MILD ALKALINE BOROHYDRIDE TREATMENT LIBERATES *N*-ACETYLGLUCOSAMINE-LINKED OLIGOSACCHARIDE CHAINS OF GLYCOPROTEINS

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Received 17 September 1981

1. Introduction

Identification of the linkage-type between the polypeptide chains and the oligosaccharides of glycoproteins is often based on the use of mild alkaline borohydride treatment that liberates the GalNAc–Ser(Thr)-linked sugars [1]. In contrast, the GlcNAc–Asn-linkage, the other major type of bonds in glycoproteins, is considered 'stable' under mild conditions, although it is known to be cleaved by alkaline borohydride under rigorous conditions [2,3].

This paper shows that both O- and N-linked oligosaccharides are liberated from glycoproteins under conditions as mild as 0.05 M NaOH/1 M $NaBH_4$ at 45°C for 16 h. Some of the N-linked glycans appear to be released as oligosaccharide alcohols with *N*-acetylglucosaminitol as the reduced monosaccharide unit.

2. Materials and methods

The glycopeptide GC-4 was a highly appreciated gift from Drs J. Montreuil and A. Cheron [4]. It was labeled with tritium in its galactose residues as in [5]. Treatment of GC-4 with 0.05 M NaOH/1 M $NaBH_4$ [6], with 0.05 M NaOH, and with 1 M $NaBH_4$ was carried out under nitrogen by using reagent volumes of 0.2–0.5 ml. The reactions were terminated with glacial acetic acid. Borate was removed by repeated addition and evaporation of a mixture of methanol and glacial acetic acid.

The PA 1 cells were cultured, and their glycoprotein oligosaccharides were labeled metabolically with [6-^{3}H]glucosamine as in [7]. Treatment of the cells with 0.05 M NaOH/1 M $NaBH_4$ was carried out by suspending them in 1.0 ml physiological saline and adding 0.25 ml 0.25 M NaOH/5 M $NaBH_4$. After 16 h at 45°C the reaction was terminated by acidifying the mixture. A small amount (2 mg) of bovine serum albumin was added as a carrier, and the proteins were precipitated by adding 1 vol. 20% and 5 vol. 10% trichloroacetic acid. The precipitate was collected by centrifuging, washed twice with cold 10% trichloroacetic acid and then with diethyl ether. The washed precipitate was treated with pronase as in [7]. The combined trichloroacetic acid supernatants were neutralized and lyophylized.

For analysis of *N*-acetylhexosamine and *N*-acetylhexosaminitol the oligosaccharide chains were hydrolyzed with 4 M HCl at 100°C for 8 h. The fraction of hexosamines and hexosaminitols was isolated by ion-exchange chromatography according to [8]. This fraction was re-*N*-acetylated as in [9] then desalted on a column of AG 50 W (H^+) and Dowex 1 (acetate).

Paper chromatography of *N*-acetylhexosaminitols and *N*-acetylhexosamines was carried out as in [10]. Briefly, Whatman no. 1 paper was moistened with 0.05 M borate (pH 8.0) that contained 0.01 M NaCl, dried overnight, spotted and developed for 24 h with ethyl acetate:isopropanol:pyridine:water (7:3:2:2), a solvent for separation of *N*-acetylglucosamine and *N*-acetylgalactosamine on thin-layer chromatography [11]. Radioactivity was measured by liquid scintillation as in [7].

Gel filtration on Bio-Gel P-10, and affinity chromatography on concanavalin A–Sepharose were carried out as in [7]. Small samples from each fraction were taken for liquid scintillation counting, and the data are presented as radioactivity of these aliquots.

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

3. Results

3.1. *Gel filtration analysis of borohydride cleavage products from a reference glycopeptide of the 'complex' type*

The first indication that treatment with 0.05 M NaOH/1 M $NaBH_4$ at 45°C for 16 h [6] liberates asparagine-linked oligosaccharides, was obtained by gel filtration of the reaction products from GC-4, a reference glycopeptide containing a relatively large peptide portion. The structure of this glycopeptide originating from bovine colostrum IgG [4] is shown in fig.1. The [^{3}H]galactose-labeled GC-4 gave a well-defined peak in Bio-Gel P-10 chromatography (fig.2A). During treatment with 0.05 M NaOH/1 M $NaBH_4$ the original GC-4 peak disappeared almost completely, and a new peak of a lower app. M_r appeared (fig.2B). The new peak migrated in Bio-Gel P-6 like the glycan liberated from GC-4 by hydrazinolysis [5] (not shown). Treatment of the new peak with acetic anhydride in saturated sodium bicarbonate, which should re-*N*-acetylate any free amino groups eventually generated by the borohydride [9], did not cause any further change in its gel filtration properties (not shown).

