Inhibition of formation of complex oligosaccharides by the glucosidase inhibitor bromoconduritol
(glycoprotein processing/α-glucosidase/influenza virus)

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Communicated by W. Beermann, July 26, 1982

ABSTRACT The α-glucosidase inhibitor bromoconduritol (6-bromo-3,4,5-trihydroxy-cyclohex-1-ene) inhibits trimming of the innermost glucose residue from the Glc2Man9GlcNAc2 precursor of high-mannose and complex oligosaccharides. This inhibition occurs both in intact cells and with a microsomal enzyme preparation. The formation of lipid-linked oligosaccharides was increased in glucosidase-inhibited cells. Inhibition of transfer of high-mannose oligosaccharides to protein was not observed. In bromoconduritol-treated virus-infected cells, trimming of mannose can occur despite incomplete removal of glucose. The glucosylated high-mannose oligosaccharides Glc2Man6GlcNAc, GlcMan5GlcNAc, and GlcMan6GlcNAc were released from viral glycoproteins after digestion with Pronase and endo-β-N-acetylglucosaminidase H. The formation of complex oligosaccharides was concomitantly inhibited. The release of infectious fowl plague virus particles (an influenza virus) was inhibited from bromoconduritol-treated infected chicken-embryo cells.

The formation of asparagine-linked oligosaccharides starts with transfer of the oligosaccharide Glc2Man8GlcNAc2 from dolichol diphosphate to the protein (1, 2). This oligosaccharide is a precursor of two basic types of asparagine-linked oligosaccharides—i.e., the high-mannose oligosaccharides (containing mannose and GlcNAc) and the complex oligosaccharides (containing also galactose, fucose, and NeuNAc).

The glucose residues in the precursor occur in the sequence Glc1–2–Glc1–3–Man1–2–Man2–3–Man . . . (2). Two neutral glucosidases have been reported to be involved in the removal of the glucose residues that occurs after transfer of the oligosaccharide from the lipid to the protein (2). These are glucosidase I (or Glc2-glucosidase), which removes the α1–2-linked glucose, and glucosidase II (or Glc2-glucosidase), which cleaves the two α1–2-linked glucose residues. The subsequent removal of up to four mannose residues gives rise to high-mannose oligosaccharides, whereas mannose trimming followed by addition of GlcNAc, galactose, fucose, and NeuNAc from the appropriate sugar donors results in the formation of complex oligosaccharides (3). Thus, trimming of glucose residues is an obligatory step in glycoprotein processing.

Most inhibitors of protein glycosylation interfere with the assembly of the dolichol-linked oligosaccharide, but some drugs are known to interfere with processing (4, 5). Bromoconduritol (6-bromo-3,4,5-trihydroxy-cyclohex-1-ene) is an active-site directed covalent inhibitor of α-glucosidases (6, 7). We have tested conduritol derivatives as inhibitors of glucosidases involved in glucose trimming of asparagine-linked oligosaccharides and, therefore, as inhibitors of formation of the high-mannose and complex oligosaccharides found on mature glycoproteins. For these investigations, cells infected with influenza virus were used. In these cells, host glycoprotein synthesis is shut off and, after infection, viral glycoprotein synthesis de novo can be followed.

EXPERIMENTAL PROCEDURES

Chemicals. Bromoconduritol was prepared as described (6). Unless indicated otherwise, a 1:1 mixture of bromoconduritol A (3,5/4,6-bromo-3,4,5-trihydroxy-cyclohex-1-ene) and bromoconduritol B (3,5,6/4,6-bromo-3,4,5-trihydroxy-cyclohex-1-ene) was used. The material was dissolved in cold water and used immediately. The following isotopic precursors were obtained from Amersham Buchler: [1-3H]fucose, [2-3H]mannose, [1-3H]galactose (all at 2–5 Ci/mmol; 1 Ci = 3.7 × 1010 becquerels), and [35S]methionine (600 Ci/mmol). Pronase P (from Streptomyces griseus) was from Serva, α-mannosidase (type III from jack beans) was from Sigma, and endo-β-N-acetylglucosaminidase H (endo H) was from Miles. Tunicamycin was a gift from G. Tamura, University of Tokyo.

