High-Energy Collision-Induced Dissociation Mass Spectrometry of Synthetic Mannose-6-Phosphate Oligosaccharides

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The high-energy collision-induced dissociation spectra of a series of linear and branched synthetic mannosyl oligosaccharides that contain 6-phosphate substituents on either or both non-reducing terminal or penultimate residues have been studied. These phosphorylated structures were designed to mimic those of naturally derived *N*-glycans (Man-6-PO₄) on lysosomal enzymes and to probe the minimally required binding motif for the Man-6-PO₄ receptors. When a phosphate group was present, the spectra were dominated by ions that arise from cleavages at the glycosidic bonds (single and double) with charge retention on the phosphate-containing fragments. The spectra of linear structures that bear the nonreducing terminal Man-6-phosphate residues were devoid of Y-type ions, unlike those with similar phosphorylation at the penultimate residue. The location of the phosphorylated residue was deduced from the presence or absence of unique B and Y ions. In neutral branched structures, the ions were formed by cleavage at the glycosidic bond at either one or both of the branch points and the aglycon, which was attached to the disubstituted mannosyl residue. Branched oligosaccharides that contained one or two terminal Man-6-PO₄ residues also showed double cleavages with charge retention on the phosphate-containing fragment. Our investigation shows that positive mode high energy collision-induced dissociation mass spectrometry can determine the location—terminal or penultimate—of Man-6-PO₄ residues in *N*-linked type oligosaccharides. (*J Am Soc Mass Spectrom* 1996, 7, 182–188)

Mannose-6-phosphate-containing oligosaccharides are important in routing newly synthesized degradative enzymes to lysosomes (for reviews, see [1] and [2]). Mannose-6-phosphate (Man-6-PO₄) residues occur at either terminal (~70%) or next to last position in *N*-linked oligomannosidic-type structures with six to nine mannose residues. A diverse array of oligosaccharides, which contain one or two Man-6-PO₄ residues, has been described [3–6], but, in many cases, sequence and branch location of the phosphorylated residues have not been determined [3–6]. By using a series of synthetic oligosaccharides [7–11], it was found that the number and sequence position of phosphorylated residues contributed significantly to the binding of oligosaccharides to both the

Ca⁺⁺ dependent and independent Man-6-PO₄ receptor [12].

Because purified, naturally occurring phosphorylated oligosaccharides are usually available only in sub-nanomole quantities, we have explored the utility of positive mode tandem mass spectrometry with high-energy collision-induced dissociation (HE-CID-MS) for their structural elucidation. We analyzed a series of linear and branched synthetic oligosaccharides that contained one or two Man-6-PO₄ residues in either terminal or penultimate positions. We found that, in contrast to neutral mannose-containing oligosaccharides, the predominant ions observed arose from single cleavages at the glycosidic bonds with charge retention on the phosphorylated fragments. A new ion with *m/z* 99 (P ion) was observed only in positive collision-induced dissociation (CID) mass spectra of compounds that contain phosphate groups. Further, discrimination between terminal and penultimate phosphorylation was determined from mass val-

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ues associated with B- and Y-type ions. In contrast to previous studies with neutral glycoconjugates [13], ring cleavages were not observed.

Experimental

Materials

Mannose-6-phosphate-containing oligosaccharides were synthesized as previously described [7-11]. All solvents were high performance liquid chromatography grade. Thioglycerol (1-thio-2,3-propanediol) was purchased from Aldrich Chemical Company (Milwaukee, WI).

