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STERIC FACTORS INVOLVED IN THE ACTION OF GLYCOSIDASES AND GALACTOSE OXIDASE

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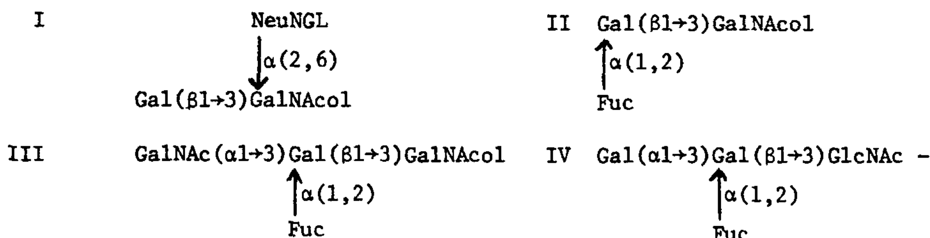
SUMMARY: α-(1→2)-L-Fucosidase, β-D-galactosidase and galactose oxidase are sterically hindered by certain types of branching in the oligosaccharide chains. 1) β-D-*Galactosidase* will not cleave galactose when the penultimate sugar carries a sialic acid residue as in I. 2) *Galactose Oxidase* will not oxidize the galactose residue in trisaccharide I but will in II. Moreover, neither galactose nor N-acetylgalactosamine, glycosidically bound as in III, is susceptible to oxidation with galactose oxidase until the α-(1→3) linkage between them is cleaved by α-N-acetylgalactosaminidase. 3) α-(1→2)-L-*Fucosidase* action is inhibited by α-(1→3)-N-acetylgalactosaminyl or galactosyl residue, as in III and IV. Removal of the terminal sugars makes the fucosyl residue susceptible to fucosidase action.



INTRODUCTION: We had previously presented evidence, derived from periodate oxidation studies (1), for the structures of the oligosaccharides from A^+, H^+ and A^-H^-hog submaxillary glycoproteins (2). Further studies by NMR spectroscopy and permethylation analysis gave results that did not substantiate

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§ *Abbreviations used:* A^+ PSM, H^+ PSM, and A^-H^- PSM from A and H active and inactive porcine submaxillary glycoproteins; OSM, ovine submaxillary mucin; B^+ oligo alditols for the mixture of oligosaccharide alditols isolated from B-active ovarian cyst fluid; Fuc, L-fucose; Gal, D-galactose; GalNAc, N-acetyl-D-galactosamine; GalNAcol, N-acetyl-D-galactosaminitol; NeuNGl, N-glycolylneuraminic acid; PNP-Gal, p-nitrophenyl-β-galactoside; PNP-GalNAc, p-nitrophenyl-α-acetyl-D-galactosaminide; u = enzyme unit, defined as μmol of sugar released per hour, incubated at 37° under optimal conditions with the appropriate substrate as indicated in Methods.

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some of these findings (3). The disagreement revolved around the attachment of NeuNGl to Gal or GalNAc.

One approach contemplated for the resolution of the problem was by the use of enzymes such as β-galactosidase and galactose oxidase. If the galactose is terminal and unsubstituted by NeuNGl, it should be susceptible to both enzymes. Conversely, resistance to the action of either enzyme would suggest substitution. As it turned out, neither statement was correct since an additional factor, that of steric hindrance, was involved. This became evident with the establishment of the structures by NMR and methylation studies (3).

We studied this steric hindrance in greater detail in view of the presence of these sugars in many biologically important compounds.

MATERIALS AND METHODS: Hog submaxillary glycoproteins with A or H reactivity, A^{+} PSM and H^{+} PSM, and OSM were prepared as previously described (4). The purified oligosaccharide alditols used in this investigation were obtained from hog subamxillary glycoproteins by β-elimination, alkaline cleavage in the presence of borohydride (1,5,6). The unseparated mixture of oligosaccharide alditols of B-active ovarian cyst fluid were also obtained by alkaline β-elimination cleavage of a phenol-insoluble fraction (7) of lyophilized B-active ovarian cyst, a gift from Dr. W.M. Watkins. The synthetic PNP-glycosides, PNP-β-Gal and PNP-α-GalNAc, were obtained from Koch-Light Laboratories Ltd. and Calbiochem, respectively. Synthetic di- and trisaccharides of established structures (8), with blood group A and B activities, were obtained as gifts through the courtesy of Dr. D.A. Baker, Chembiomed Ltd.

