Structural Characterization of Picket-fence Porphyrinatoiron with ω -Hydroxy-2,2-dimethylicosanoyl Side-chains *vs.* ω -Cholinephosphoryl-2,2-dimethylicosanoyl Side-chains by FAB High-energy-CID MS/MS

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Applicability of FAB high-energy-CID MS/MS to structural characterization of two types of tetraphenylporphyrin (TPP)-derivatives was examined. These tetraphenylporphyrin (TPP)-derivatives, 20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron and 20-cholinephosphoryl-2,2-dimethylicosanoyl picket-fence porphyrinatoiron have four 2,2-dimethylicosanamido long-chains in common in their structures, which keep a balance between hydrophobic and hydrophilic properties. The structural difference between the two compounds is that the former has a hydroxyl group at the remote end of the 2,2-dimethylicosanamido long-chain, while the latter has a phosphocholine ester functional group at the remote-end. This structural difference gives rise to different features as well as some analogies in their FAB and the high-energy-CID MS/MS spectra. Differences and analogies in those spectra were also noted between the long-chain porphyrin derivatives we are describing here and the pivaloyl-type porphyrins we reported previously [T. Shibue, H. Kambe, H. Nishide, S. Akashi, N. Dohmae, and Y. Ohashi, *Eur. Mass Spectrom.*, **3**, 291–299 (1997)]. By selecting a precursor ion at m/z 476, which corresponds to the 20-cholinephosphoryl-2,2-dimethylicosanoyl group, charge-remote fragmentation was observed. This observation reminded us our previous study on the imidazole-tailed picket-fence porphyrin fragmentations.

1. Introduction

Hemoglobin is a conjugated protein, for which the molecular weight is 64,450 and consists of four polypeptide chains. These chains have a compact conformation, and the porphyrinatoiron is embedded in the cavity of these polypeptide chains.

The hydrophobic character of the space inside of the heme pocket relates to the oxygen-binding function of hemoglobin and myoglobin.

Collman reported a picket-fence porphyrinatoiron: 5, 10, 15, 20-tetrakis-2 (pivalamido) phenylporphyrinatoiron,¹⁾ which has a protective enclosure for oxygen binding on one side of the porphyrin plane, whereas the other side is left unhindered so as to bind a base.

Tsuchida and co-workers utilized a phospholipid liposome instead of the globin protein.²⁾

Thus, 20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron: 5,10,15,20-tetrakis-2 (20-hydroxy-2,2-dimethylicosanamido) phenylporphyrinatoiron (1) and 20-cholinephosphoryl-2,2-dimethylicosanoyl picket-fence porphyrinatoiron: 5,10,15,20-tetrakis-2 [20-(2-trimethylammonioethyl)phosphonatoxy]-

2,2-dimethylicosanamido]phenylporphyrinatoiron (2) were synthesized (Scheme 1). These compounds showed proper balance between hydrophobic and hydrophilic properties in the liposome. Each of these two porphyrinatoirons has either four 20-hydroxy-2,2-dimethylicosanamide groups or four 20-cholinephosphoryl-2,2-dimethylicosanamide groups on the porphyrin plane. They are dissolved in the phospholipid liposome to implement the reversible oxygen binding in the aqueous solution.

Such techniques as ours to modify the porphyrin plane have become ever more sophisticated and the complexity of these structures has accordingly increased. Thus, the difficulty of their structural analyses also has increased.

Recently, we reported the structural characterization of picket-fence porphyrins and tailed picket-fence porphyrins by EI and FAB high-energy-CID MS/MS experiments, respectively. These experiments clearly showed the number of pivaloyl substituents which construct a picket-fence around the porphyrin ring system, and represented the detailed side-chain structure of the tail.^{3)–5)}

In this paper, we report the applicability of FAB ionization and high-energy-CID MS/MS to structural characterization of the 20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron and 20-cholinephosphoryl-2,2-dimethylicosanoyl picket-fence porphyrinatoiron. Each compound has a partially but definitely different functional group and showed quite different mass spectral features from each other as well as from similar spectra of previously studied compounds.