Mass Spectrometry

The Kratos Concept IHH four sector tandem mass spectrometer (Kratos Analytical Ltd., Manchester, UK), under Mach 3 control, was equipped with a multichannel array detector using a rapid scanning charge-coupled device (CCD) [14]. Precursor ions were formed

by using a 0.2- μ A Cs⁺ beam at an energy of 10 kV and an acceleration voltage of 8 kV. The collision cell was filled with He and floated at 4 kV, which resulted in a collision energy of 4 keV. The [¹²C] isobar of the [M + H]⁺ ion of each sample was selected by MS1. MS2 was scanned continuously on the CCD array at a scan rate of 10 s/decade through a range of 3000-38 mass units. The samples were introduced into MS1 with an ABI 140A solvent delivery system (Applied Biosystems, Foster City, CA) and a Rheodyne model 8125 injection valve (Rheodyne Inc., Cotati, CA) through a continuous flow probe at 3 μ L/min. The matrix used was a mixture of 2% thioglycerol (1-thio-2,3-propanediol)/5% acetonitrile/0.1% trifluoroacetic acid in water. One nanomole of each linear oligosaccharide (20 μ L of a 50- μ M solution) or 4 nmol of each branched oligosaccharide (20 μ L of a 200- μ M solution) were dissolved in the flow probe matrix and injected. Eight to nine CID spectra were summed to obtain each spectrum. For the sensitivity measurements, serial dilutions of 2 (Table 1) that ranged from 25- μ M to 375-pM solutions were injected in 20 μ L. The instru-

Table 1. Synthetic neutral and Man-6-PO₄-containing oligosaccharides^a

Structure	Calculated [M + H] ⁺ ion (u)	Measured [M + H] ⁺ ion (u)
1 Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ OR	513.2	513.1
2 ManP α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ OR	593.2	593.3
3 Man α 1 $\rightarrow$ 2ManP α 1 $\rightarrow$ OR	593.2	593.3
4 ManP α 1 $\rightarrow$ 2ManP α 1 $\rightarrow$ OR	673.2	672.8
5 ManP α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ OR	593.2	593.0
6 ManP α 1 $\rightarrow$ 6Man α 1 $\rightarrow$ OR	593.2	593.4
7 ManP α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man β 1 $\rightarrow$ OR	755.2	755.3
8 ManP α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ OR	755.2	755.4
9 ManP α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 6Man α 1 $\rightarrow$ OR	755.2	755.4
10 Man α 1 $\rightarrow$ 2ManP α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ OR	755.2	755.4
11 Man α 1 $\rightarrow$ 2ManP α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ OR	755.2	755.3
12 Man α 1 $\rightarrow$ 2ManP α 1 $\rightarrow$ 6Man α 1 $\rightarrow$ OR	755.2	755.4
Man α 1 $\rightarrow$ 2Man α 1 ↓6		
13 Man α 1 $\rightarrow$ OR ↑3	999.4	999.6
Man α 1 $\rightarrow$ 2Man α 1 ManP α 1 $\rightarrow$ 2Man α 1 ↓6		
14 Man α 1 $\rightarrow$ OR ↑3	1079.3	1079.6
Man α 1 $\rightarrow$ 2Man α 1 Man α 1 $\rightarrow$ 2Man α 1 ↓6		
15 Man α 1 $\rightarrow$ OR ↑3	1079.3	1087.1 ^b
ManP α 1 $\rightarrow$ 2Man α 1 ManP α 1 $\rightarrow$ 2Man α 1 ↓6		
16 Man α 1 $\rightarrow$ OR ↑3	1159.3	1159.5
ManP α 1 $\rightarrow$ 2Man α 1		

^a ManP = mannose-6-phosphate; R = (CH₂)₆COOMe.

^b The signal at *m/z* 1087.1 corresponds to the [M + Na]⁺ ion of this structure where the aglycon has lost the methyl group, that is, R = (CH₂)₆COOH. The M + H ion was not observed.

ment was then scanned at a rate of 3 s/decade through the above described mass range. Five CID spectra were summed to obtain each spectrum.