The following enzymes were used in these studies: β-galactosidase (3.2.1.23) from bovine testes (9) was a gift from Dr. G.W. Jourdian. α-(1→2)-L-Fucosidase purified from *Ruminococcus* (10) was a gift from Dr. L.C. Hoskins. This material also contained α-galactosidase activity. Yet another preparation of α-L-fucosidase (3.2.1.51), a gift from Dr. G.W. Jourdian, was a contaminant of partially purified β-galactosidase from bovine testes. α-N-Acetylgalactosaminidase (3.2.1.49) was isolated from *Cl. perfringens* (11). Sialidase (3.2.1.18), which requires Ca^{++}, was prepared from *Vibrio cholerae* (12). α-Galactosidase (3.2.1.22) was prepared from *Cl. sporogenes* (13). Galactose Oxidase (1.1.3.9) was contained in the Galactostat reagent, a commercial preparation from Worthington.

Buffers were used in these experiments: buffer A, 0.1 M sodium acetate, pH 4.5, and buffer B, 0.1 M sodium citrate-phosphate, pH 6.0.

Free GalNAc was measured by a modification (14) of the Morgan-Elson reaction (15). Free Gal was determined with galactose dehydrogenase (1.1.1.120, Boehringer-Mannheim) (16). The oxidation of Gal, GalNAc or their derivatives was followed by the Galactostat reagent (Worthington). Total sialic acid was assayed by a modification of the Svennerholm resorcinol procedure (17) with 1-butanol replacing isoamyl alcohol for the development of color (18); and

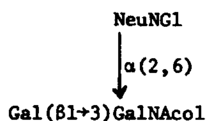
free sialic acid by the thiobarbituric acid procedure (19). Total fucose was determined after a 10 min heating period (20), while fucose dehydrogenase (1.1.1.122, Sigma) was used to determine the amount of free fucose (16). The activities of β-galactosidase, α-fucosidase, and α-N-acetylgalactosaminidase were determined by the action of these enzymes on their respective PNP-substrates using the procedure of McGuire et al (22) for the measurement of free PNP. B-Active ovarian cyst alditols were used to determine the activity of the α-galactosidase from *Ruminococcus* and the α-galactosidase from *Cl. sporogenes*; OSM for sialidase; and Gal for galactose oxidase.

RESULTS AND DISCUSSION: Action of β-Galactosidase on Acidic Oligosaccharide Alditols from A^+, H^+ and A^-H^- Glycoproteins. The acidic trisaccharide alditols released from each of the three phenotype glycoproteins (2) A^+,$H^+$and A^-H^-, gave similar results, Table 1. The release of NeuNGl from the GalNAcol is necessary before the release of Gal by β-galactosidase can occur. With the acidic tetrasaccharide alditols, Table 2, the release of both fucose and NeuNGl by their respective enzymes is required before the action of the β-galactosidase can occur.

Action of α-(1→2)-L-Fucosidase on Various Substrates. A re-examination of the action of α-fucosidase on intact glycoproteins (21) was undertaken to establish the characteristics of steric hindrance associated with fucosidase. It is readily apparent, Table 3, that all of the fucose in H^+ PSM glycoprotein was susceptible to the action of α-fucosidase (21). In contrast, only 22% of the total fucose present in the A^+ PSM glycoprotein was susceptible to the action of α-fucosidase (Table 3). This compares well with results previously presented (21). The addition of either sialidase, β-galactosidase, or more α-fucosidase to individual 600 μl aliquots of the original incubation mixture did not increase the amount of fucose released. However, when α-N-acetylgalactosaminidase was added to the incubation mixture containing fucosidase, almost all the fucose was released within 16 additional hrs. The results indicate that the terminal non-reducing GalNAc is involved in the inability of α-fucosidase to hydrolyze all the fucose from the A^+ PSM glycoprotein. This was further confirmed by the results obtained with the pure isolated acidic pentasaccharide alditol (6) as substrate. Again, only with the addition of α-N-acetylgalactosaminidase was the fucose