In contrast to the experiment with 0.05 M NaOH/1 M $NaBH_4$, a treatment with 0.05 M NaOH for 16 h at 45°C did not cause any change in the gel filtration properties of GC-4 in the absence of borohydride (not shown). A treatment with 1 M $NaBH_4$ at 45°C for 16 h in turn caused an almost complete cleavage of GC-4 even in the absence of any added alkali (not shown). These findings show that the borohydride, which was originally added to the reaction mixture [6] to protect the released oligosaccharide against the 'peeling reactions' [12], causes unexpected cleavage reactions itself.

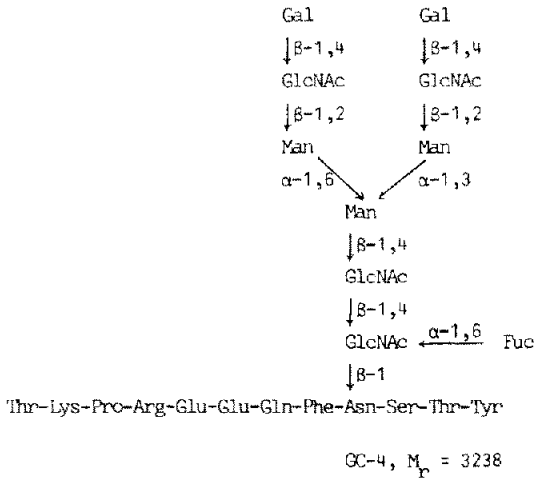

Fig.1. Structure of GC-4.

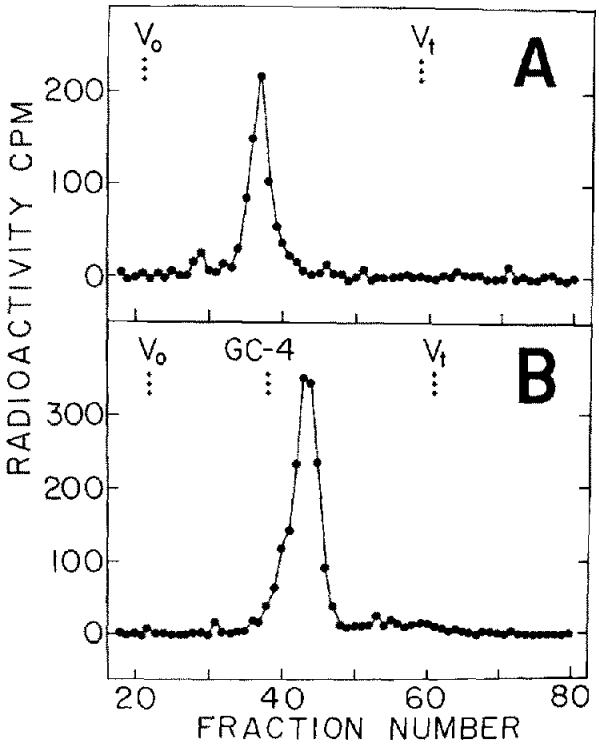

Fig.2. Gel filtration of GC-4 (A) before and (B) after mild alkaline borohydride treatment. The arrows labeled V_o and V_t show the positions of blue dextran 2000 and [^{3}H]mannose, respectively.

3.2. *Release of 'high mannose'-type oligosaccharides from intact proteins by mildly alkaline borohydride*

The presence of 'high mannose' oligosaccharides in the glycoproteins of PA 1 cells has been established in [7], M.-L. R., O. R. unpublished): Bio-Gel P-10 chromatography of pronase digested PA 1 cells gave a fraction of small-sized molecules (fraction C; M_r 850–2200) that contained the 'high mannose' glycopeptides. These were isolated in pure form (fraction C–con A III) by affinity chromatography on a column of concanavalin A–Sepharose. They were characterized by reactivity with endo-β-*N*-acetyl-glucosaminidase H (EC 3.2.1.96) and with α-mannosidase (EC 3.2.1.24), and their linkage monosaccharide was

firmly identified as *N*-acetylglucosamine. The glycoproteins of PA 1 cells bearing these 'high mannose' type oligosaccharides served as another convenient model in the borohydride degradation experiments.

