtration prior to analysis, $(M+Na^+)$ adducts of the major components were always observed. In the case of the bound substrate, traces of a fucosylated product were observed along with small amounts of $(M+H^+)$ species corresponding to products of a mannosidase II catalyzed removal of one Man residue from both the substrate (a, observed m/z: 1731) and the product (b, observed m/z: 1877). These components were not apparent when the same samples were subjected to chromatography. The experimental conditions are described in the text.

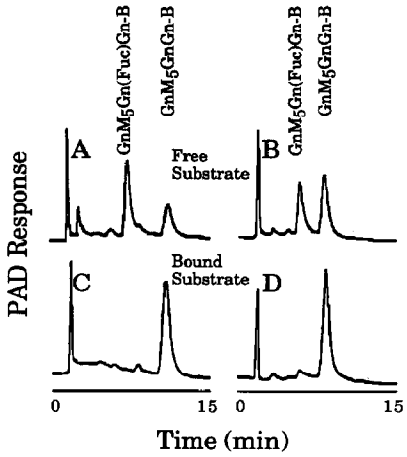

Fig. 3. Ion-exchange chromatographic patterns of fucosylation reaction mixtures having GlcNAc-Man_5GlcNAcGlcNAc-B as the substrate in the presence of biotin-saturated streptavidin (free substrate) (top traces) and streptavidin alone (bound substrate) (lower traces). B is either biotinylAsn (**A** and **C**) or 6-biotinamidohexanoylAsn (**B** and **D**). The experimental conditions are described in the text.

free substrate, and, in agreement with the data in Table III, under conditions where ~ 60% of the free substrate was fucosylated, a small, but significant amount of fucosylation (~ 20% of that of the free substrate) was also observed. Table V shows the results obtained with the substrate $GlcNAc_2Man_3$-R(R'). Even with only 30% fucosylation of the free substrates, the total absence of any fucosylated derivative from the proximal complex and the fucosylation of a maximum of 10% of that of the free substrate in the distal complex is considered to represent significant support for the general model in Fig 1.

In addition to shedding some light on the specificity of the fucosyltransferase, the results obtained in this study will also significantly influence future plans and actions in the exploration of protein matrix effects with the streptavidin model system. Even at this preliminary level, it appears that the model has general validity and should allow us to explore key steps in glycan processing. By varying the length of the extension arm between biotin and the glycan it should be possible to approach the question of how the glycan and the protein matrix actually interact. Ultimately, success in crystallizing and determining the actual structures involved in these model glycoproteins will provide the only proper basis for the interpretation of substrate quality data such as that presented here. In contrast to natural glycoproteins, these model glycoproteins contain completely homogeneous glycans, and their 3D structural analysis thus seems to be well worth pursuing.

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