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2. Experimental

2.1 Materials

Porphyrin derivatives examined: 5, 10, 15, 20-tetrakis-2 (20-hydroxy-2,2-dimethylicosanamido) phenylporphyrinatoiron (1) (monoisotopic molecular mass 2081.48) and 5,10,15,20-tetrakis-2 [20-(2-trimethylammonioethylphosphonatoxy)-2,2-dimethylicosanamido] phenylporphyrinatoiron (2) (monoisotopic molecular mass 2741.71) were prepared according to the procedures of Tsuchida *et al.*,²⁾ and they are shown in Scheme 1. 3-Nitrobenzyl alcohol was purchased from Tokyo Kasei (Tokyo, Japan), and used without further purification.

2.2 Mass spectrometry

B/E constant linked-scan experiments were performed using a JMS-SX102A double-focusing reversed-geometry mass spectrometer (JEOL, Tokyo, Japan), while tandem mass spectrometry was carried out using a JMS-HX110/HX110 of *EBEB* geometry four sector tandem mass spectrometer equipped with an array detector (JEOL, Tokyo, Japan).

Fast atom bombardment ionization (FAB) was carried out using xenon as the primary beam with an

Scheme 1. Structures of compounds used for the study. 1: 5,10,15,20-tetrakis-2 (20-hydroxy-2,2-dimethylicosanoyl)phenylporphyrinatoiron (monoisotopic molecular mass 2081.48). (20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron) 2: 5,10,15,20-tetrakis-2 [20-(2-trimethylammonioethylphosphonatoxy)-2,2-dimethylicosanoyl]phenylporphyrinatoiron (monoisotopic molecular mass 2741.71). (20-Cholinephosphoryl-2,2-dimethylicosanoyl picket-fence porphyrinatoiron.)

Fig. 1. (+) FAB mass spectrum of 20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron using JMS-HX110/HX110 with an array detector.

energy of 6 keV in the positive-ion mode, and the ion acceleration voltage applied was 10 keV. Four sector tandem mass spectrometer (JMS-HX110/HX110) used 8 keV potential floated gas cell. One microliter of a sample solution in an appropriate solvent, such as methanol, was mixed with 3-nitrobenzyl alcohol, which was dispersed on a clean stainless-steel probe tip beforehand; the probe was fixed onto the sample holder of the direct inlet system.

Molecular-related ions were selected as precursor ions for CID *B/E* constant linked scan and high-energy-CID MS/MS studies. Helium was introduced as the collision gas until the intensity of the molecular-related ions was reduced to 50%.

3. Results and Discussion

3.1 20-Hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron

The positive-ion FAB mass spectrum of 20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron is shown in Fig. 1. Ions at *m/z* 2081.2 and 2082.2 correspond to the molecular ion $[M]^+$ and the protonated molecule $[M+1]^+$ with the natural abundance of ^{13}C of $[M]^+$, respectively. We already reported that a series of picket-fence type porphyrin compounds showed the oxidation product $[M]^+$, as well as the protonated molecule $[M+1]^+$.⁵⁾ Here, the doubly-charged ion $[M+2H]^{2+}$ was not observed in the spectrum. Other peaks at *m/z* 1743.2, 1404.8, 1066.6, and 729.2 were produced by successive eliminations of the 20-hydroxy-2,2-dimethylicosanoyl group from the molecular ion.

We studied its fragmentation features first by the *B/E*-constant linked-scan technique using a double-focusing reversed-geometry mass spectrometer (JMS-SX102A). Figure 2 shows a (+) FAB *B/E*-constant linked-scan spectrum of 20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron (E_{lab} : 10 keV, E_{com} : 19.2 eV).

The characteristic peaks of *m/z* 1743, 1405, 1067, and 729, which much resemble the ions observed by HX110/HX110 tandem mass spectrometry except for less accuracy, again, indicate that four side-chains are eliminated from the molecular-related ions.