Results and Discussion

We have determined the high-energy CID (HE-CID) mass spectra of 16 synthetic oligosaccharides that contain structural features which represent naturally derived neutral and phosphorylated oligomannosidic glycosylation linked to asparagine residues in glycoproteins. Figure 1 shows the HE-CID spectrum of the synthetic neutral disaccharide **1** (Table 1), which constitutes the nonreducing terminus of naturally derived oligomannosidic structures. The MH^+ ion (m/z 513.1) agrees with the expected calculated value of 513.2 u that arises from two Hex residues and the aglycon- $(CH_2)_8COOMe$. Sequential losses of Hex residue values from the molecular ion at m/z 351 (Y_1) and 189 (Y_0) are also consistent with the linear disaccharide **1**. The ion fragments are designated according to the nomenclature of Domon and Costello [13] (Scheme I) and the mechanisms for the formation of the B, C, and Y ions are as proposed by Carr et al. [15]. The complementary B ions at m/z 163 (B_1) and 325 (B_2) are observed at similar intensities. Sequential losses of two elements of water from B_1 gave ions at m/z 145 and 127. Losses of water and methanol from the aglycon Y_0 ion gave ions at m/z 171 and 157, respectively.

Figure 2 shows the HE-CID spectrum of a synthetic disaccharide with either a terminal (2; panel a) or penultimate (3; panel b) Man-6- PO_4 residue. Both spectra are dominated by phosphorylated fragment ions: the only unmodified ions are observed from the aglycon. The presence of Man-6- PO_4 residues in these compounds is confirmed by the ion at m/z 243 in both spectra. Sequential losses of water and/or phosphoric acid from the Man-6- PO_4 fragment ions resulted in ions at m/z 225, 207, 145, and 127. An ion at m/z 99 was present only in the spectra of phosphorylated compounds. We propose that it is formed by elimination of a protonated phosphoric acid moiety via a

Scheme I. Fragmentation nomenclature proposed by Domon and Costello [13]. Reducing terminal fragments are designated Y and Z; nonreducing terminal fragments are indicated by B and C.

McLafferty-type rearrangement [15, 16] (Scheme II). The spectrum of compound **2** (Figure 2a) gave B ions at m/z 405 and 243. In this compound, the absence of a Y_1 ion prevented the localization of the Man-6- PO_4 residue to either a terminal or penultimate position. However, a prominent Y_1 ion (m/z 431) was observed in the isomer **3** (Figure 2b). The B_2 ion was conspicuously absent in the spectrum of **3**, but a B_1 ion at m/z 163, consistent with a nonphosphorylated terminal

Figure 1. Tandem HE-CID mass spectrum of mannobiose coupled to $(CH_2)_8COOMe$ (**1**, Table 1).

Figure 2. Tandem HE-CID mass spectra of (A) terminally and (B) penultimately phosphorylated mannobiose coupled to $(CH_2)_8COOMe$ (Table 1).

Scheme II. Elimination of the phosphate group from Man-6-PO₄ residues.

mannose residue, was seen. The ion at m/z 243, indicative of a mannosyl phosphate residue, was observed in the spectrum of **2** (B-type cleavage) and as an isomeric "internal" fragment in the spectrum of **3**, formed from a double cleavage (B_2Y_1). In linear trimannosyl structures (**10**–**12**) the location of penultimate Man-6-PO₄ was determined from the B_2 and Y_2 ions, m/z 405 and 593, respectively (data not shown), where the Y_2 ion is formed by cleavage at the glycosidic bond on the nonreducing end of the Man-6-PO₄ residue. The presence of a terminal Man-6-PO₄ residue was deduced by the absence of the Y_2 ion and the presence of a B_2 ion, showing a nonreducing terminal hexose residue.

The spectrum of the diphosphorylated disaccharide (**4**; Figure 3) clearly demonstrated the almost exclusive charge retention of phosphorylated fragments. Localization of the phosphate groups to particular carbohydrate moieties was apparent from the Y_0 ion at m/z 189 and the B_2 ion at m/z 485. A strong signal at m/z 431 (Y_1 ion) was present also. This Y_1 ion confirms the presence of phosphate groups on both Hex residues. Primary and secondary fragment ions from the mannosyl phosphate residue also were observed at m/z 243, 225, 207, 145, and 127, respectively, together with a P ion at m/z 99, as described in the foregoing text.