Intact PA 1 cells, which had been metabolically labeled with [^{3}H]glucosamine, were treated with 0.05 M NaOH/1 M $NaBH_4$ for 16 h at 45°C and then precipitated with trichloroacetic acid. The resulting supernatant contained the 'high mannose' oligosaccharides liberated from the glycoproteins in the form of glycopeptides or oligosaccharide alcohols, and the precipitate contained the bound 'high mannose' oligosaccharides that had not been released from the proteins by the alkaline borohydride.

The trichloroacetic acid supernatant was neutralized and then fractionated by Bio-Gel P-10 chromatography to obtain fraction C (fig.3A). This fraction was finally chromatographed on a column of concanavalin A–Sepharose to obtain fraction C–con A III (fig.4A) that represents pure 'high mannose' glycopeptides and oligosaccharides.

The trichloroacetic acid precipitate was solubilized by exhaustive pronase digestion and then fractionated by Bio-Gel P-10 chromatography to obtain fraction C (fig.3B). This fraction, too, was finally chromatographed on concanavalin A–Sepharose to obtain the pure 'high mannose' glycopeptides as fraction C con A III (fig.4B).

Calculations based on the data (in fig.3,4 revealed that a ~85% of the total 'high mannose'-type oligosaccharides had become soluble during the treatment with 0.05 M NaOH/1 M $NaBH_4$. Only 15% of the total was recovered from the trichloroacetic acid precipitate (table 1).

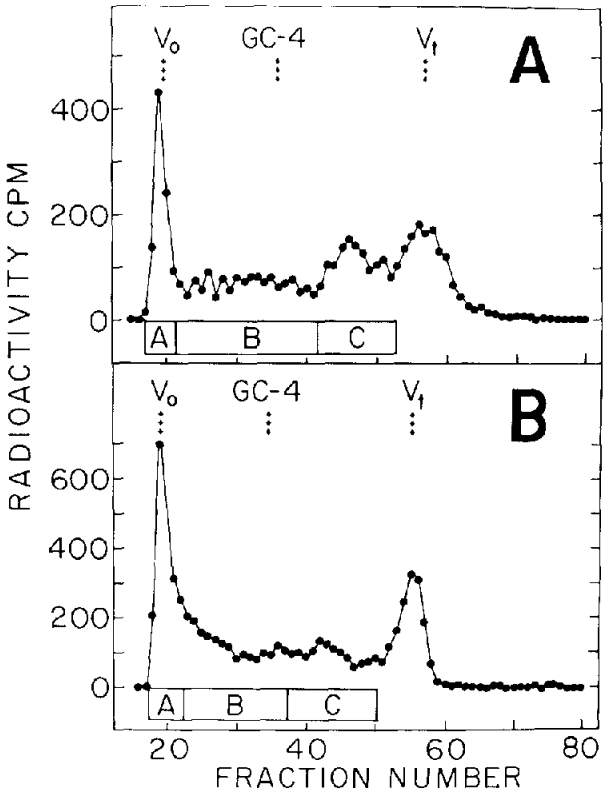

Fig.3. Bio-Gel P-10 chromatography: (A) trichloroacetic acid supernatant from PA 1 cells treated with 0.05 M NaOH/1 M $NaBH_4$; (B) trichloroacetic acid precipitate (after pronase digestion) from PA 1 cells treated with 0.05 M NaOH/1 M $NaBH_4$.