Table I

Free Sugars Released by Enzymatic Hydrolysis of Acidic Trisaccharides



% Sugar Released

Source of Trisaccharide 0.125 μmol	Sialidase (1.2u) NeuNGl	Sialidase (1.2u) Gal	β-Galactosidase (0.4u) NeuNGl	β-Galactosidase (0.4u) Gal	β-Galactosidase (0.4u) Sialidase (1.2u) NeuNGl	β-Galactosidase (0.4u) Sialidase (1.2u) Gal
A^+	85	0	0	0	85	69
H^+	78	0	0	0	64	66
A^-H^-	64	0	0	0	56	51

Incubations for 16 hr at 37° in 200 μl of buffer A containing 10mM $CaCl_2$

almost completely released within an additional 16 hrs.

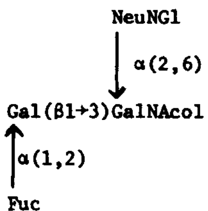
A parallel series of experiments was performed on B-active ovarian cyst alditols. During the initial incubation of the substrate with fucosidase, 22% of the total fucose was released (Table 3). This most likely resulted from the release of fucose from the "biosynthetically incomplete" oligosaccharides which did not contain terminal non-reducing α-galactosyl residues - results comparable to those obtained after treatment of A^+ PSM with α-fucosidase (21). After 48 hrs, α-galactosidase (0.3 u) was added, resulting in the release of an additional 18% of the fucose.

These observations were further confirmed by the action of α-(1→2)-L-fucosidase on chemically synthesized oligosaccharides of the A and B blood group type (Table 4). Fucose was released by α-fucosidase only after the terminal α-GalNAc or α-Gal, blood group A and B determinants, were removed by the respective exoglycosidase.

Action of Galactose Oxidase on Various Oligosaccharides. The results, Table 5, illustrate that the cleavage of NeuNGl was again necessary before the Gal could be oxidized, 47%. Low values were also obtained with the

Table 2

Free Sugars Released by the Enzymatic Hydrolysis of Acidic Tetrasaccharides



% Sugar Released

Source of Tetrasaccharide	Fucosidase (1.8u) Sialidase (1.8u)			Fucosidase (1.8u) β-Galactosidase (0.6u)			Sialidase (1.8u) β-Galactosidase (0.6u)			Fucosidase (1.8u) Sialidase (1.8u) β-Galactosidase (0.6u)		
	Fuc	NeuNGl	Gal	Fuc	NeuNGl	Gal	Fuc	NeuNGl	Gal	Fuc	NeuNGl	Gal
A^+	41	39	0	59	0	0	0	85	0	63	39	39
H^+	56	47	0	66	0	0	0	78	0	70	44	42
A^-H^-	53	47	0	64	0	0	0	78	0	70	41	39

Incubations for 16 hr at 37° in 300 µl of buffer A containing 10mM $CaCl_2$

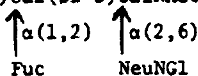
Table 3

Action of α-Fucosidase Alone on Various Substrates and with Subsequent Additives

		% of Total Bound Fucose Released from Substrate			
		H^+ PSM 2.3 μmol	A^+ PSM 2.5 μmol	Acidic Pentasaccharide Alditol* 3.0 μmol	B-Active Ovarian cyst Alditols
Fucosidase alone for 24 hrs.		97	21	0	22
Plus Following additives for further 16 hrs.					
α-Fucosidase	1.8u	98	21	0	ND
Sialidase	6.0u	98	21	0	ND
α-Galactosidase (48 hr)	0.3u	ND	ND	ND	40
β-Galactosidase	0.9u	98	21	0	ND
α-N-Acetylgalactosaminidase	0.7u	98	97	97	ND
More substrate		ND	ND	0	ND

The 24 hr. incubation with fuc'ase (1.8u) in buffer B(5 ml) was also followed by subsequent treatment of 600 μl aliquots of incubation mixture with enzymes as indicated below. Their effects on % of total fucose released after a further 16 hr. incubation was noted.

* GalNAc(α1→3)Gal(β1→3)GalNAcol



ND, Not Determined

disaccharide of the antifreeze glycoprotein (23). However, unlike the action of β-galactosidase, the presence of fucose bound to the Gal did not significantly affect the ability of galactose oxidase to oxidize the Gal residue in the tetrasaccharide, as had been observed after NeuNGl had been previously cleaved by sialidase.