Glc3Man9GlcNAc labeled in its glucose or mannose moiety was prepared from lipid-linked oligosaccharides formed in intact cells after labeling with [3H]galactose or [3H]mannose as described (8). Glc2Man8GlcNAc, Man2GlcNAc, Man3GlcNAc, and Man4GlcNAc labeled with [14C]mannose were prepared from their respective dolichol diphosphate derivatives followed by treatment with endo H as described (9). [3H]Glc1Man5GlcNAc was isolated after Pronase treatment and digestion with endo H of glycoproteins from virus-infected cells that had been labeled with [3H]galactose for 1 hr and then further incubated for 2 hr. Characterization was done by chromatography on Bio-Gel P-4 columns, resistance against α-mannosidase (9), and susceptibility to α-glucosidase (see Results, Fig. 6A). β-Man-GlcNAc was obtained by α-mannosidase digestion of Man2GlcNAc as described (10).

Tissue Culture and Labeling Procedures. For labeling, in vivo confluent monolayer cultures of primary chicken embryo cells in 9-cm-diameter Petri dishes were used. The cells were infected with fowl plague virus (strain Rostock) or PR8 (a human influenza virus) as described (9). After infection, the cells were maintained in 2.5 ml of Earle's medium containing 10 mM pyruvate. Radioactive isotopes were added 4 hr after infection.

Separation Techniques. Extraction of lipid-linked oligosaccharides and preparation of glycopeptides was as described (8). Bio-Gel P-4 columns were calibrated as described (10). Round gel electrophoresis was as described (8). Separation of virus particles on sucrose density gradients was as described (11).

Other Procedures. Release of glucose from oligosaccharides was accomplished as described by Grimmel and Robbins (12), except that the assay mixture was modified by addition of 10 mM L-glutamine to the cells. The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: endo H, endo-β-N-acetylglucosaminidase H.

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EDTA to inhibit the residual α-mannosidase activity shown by the inhibition of release of radioactive mannose from Glc3[^14C]Man9GlcNAc and [[^14C]Man5]GlcNAc; no effect on the activity of the α-glucosidase was detected. For inhibition of glucosidase with bromoconduritol, see Fig. 3. Glycosylation in vitro was done as described (8). Pronase digestion of glycoproteins and treatment with endo H and α-mannosidase were as described (9). Virus quantitation was done by the plaque assay (13).

RESULTS

Effect of Bromoconduritol on Glycosylation in PR8-Infected Cells. The residue obtained after extracting virus-infected cells with organic solvents and water (8) contains the virus-specific glycoproteins. The virus used in this particular experiment was the influenza virus strain PR8, which has a high proportion of complex oligosaccharides (14). An electropherogram of this residue from chicken embryo cells infected with virus and labeled with [^3H]mannose is shown in Fig. 1A (Inset). The two glycoproteins hemagglutinin and neuraminidase were detected. Digestion of this residue with Pronase gave rise to glycopeptides of the high-mannose and complex types. Subsequent treatment with endo H cleaved high-mannose oligosaccharides from the peptide to yield free oligosaccharides lacking the GlcNAc residue that was attached to asparagine. Complex glycopeptides are not cleaved by the enzyme. The elution pattern from a Bio-Gel P-4 column of glycopeptides and oligosaccharides obtained after endo H treatment of the Pronase digest is shown in Fig. 1A. The complex glycopeptides eluted before the marker ([[^14C]Glc3]Man9GlcNAc arrow), and the high-mannose oligosaccharides eluted after this marker.

The results obtained from PR8-infected cells treated with the α-glucosidase inhibitor bromoconduritol are shown in Fig. 1B. The inhibitor (2 mM) was added to the cells 3.5 hr after infection (i.e., 30 min before addition of [^3H]mannose) and was present during the labeling period. The incorporation of [^3H]mannose into the viral glycoproteins was inhibited slightly, but the same glycoproteins were detected in the same ratio (Inset). However, the analysis on Bio-Gel P-4 columns of the endo H-treated Pr

Fig. 1. Bio-Gel P-4 chromatography of [^3H]mannose-labeled oligosaccharides and glycopeptides from nontreated (A) or bromoconduritol-treated cells (B). Cells infected with influenza virus PR8 were labeled with [^3H]mannose 4–7 hr after infection in the absence (A) or presence (B) of 2.5 mM bromoconduritol (added 30 min prior to addition of the label). One portion of the lipid-free residue was subjected to electrophoresis (Inset) and another portion was digested with Pronase and then with endo H. Arrows: 1, elution position of the standard Glc3Man3GlcNAc; V0, void volume (bovine serum albumin).