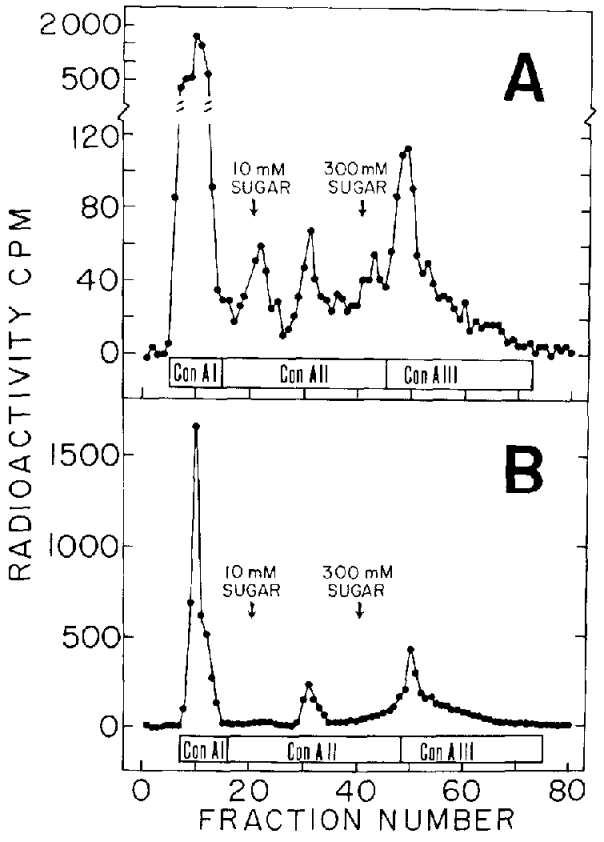

Fig.4. Concanavalin A–Sepharose chromatography: (A) fraction C from the trichloroacetic acid supernatant from NaOH/$NaBH_4$-treated PA 1 cells; (B) fraction C from the trichloroacetic acid precipitate from NaOH/$NaBH_4$-treated PA 1 cells. Fractions 1–20 were eluted with buffer, fractions 21–40 with buffer containing 10 mM α-methyl mannoside and fractions 41–80 with buffer containing 300 mM α-methyl mannoside.

Table 1
Liberation of 'high mannose'-type oligosaccharides and glycopeptides from PA 1 cell glycoproteins by mild alkaline borohydride treatment[a]

Stage	Trichloroacetic acid supernatant (cpm/dish)	Trichloroacetic acid precipitate (cpm/dish)
Crude trichloroacetic acid supernatant and trichloroacetic acid precipitate	2 030 000	197 000
'Fraction C' isolated by gel filtration	480 000	32 000
'Fraction C–con A III' isolated by con A- chromatography	60 400	10 600

[a] PA 1 cells were labeled metabolically with [^{3}H]glucosamine as in section 2, then treated with 0.05 M NaOH/1 M $NaBH_4$ for 16 h at 45°C. The proteins were next precipitated with trichloroacetic acid. The supernatant was subjected to gel filtration on Bio-Gel P-10 to isolate 'fraction C' (app. M_r ~700–2000). This fraction was then chromatographed on con A–Sepharose to isolate the liberated 'high mannose' oligosaccharides and glycopeptides as 'fraction C–con A III'. The trichloroacetic acid precipitate was digested with pronase and then analyzed in the same way as the supernatant; 'fraction C–con A III' obtained represents the 'high mannose' chains that were not liberated by NaOH/$NaBH_4$

3.3. *Analysis of* N-*acetylhexosamines and* N-*acetylhexosaminitols of the liberated 'high mannose' chains*

To see whether the liberated 'high mannose' oligosaccharide chains were released as oligosaccharide alcohols or as glycopeptides by the alkaline borohydride, they were hydrolyzed with acid to monosaccharides and analyzed by paper chromatography.

When fraction C–con A III from the trichloroacetic acid supernatant was hydrolyzed and then re-*N*-acetylated and analyzed by paper chromatography in the system of [10], both *N*-acetyl-[^{3}H]glucosaminitol and *N*-acetyl-[^{3}H]glucosamine were found (fig.5). The amount of *N*-acetyl-[^{3}H]glucosaminitol was ~1/6th of that of *N*-acetyl-[^{3}H]glucosamine. This is the result expected from a mixture consisting of 30% of oligosaccharide alcohols and 70% of glycopeptides, if the specific radioactivities of the different *N*-acetylglucosamine groups were identical.