We propose that simply by using the *B/E* constant

Fig. 2. (+) FAB *B/E*-constant linked-scan spectrum of $[M+1]^+$ of 20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron using a JMS-SX 102A.

linked-scan data, the size of side-chains can be determined with a successive mass difference starting from the incompletely resolved molecular-related ions and the following series of major product ions. The *B/E* constant linked-scan spectrum also shows the number of 20-hydroxy-2,2-dimethylicosanoyl groups on the porphyrin ring system.

We next compared the virtue of FAB high-energy-CID MS/MS using an *EBEB*-type four-sector tandem mass spectrometer (JMS-HX110/HX110) equipped with an array detector with the correspondent linked scan using a double-focusing reversed-geometry instrument (JMS-SX102A). *B/E* constant linked-scan was adequate for studying fragmentation, although resolution is not sufficient to separate molecular-related ions at *m/z* 2081.2 from 2082.2 and 2082.2 from 2083.2 in order to strictly select the precursor ion for CID-product ion scan studies. On the other hand, the four-sector instrument has a high resolution, which easily separates those ions from each other. The four-sector instrument with an array detector also provided high ion intensities in recording product ion spectrum. Figures 3 [A] and 3 [B] show partial spectra of (+) FAB *B/E*-constant linked-scan (E_{lab} : 10 keV, E_{com} : 19.2 eV) and (+) FAB high-energy-CID MS/MS (E_{lab} : 2 keV, E_{com} : 3.8 eV) of $[M+1]^+$ of 20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron. Comparison of Fig. 3[A] with Fig. 3[B] provides a clear difference between the two. It

Fig. 3. [A] Partial spectrum of (+) FAB *B/E*-constant linked-scan of $[M+1]^+$ of 20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron using a JMS-SX102A. [B] Partial spectrum of (+) FAB high-energy-CID MS/MS of $[M+1]^+$ of 20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron using a JMS-HX110/HX110 with an array detector.

is interesting that this spectrum evidently shows charge-remote product ions, representing the detailed structure of the side-chain.

3.2 20-Cholinephosphoryl-2,2-dimethylicosanoyl picket-fence porphyrinatoiron

The positive-ion FAB mass spectrum of 20-cholinephosphoryl-2,2-dimethylicosanoyl picket-fence porphyrinatoiron is shown in Fig. 4. Ions at *m/z* 2741.9 and 2743.0 correspond to the molecular ion $[M]^+$ and the protonated molecule $[M+1]^+$ including the natural abundance of ^{13}C of $[M]^+$, respectively.

We were interested in that ions around *m/z* 1371.9, corresponding to the doubly-charged ion $[M+2H]^{2+}$, were observed in this case as discussed later.

The spectrum also shows a characteristic fragment ion at *m/z* 476.4, which corresponds to the 20-cholinephosphoryl-2,2-dimethylicosanoyl group. In contrast to the case of 20-hydroxy-2,2-dimethylicosanoyl picket-fence porphyrinatoiron, most of the fragment ions were observed in the lower mass region. We studied its fragmentation feature by the FAB high-energy-CID MS/MS of $[M+1]^+$ using the JMS-HX110/HX110 instrument. Figure 5 shows a (+) FAB high-energy-CID MS/MS spectrum of $[M+1]^+$ of 20-cholinephosphoryl-2,2-dimethylicosanoyl picket-fence porphyrinatoiron (E_{lab} : 2 keV, E_{com} : 2.9 eV). The peaks of *m/z* 2683.6, 2626.3 and 2566.6 together indicate that three trimethylamine groups were eliminated from the $[M+1]^+$ (see Fig. 5). The branch trimethyl amino group then

Fig. 4. (+) FAB mass spectrum of 20-cholinephosphoryl-2,2-dimethylicosanoyl picket-fence porphyrinatoiron using a JMS-HX110/HX110 with an array detector.

Fig. 5. (+) FAB high-energy-CID MS/MS spectrum of $[M+1]^+$ of 20-cholinephosphoryl-2,2-dimethylicosanoyl picket-fence porphyrinatoiron using a JMS-HX110/HX110 with an array detector.