Fig. 2. Effect of bromoconduritol on the incorporation of [^3H]mannose and [^14C]labeled amino acids (A) and of [^3H]fucose (B) into viral glycoproteins. Virus-infected cells were treated with bromoconduritol 30 min before addition of the labeled sugars or 2 hr before addition of the isotope. The radioactivity in the lipid-free glycoprotein-containing fraction was determined. Labeling time was 15 min ([^14C]labeled amino acids) or 60 min ([^3H]fucose). (A) ○, [^3H]Labeled amino acids; ○, [^3H]mannose. (B) ●, Cells were treated with bromoconduritol for 30 min; ○, cells were treated with bromoconduritol for 120 min.
trimming of glucose residues from the precursor oligosaccharide Glc$_3$Man$_9$GlcNAc, a microsomal preparation from rat liver was obtained (12) and incubated with this oligosaccharide labeled in the glucose residues. It is shown in Fig. 3 that glucose is removed from the oligosaccharide in a pH 6.8 sodium phosphate buffer. At pH 4.6 (in citrate phosphate buffer), no glucose release was detected after incubation for 90 min (not shown) whereas, in this buffer at pH 6.5, 47% of the glucose in the original oligosaccharide was released after 90 min as free glucose. This result indicates that the trimming of glucose residues was caused by the neutral glucosidases rather than by the lysosomal acid α-glucosidase.

The addition of bromoconduritol decreases the rate of glucose release (Fig. 3). Of the two isomers (bromoconduritol A and bromoconduritol B), bromoconduritol A was the stronger inhibitor (Fig. 3). This may be related to the fact that the half-life for decomposition of bromoconduritol A into conduritol B and F in water at pH 7.3 and 37°C (16 min) is higher than that of bromoconduritol B (2 min). Bromoconduritol A and B were each contaminated with about 5% of the other isomer, and we ascribe the smaller difference in the inhibitory effects of the higher concentration to this fact. Other derivatives, conduritol B and conduritol epoxide (up to 5 mM), had no effect on glucose release.

The analysis on Bio-Gel P-4 columns of the oligosaccharides after release of 40% or 60% of the glucose as free glucose showed that only the original material was present (Fig. 4A). Incubation of the microsomal enzyme preparation with Glc$_3$Man$_9$GlcNAc labeled in the mannose residues did not result in the release of significant amounts of free mannose, and analysis of the oligosaccharides (Fig. 4B) shows some Glc$_3$Man$_9$GlcNAc but mainly Man$_9$GlcNAc. Thus, under the particular conditions of the assay, the reaction catalyzed is the conversion of Glc$_3$Man$_9$GlcNAc to Man$_9$GlcNAc.

The analysis of the oligosaccharides obtained after glucosidase treatment of [3H]Glc$_3$Man$_9$GlcNAc in the presence of 5 mM bromoconduritol is shown in Fig. 4C. Three major oligosaccharides are seen, differing from one another by one hexose unit. Because of the absence of mannosidase activity, the oligosaccharides are Glc$_3$Man$_9$GlcNAc, Glc$_3$Man$_9$GlcNAc, and Glc$_3$Man$_9$GlcNAc. After incubation of Glc$_3$Man$_9$GlcNAc in the presence of 5 mM bromoconduritol, the pattern of Fig. 4D is obtained. It shows the presence of Glc$_3$Man$_9$GlcNAc, Glc$_3$Man$_9$GlcNAc, Glc$_3$Man$_9$GlcNAc, and Man$_9$GlcNAc in the molar proportions 1.8:5.6:10.9:1.0. Clearly, in the presence of bromoconduritol, the trimming of the innermost glucose residue is inhibited.

Inhibition of Glucose Trimming in Vitro. To detect glucosylated high-mannose oligosaccharides attached to viral glycoproteins, primary chicken embryo cells infected with fowl plague virus were labeled with [3H]mannose or [3H]galactose for 40 min and then chased for 2 hr. The inhibitor (2.4 mM) was added 90 min before the isotopes and was also present in the chase medium. The viral glycoproteins were isolated and digested with Pronase and then with endo H. The mixture of glycopesides and oligosaccharides was separated on a Bio-Gel P-4 column (Fig. 5).