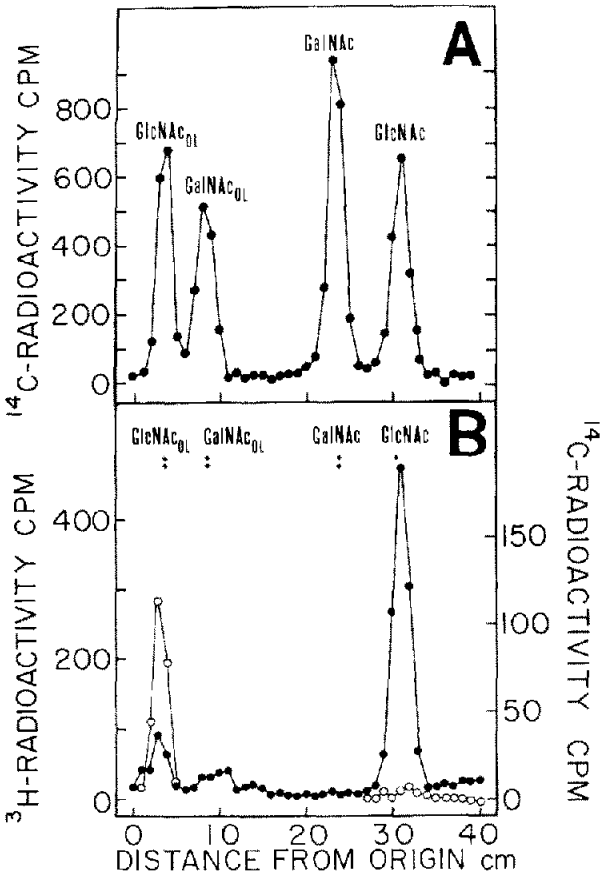

Fig.5. Paper chromatography of *N*-acetylhexosaminitols and *N*-acetylhexosamines: (A) separation of ^{14}C-labeled *N*-acetylglucosaminitol (GlcNAc$_{ol}$), *N*-acetylgalactosaminitol (GalNAc$_{ol}$), *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc). (B) a chromatogram of [^{3}H]glucosamine labeled *N*-acetylhexosaminitols and *N*-acetylhexosamines from the librated fraction C–con A III (•); *N*-acetyl-[^{14}C]glucosaminitol added as an internal marker (○).

4. Discussion

Treatment of glycoproteins with 0.05 M NaOH/1 M $NaBH_4$ was devised for liberation of GalNAc–Ser-(Thr)-linked oligosaccharides from glycoproteins [6]. The high borohydride content of the reagent is necessary to protect the released oligosaccharides against the 'peeling reactions'. These data show that even GlcNAc–Asn-linked oligosaccharides are released under these conditions. This is true both for the oligosaccharides of the 'complex' type as well as for those of the 'high mannose' type.

The cleavage of the GlcNAc–Asn-linked saccha-

rides from proteins appears to be caused by the borohydride of the reagent, and not by the alkali. This suggests that the GalNAc–Ser(Thr)-linked saccharides could be liberated without degrading the GlcNAc–Asn-linkage if alkali alone, or alkali with a low concentration of borohydride, were used. We have treated [^{3}H]glucosamine-labeled PA 1 cells with 0.1 M NaOH/0.1 M $NaBH_4$ for 24 h at 23°C, under conditions known to liberate Ser(Thr)-linked saccharide chains [13]. In this experiment only 18% of the 'high mannose' oligosaccharide chains were liberated into the trichloroacetic acid supernatant (M.-L. R., O. R., unpublished). In contrast, there was 85% liberation of these chains in ther experiment with 0.05 M NaOH/1 M $NaBH_4$ as shown in table 1.

The 'high mannose' sugar chains liberated from the glycoproteins by 0.05 M NaOH/1 M $NaBH_4$ appeared to contain oligosaccharide alcohols and glycopeptides. The oligosaccharide alcohols were identified by isolating the *N*-acetylglucosaminitol units after strong acid hydrolysis. The yield was, however, only 30% of that expected, suggesting that most of the oligosaccharide chains had been liberated in some other form, possibly as glycopeptides. Proteolytic cleavage caused by sodium borohydride under mild conditions has been observed [14], and some of the peptide bonds in the proximity of the linkage asparagine residue may be particularly reactive.

The possibility of releasing both major types of sugar chains of glycoproteins as oligosaccharide alcohols with borohydride under mild alkaline conditions provides samples well suited for identification of the linkage sugar units. The linkage sugars are released as hexosaminitols when the reduced oligosaccharides are hydrolyzed with acid, and they are easily identified by ion-exchange chromatography [15,16], or with the newly developed paper chromatographic system that is particularly well suited for separating labeled *N*-acetylglucosaminitol and *N*-acetylgalactosaminitol [10].

Acknowledgements

The authors wish to extend special thanks to Ms Leena Penttilä and to Ms Raija Oinonen for skilled and dedicated assistance. The work was supported by grants from the Finnish Academy and from Ida Montin Foundation.

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