Naturally derived Man-6-PO₄ containing oligosaccharides are branched structures and their mimetics are represented by compounds **14**–**16** (Table 1). The spectrum of their neutral counterpart, **13**, is shown in Figure 4a. In this spectrum the complements of expected B ions were observed at m/z 325 and 163.

Figure 3. Tandem HE-CID mass spectrum of diphosphorylated mannosyl coupled to (CH₂)₈COOMe (Table 1).

Figure 4. Tandem HE-CID mass spectra of (a) neutral and (b) phosphorylated pentamannosyl compounds (Table 1, **13** and **14**). The ions at m/z 127, 145, 163, 325, 487, 675, and 837 (panel a) also can be formed by cleavage of the 3-branch. For simplicity only the cleavages of the 6-branch are shown.

Sequential losses of water from B_1 gave ions at m/z 145 and 127. Sequential losses of 1-, 2-, and 5-Hex residues were evident from ions observed at m/z 837 (Y_2), 675 (Y_1), and 189 (Y_0), respectively. Figure 4b shows the monophosphorylated pentasaccharide with a molecular ion at m/z 1079.6, which is consistent with the structure for **14**. As in the case of the linear anionic oligosaccharides discussed previously, the fragmentation of this dibranched pentasaccharide was dominated by phosphorylated fragments, and a similar fragmentation pattern was obtained for the Man-6-PO₄-containing branch (Figure 2, **3**). The only fragment observed from the branch that contained neutral mannose residues was a B_1 ion at m/z 163. For the monophosphorylated structure **14**, Y ions were observed at m/z 755 ($Y_{1\beta}$) and 189 (Y_0).

Internal fragments were observed in both the neutral and phosphorylated branched compounds **13**–**16** (Table 1). Excision of dimannosyl units from both of the branches of **13** gave an ion at m/z 351 (Y_{1Y_1}), which was not observed in the monophosphorylated pentasaccharide (Figure 4b). A cleavage at either branch and at the glycosidic bond to the aglycon gave B_3Y_1 ions at m/z 487 from the neutral compound **13**. The same type of process for the phosphorylated com-

pound **14** gave a signal at m/z 567 ($B_3Y_{1\beta}$) from cleavages at the $\alpha 1 \rightarrow 3$ branch and the aglycon. The nonphosphorylated fragment m/z 487, from cleavage at the $\alpha 1 \rightarrow 6$ branch and the glycosidic bond of the aglycon ($B_3Y_{1\alpha}$), was not observed, and thus the branch location of the Man-6- PO_4 could not be determined from these spectra. The HE-CID spectra of the various linkage isomers ($\alpha 1 \rightarrow 2$, $\alpha 1 \rightarrow 3$, and $\alpha 1 \rightarrow 6$) (Table 1) all gave spectra that did not reveal linkage information (data not shown).

To determine the sensitivity for HE-CID analysis of this class of compounds, serial dilutions of **2** (Table 1) were analyzed. The smallest amount of sample that gave an $[M + H]^+$ ion of sufficient intensity to be recognized above background in MS1 was ~ 370 fmol/ μ L. Figure 5a shows the resulting spectrum. When the spectrum was compared to the background spectrum (Figure 5b) all the major cleavage ions were present except for the Y_0 ion at m/z 189 and two secondary fragment ions at m/z 145 and 171. The presence of the B_1 , B_2 , and P ions combined with the absence of a Y_1 ion indicates that the oligosaccharide is terminally phosphorylated as previously discussed. Because only five CID spectra were summed to obtain the spectrum, it represents approximately 950 fmol of material. However, because it takes a few seconds for the peak to rise to a sufficient intensity in MS1 and the

instrument then has to be switched to the tandem mode, the true lowest amounts needed to obtain reasonable spectra is a few picomoles.