The formation of [3H]mannose-labeled complex oligosaccharides (eluted between fractions 90 and 120) was inhibited 60% in this particular experiment in cells treated with bromoconduritol, whereas the formation of high-mannose oligosaccharides was increased 1.4-fold. Furthermore, the pattern of high-mannose oligosaccharides from inhibitor-treated cells was clearly different from that of untreated cells (Fig. 5 A and B). The fractions under peaks a, b, c, d, e, and f were pooled separately, and the oligosaccharides were analyzed by glucosidase and α-mannosidase treatment. After glucosidase treatment (using the rat liver enzyme preparation), the oligosaccharides were mixed with 14C-labeled standards—Glc$_3$Man$_9$GlcNAc,
was detected on hexose and glycopeptides from \[^{[H]}\]mannose-labeled (A and B) or \[^{[H]}\]galactose-labeled (C) virus-infected cells treated or not treated with bromoconduritol. Cells infected with fowl plague virus were incubated for 90 min with (B and C) or without (A) 2.4 mM bromoconduritol before the addition of \[^{[H]}\]mannose or \[^{[H]}\]galactose. After 40 min, the labeling medium was removed and the cells were incubated for 2 hr in unlabeled medium. The glycoprotein fraction was isolated and digested with Pronase and then with endo H. \(V_0\), elution position of bovine serum albumin. For peaks a-f, see Fig. 6 and the text.

\(\text{Man}_{9}\text{GlcNAc}, \text{Man}_{7}\text{GlcNAc}, \text{Man}_{9}\text{GlcNAc}\), and \(\text{Man}_{7}\text{GlcNAc}\)—and then separated on Bio-Gel P-4 columns.

Oligosaccharide d, untreated, eluted at the position of Glc-Man\(_9\)GlcNAc and, after glucosidase treatment, was shifted one hexose unit on the column to coelute with Man\(_9\)GlcNAc (Fig. 6A). Consistent with the identity of d as GlcMan\(_9\)GlcNAc is the result that, after \(\alpha\)-mannosidase treatment, no \(\beta\)-ManGlcNAc was detected on TLC of the digest (cf. ref. 9). Fig. 6A also shows that glucose removal by the glucosidase is almost quantitative. Oligosaccharide e, untreated, eluted slightly ahead of Man\(_9\)GlcNAc and, after glucosidase treatment, a part was shifted one hexose unit to coelute with Man\(_9\)GlcNAc (Fig. 6B). Thus, oligosaccharide e contains GlcMan\(_9\)GlcNAc. After \(\alpha\)-mannosidase digestion, 2% of the radioactivity was found in \(\beta\)-ManGlcNAc, indicating the presence of some nonglucosylated species, possibly Man\(_9\)GlcNAc. Oligosaccharide f, untreated, eluted slightly ahead of the standard Man\(_9\)GlcNAc and, after glucosidase treatment, a part was shifted one hexose unit to coelute with Man\(_9\)GlcNAc (Fig. 6C). Thus, oligosaccharide f contains GlcMan\(_9\)GlcNAc. The fact that glucosidase treatment of oligosaccharides e and f does not go to completion is consistent with the result (15) that glucosidase activity is hampered by the removal of the peripheral mannose residues. Treatment of oligosaccharide f with \(\alpha\)-mannosidase gives 4% of the radioactivity as \(\beta\)-ManGlcNAc, indicating the presence of some nonglucosylated oligosaccharide. Oligosaccharide a behaved as oligosaccharide d, and oligosaccharides b and c, coeluting with Man\(_9\)GlcNAc and Man\(_7\)GlcNAc, respectively, had the same mobilities after glucosidase treatment.

Consistent with the presence of glucose in oligosaccharides a, d, e, and f is the fact that they can be labeled with \[^{[H]}\]galactose (Fig. 5C). Incubation of these oligosaccharides, labeled in the glucose residues, with the rat liver glucosidase in the presence of bromoconduritol completely inhibited glucose release. Taken together, these results indicate that the mechanism of inhibition of bromoconduritol is the same in intact cells and in a rat liver enzyme preparation.