Next, the effect of galactose oxidase on the oxidation of a GalNAc residue at the non-reducing end of the tetrasaccharide, compound III, was examined. No oxidation had occurred even after 24 hrs. However, the addition of α-N-acetylgalactosaminidase to hydrolyze the terminal GalNAc resulted after a further 42 hrs in 98% oxidation of both the free GalNAc and the now exposed terminal Gal. Thus, the presence of GalNAc at the non-reducing end of the oligosaccharide chain prevents the oxidation of both the terminal GalNAc and penultimate Gal residues. In like manner, there was negligible oxidation of B cyst alditols on treatment with galactose oxidase. Pre-

Table 4

Enzymatic Cleavage of Synthetic Oligosaccharides

% Sugar Released

	α-N-acetylgalactosaminidase Alone		α-Fucosidase Alone		α-Fucosidase and α-N-acetylgalactosaminidase	
Substrate	GalNAc	Fuc	GalNAc	Fuc	GalNAc	Fuc
1. GalNAc(α1→3)Gal β→R with Fuc α(1,2)→Gal	48	0	0	0	100	100
2. GalNAc(α1→3)Gal β→R	100	ND	0	ND	100	ND
	α-Galactosidase Alone		**α-Fucosidase Alone**		**α-Fucosidase and α-Galactosidase**	
Substrate	Gal	Fuc	Gal	Fuc	Gal	Fuc
3. Gal(α1→3)Gal β→R with Fuc α(1,2)→Gal	100	0	0	0	100	100
4. Gal(α1→3)Gal β→R	100	ND	0	ND	100	ND

ND, Not Determined

treatment of the mixture of alditols with α-galactosidase resulted in 100% oxidation of galactose released.

Exoglycosidases have been recommended by a number of authors as valuable tools for structural studies (24,25). Previous reports of steric hindrance in the action of glycosidases involved inhibition of the action of sialidase (26-28) and of α-mannosidase (29). The studies outlined here indicate that steric hindrance is more widespread. Indeed, the inability to oxidize GalNAc α(1→3), the blood group A determinant, and Gal α(1→3), the B determinant, substantiates our previously unpublished observation that galactose oxidase has no effect on the A and B blood group activities (see also 30,31). These findings emphasize the need for greater caution in the interpretation of results obtained in structural investigations and in studies of biological effects of exoglycosidases and galactose oxidase on soluble glycoconjugates and cell surfaces. Our findings can be summarized as follows:

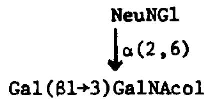
	Substrate	Blood Group Specificity	Susceptibility to Enzyme		
			α-Fucosidase	β-Galactosidase	Galactose Oxidase
I	NeuNGl ↓α(2,6) Gal(β1→3)GalNAcol	$A^-B^-H^-$	No	No	No
II	Gal(β1→3)GalNAcol ↑α(1,2) Fuc	$A^-B^-H^+$	Yes	No	Yes
III	GalNAc(α1→3)Gal(β1→3)GalNAcol ↑α(1,2) Fuc	$A^+B^-H^-$	No	No	No
IV	Gal(α1→3)Gal(β1→3)GlcNAc – ↑α(1,2) Fuc	$A^-B^+H^-$	No	No	No

Table 5

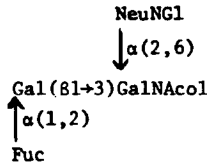
Oxidation of Galactose by Galactose Oxidase

in

Acidic Trisaccharide



Acidic Tetrasaccharide



		% Oxidation		
		Galactose Oxidase Alone	Galactose Oxidase Sialidase (3u)	Galactose Oxidase Sialidase (3u) Fuc'ase (1.8u)
Galactose		100	ND	ND
GalNAc		100	ND	ND
GalNActitol		0	ND	ND
Acidic Tri-A^+	0.1 μmol	0	47	ND
H^+		0	47	ND
A^-H^-		0	47	ND
Acidic Tetra-A^+	0.2 μmol	0	40	47
H^+		0	31	45
A^-H^-		0	34	47

ND, Not Determined
Two ml of Galactostat reagent in a total volume of 3 ml and containing 3 mM $CaCl_2$.
The amount of Gal or GalNAc oxidized was determined after 16 hr at 37°.

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