Positively charged ions from CID fragmentation of synthetic and naturally derived glycoconjugates are the result of cleavages of one or two bonds [8, 15, 17-20] that generate the B, C, Y, and Z ion series [8] or ring cleavage products that involve the scission of two bonds—X ions. These latter fragments arise by cleavage of at least one C—C bond and/or a C—O bond to give rise to the A and X series of ions [8]. Internal fragments that arise from double cleavages involving two glycosidic bonds have not been reported previously in the positive ion mode. We found that internal Man-6- PO_4 residues readily produce double cleavages at glycosidic bonds with charge retention on the phosphorylated fragment. Branched oligosaccharides, containing one (Figure 4b) or two (data not shown) nonreducing terminal Man-6- PO_4 residues, also showed double cleavage with charge retention on the phosphate-containing fragment. Formation of intense fragments also was observed in similar neutral branched structures involving excision of the disubstituted mannose residue linked to the aglycon.

In all spectra of these phosphate-containing mannosyl glycoconjugates, the ion current was carried almost exclusively by phosphorylated fragments, whether they arose from the reducing or nonreducing end. Analogously, in a previous study in which the phosphate group was located on the aglycon (phosphatidylethanolamine derivatives), almost all of the ion current also was carried by phosphorylated fragments [18]. In this study [18], only single glycosidic bond cleavages occurred in the carbohydrate moiety, thus forming Y ions that contained the phosphorylated aglycon, which dominated the spectrum. Our data are in agreement with this observation. Neutral or sialylated reducing oligosaccharides exhibit equal abundances of B- and Y-type ions with a predominance of fragments that contain *N*-acetyl hexosamine residues [15, 19].

The potential for the aglycon to carry charge can have a marked effect on the fragments observed in HE-CID-MS. In the case of compounds with aglycons that contain a secondary amine, only Y ions are observed [15, 19] and the charge is apparently localized to the amine. However, in asialo-biantennary lactosamine-type oligosaccharides linked to a single Asn, B ions were observed in equal abundance as the aglycon-containing Y ions [8].

We observed no ring cleavages in the HE-CID of neutral or phosphorylated mannosyl oligosaccharides. This is in contrast to that observed in neutral glycosphingolipids [20] and linear malto-oligosaccharides derivatized with 2-amino pyridine [15, 21]. In the former compounds, the intraring cleavage $^{1,5}X$ ions are the only ions formed from the carbohydrate moiety, whereas in the latter structures the $^{1,5}X$ ions were of abundance equal to the Y ions.

Figure 5. (a) Tandem HE-CID mass spectrum of low amounts of ManPa1 $\rightarrow$ 2Man α 1 $\rightarrow$ (CH₂)₈COOMe. (b) A background spectrum obtained by blank injection of sample solvent.

In this study, fragment ions formed by double cleavages of glycosidic bonds were prominent in the spectra of synthetic oligosaccharides. These ions were found in the spectra of both linear and dibranched structures, with and without phosphorylated residues. In the case of phosphorylated structures, all the double-cleavage ions contained the Man-6-PO₄ moiety. Neutral structures gave double cleavage ions, which all retained the 3,6-disubstituted mannosyl residue. These ions, generated by double cleavages at glycosidic bonds, have not been described previously.

The intense ion at m/z 243 from Man-6-PO₄ residues and a characteristic pattern of secondary fragments (sequential losses of water and/or phosphoric acid at m/z 225, 227, 145, and 127) were found in all the phosphorylated oligosaccharides. The ion at m/z 243 was produced either by single cleavage (B ions) or by double cleavages (BY ions). A positive ion—diagnostic for the presence of a phosphate group attached to the 6-position of a pyranose ring—was observed at m/z 99 (H₄PO₄⁺). This ion likely is formed by a McLafferty-type of rearrangement (Scheme II) and has not been described previously in positive mode HE-CID of either phosphorylated carbohydrates or peptides. Ions at m/z 97 and 79, which correspond to H₂PO₄⁻ and PO₃⁻, respectively, have been described in the negative mode for lipo-oligosaccharides [22] and for phosphopeptides [23].