**Effect of Bromoconduritol on Virus Formation.** Three hours after infection, chicken embryo cells infected with fowl plague virus were treated with bromoconduritol (2.4 mM and 4.8 mM) or tunicamycin at 2 \(\mu\)g/ml or not treated. One hour later, 50 \(\mu\)Ci of \(^{[35S]}\)methionine was added. The virus was harvested 8 hr after infection and layered on a sucrose gradient. In a separate experiment, the infectivity was determined. Fig. 7 shows that incorporation of radioactive into virus particles (fractions 5, 6, and 7) is inhibited 75% by 2.4 mM bromoconduritol (Fig. 7B) and completely by 4.8 mM bromoconduritol (Fig. 7C) and by tunicamycin (Fig. 7D). In parallel, a 90–98% inhibition of infectivity (measured as plaque-forming units per ml) was measured with 2.4 mM bromoconduritol. Apparently, the release of infectious virus is inhibited when the formation of complex oligosaccharides is inhibited. It remains to be established whether the lack of complex oligosaccharides per se or secondary effects caused by inhibition of processing (such as those resulting in increased proteolytic susceptibility of the viral glycoproteins) are responsible for the inhibition of formation of infectious virus.

**DISCUSSION**

The \(\alpha\)-glucosidase inhibitor bromoconduritol inhibits trimming of glucose residues from the glucosylated protein-linked precursor oligosaccharide for high-mannose and complex oligosaccharide side chains of viral glycoproteins. Also, by using a microsomal glucosidase, it could be shown directly that bromoconduritol inhibits the deglucosylation of Glc\(_3\)Man\(_9\)GlcNAc to Man\(_9\)GlcNAc. There is no indication that bromoconduritol in-
Fig. 7. Sucrose density gradient analysis of virus particles labeled with [35S]methionine. Fowl plague virus-infected cells were labeled with [35S]methionine from 4 to 8 hr after infection; 2.4 mM (B) or 4.8 mM (C) bromoconduritol or tunicamycin at 2 μg/ml (D) was added 1 hr before addition of the isotope. (A) Control (no sugar analog or drug). (Note the different ordinate scales.) The virus was pelleted from the medium by centrifugation at 100,000 × g for 1 hr, layered on a 10-mi sucrose gradient (15–40%) containing a cushion of 1 ml of 50% sucrose at the bottom, and centrifuged for 2 hr at 40,000 rpm (SW 41 rotor).

... inhibits the formation of the lipid-linked oligosaccharide or the transfer of the oligosaccharide from the lipid to the protein. Thus, the observed inhibition of formation of complex oligosaccharides in bromoconduritol-treated cells is probably caused by inhibition of glucose trimming.

... Indeed, glucosylated high-mannose oligosaccharides were isolated from bromoconduritol-treated cells after endo H treatment and their compositions were determined to be GlcMan9GlcNAc, GlcMan8GlcNAc, and GlcMan7GlcNAc. This result has three implications. (i) Mannose trimming can occur without complete removal of glucose. This phenomenon has been observed previously as a minor pathway (16). (ii) The glucosylated glycoprotein can leave the rough endoplasmic reticulum, because the α(1→2)mannosidase is located in the Golgi complex (2). (iii) Bromoconduritol does not inhibit this mannosidase activity.

... The results indicate that bromoconduritol does not inhibit the activity of glucosidase I (Glc₂-glucosidase), because oligosaccharides containing three glucose did not accumulate. However, if bromoconduritol were to affect only the catalytic activity of glucosidase II (Glc₂-glucosidase), oligosaccharides containing two glucose residues rather than one should be found. The active site of glucosidase II may contain at least two glucose-binding sites since nigerose is a competitive inhibitor of this enzyme (17). Possibly, therefore, bromoconduritol interferes with the active site of the enzyme, preventing binding of the oligosaccharide after release of the glucose group from Glc₂Man₉GlcNAc, rather than with the catalytic site of the enzyme. This proposal avoids postulating a third glucosidase.

In tunicamycin-treated fowl plague virus-infected chicken embryo cells, the hemagglutinin is not glycosylated and virus formation is blocked (11). In bromoconduritol-treated fowl plague virus-infected chicken embryo cells, the hemagglutinin is glycosylated, be it with mainly high-mannose oligosaccharides, and yet the release of infectious virus is inhibited. This suggests a role for glycoprotein processing in virus formation in this particular system. This conclusion seems to be in accord with the results from a survey of 19 different influenza virus strains grown in chicken cells (14), in which it was shown that hemagglutinin molecules containing only high-mannose oligosaccharides do not occur in intact viruses.

We acknowledge the technical assistance of Kirstin Schallner and Susanne Fitz and thank Drs. R. Rott and C. Scholtissek for their encouragement. The research was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 47 and a Heisenberg Stipendium to R.T.S.) and a grant from Stiftung Volkswagenwerk.

P.A.R. holds a fellowship from the Alexander von Humboldt Stiftung.