Determination of terminal and penultimate phosphorylation was complicated by the occurrence of two glycosidic bond cleavages at internal Man-6-PO₄ residues. This yielded a B₂Y₁ ion which is isobaric with the terminal Man-6-PO₄ B₁ ion. Therefore, this ion cannot be used to determine the location of the phosphate residue. In the case of the monophosphorylated dimannosyl derivatives, a penultimate Man-6-PO₄ residue was deduced from a strong Y₁ ion, which contained the phosphate group, and a B₁ ion from the terminal mannose residue. In the terminally phosphorylated compound the Y ion was absent and thus the interpretation of its spectrum was ambiguous without knowledge of the spectra of the other compounds. A B₂ and a Y₂ ion were used to locate the phosphorylated residue in monophosphorylated trimannosyl derivatives, where the phosphate group could be on any of three mannose residues. In the diphosphorylated dimannosyl compound the number of phosphate groups was furnished by the molecular ion and a B₂ ion. Localization of the phosphate groups to the carbohydrate moiety was given by a B₂ ion, which together with a Y₂ showed that each mannosyl residue was singly phosphorylated.

The origin of the ions at m/z 85 and m/z 109 is not known. The m/z 85 ion is present in spectra of both neutral and phosphorylated oligosaccharides. It is not derived from the aglycon because an ion at m/z 85 was also present in the HE-CID of Man α (1 $\rightarrow$ 3)Man β (1 $\rightarrow$ 4)GlcNAc derivatized with *p*-amino benzoic acid octyl ester (P. Lipniunas, unpublished data).

The only common structural feature among these compounds is the presence of mannosyl residues. In our laboratory, the ion at m/z 109 has not been observed in HE-CID spectra of derivatized or underivatized glycoconjugates other than the Man-6-PO₄-containing structures described in this study. Thus it is unlikely that the signal at m/z 109 is from the protonated molecular ion of thioglycerol, which is a part of the flow probe matrix solution. Furthermore, none of the selected Man-6-PO₄-containing oligosaccharide MH⁺ precursor ions corresponded to thioglycerol cluster MH⁺ ions (the thioglycerol cluster ions $n = 5-11$ would be m/z 541.2, 649.2, 575.2, 865.3, 973.3, 1081.3, and 1189.4). Also, the mass increment (either 162 mass units for an additional Hex residue or 80 mass units for an additional phosphate group) between the MH⁺ of the phosphorylated structures do not correspond to a thioglycerol unit.

In conclusion, our investigation demonstrates that positive mode HE-CID can be used to identify phosphorylated oligosaccharides by the presence of ions at m/z 243 (from the Man-6-PO₄ residues and its secondary fragments) and with P ions at m/z 99 (from elimination of a protonated phosphoric acid). Furthermore, positive mode HE-CID-MS can determine whether a Man-6-PO₄ residue is at the terminal or penultimate position by the presence or absence of ions from neutral terminal mannosyl residues (B ions) and those formed by cleavage at the glycosidic bond on the nonreducing side of the Man-6-PO₄ residue (Y ions). Because internal Man-6-PO₄ residues readily undergo double cleavage at glycosidic bonds, the phosphorylated B₁ ion at m/z 243 cannot be used to deduce terminal phosphorylation. Instead, branched oligosaccharides, which contain one or two terminal Man-6-PO₄ residues, showed double cleavages with charge retention on the phosphate-containing fragment. Double cleavages also were observed in neutral branched structures. These fragments were formed by cleavage of either both branches or one branch and the aglycon with charge retention on the fragment that carried the disubstituted mannosyl residue. The amount of sample needed to obtain spectra to distinguish between terminal and penultimate phosphorylation was shown to be in the range of a few picomoles.

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