Fig. 6. (–) FAB *B/E*-constant linked-scan spectrum of a fragment ion m/z 476 of 20-cholinephosphoryl-2,2-dimethylcosanoyl picket-fence porphyrinatoiron using a JMS-SX102A.

Scheme 2. Plausible structures of fragment ions of m/z 476 of 20-cholinephosphoryl-2,2-dimethyl-icosanoyl picket-fence porphyrinatoiron appearing in the low mass range.

remains as the cation site. Otherwise, the FAB high-energy-CID MS/MS spectrum of $[M+1]^-$ of 20-cholinephosphoryl-2,2-dimethylcosanoyl picket-fence porphyrinatoiron did not show so much structural information as that of the corresponding 20-hydroxy analog spectra.

In addition to the difference in $E_{\alpha,ms}$ between the two compounds, consideration should be given to their chemical properties.

The structure **2** represents a quaternary ammonium, from which a CH_3^- elimination may occur, but a cleavage of a neutral methyl radical on the nitrogen is unlikely. Therefore, although protonation at the FAB ionization makes phosphate anion ($-\text{PO}_4^-$) to the neutral ($-\text{PO}_4\text{H}-$), the moiety ($\text{M} + \text{H} - 15$) is still neutral and not a positive ion. On the other hand, in $1 [\text{M} + \text{H} - \text{OH}]^+$ is actually a starting ion of charge-remote fragmentation.

Yet the FAB B/E -constant linked-scan spectrum of the above-stated fragment ion at m/z 476 (E_{lab} : 10 keV, E_{com} : 83.3 eV) clearly showed charge-remote product ions representing the detailed side-chain structure of the 20-cholinephosphoryl-2,2-dimethylcosanoyl group of the molecule. (Fig. 6 and Scheme 2) The quaternary ammonium cation forms a stable charge-site for the

charge-remote fragmentation.^{6i-10j}

In the case of doubly-charged ions of 20-choline-phosphoryl-2,2-dimethylcosanoyl picket-fence porphyrinatoiron, one of the two charges is held by one phosphocholine group, while the other may be located on another phosphocholine group. One more special feature showing the 2,2-dimethyl branching is that high-energy-CID MS/MS of m/z 476 has two multiplet ions, one around m/z 460 and another around m/z 449 before clear charge-remote fragmentation begins.

4. Conclusions

20-Hydroxy-2, 2-dimethylicosanoyl picket-fence porphyrinatoiron showed molecular ion $[M]^{++}$ as well as the protonated molecule $[M+1]^+$ in the positive ion FAB mode. The FAB high-energy-CID MS/MS spectrum, using the B/E -constant linked-scan technique, showed the size and the number of the 20-hydroxy-2,2-dimethylicosanoyl groups. This spectrum showed charge-remote product ions representing the detailed side-chain structure of the 20-hydroxy-2,2-dimethylicosanoyl group (E_{lab} : 10 keV, E_{cm} : 19.2 eV).

20-Cholinephosphoryl-2,2-dimethylcosanoyl picket-fence porphyrinatoiron showed molecular ion $[M]^{++}$ as well as the protonated molecule $[M+1]^+$ in the positive ion FAB mode. In addition, 20-cholinephosphoryl-2,2-dimethylcosanoyl picket-fence porphyrinatoiron showed doubly-charged ions in the positive ion mode, which do not appear in the case of the 20-hydroxy analog.

The FAB B/E -constant linked-scan spectrum of a fragment ion at m/z 476, corresponding to the 20-cholinephosphoryl-2,2-dimethylcosanoyl group in the molecule, clearly showed charge-remote product ions representing the detailed side-chain structure of the 20-cholinephosphoryl-2,2-dimethylcosanoyl picket-fence porphyrinatoiron (E_{lab} : 10 keV, E_{cent} : 83.3 eV).

This CID spectrum also proves the phosphocholine head group structure as shown in scheme 2.¹¹ In the case of cholinephosphoryl compound, information of the cholinephosphate structure is given by FAB *B/E*-constant linked-scan spectrum of the fragment ion of the side-chain, owing to the strong charge retained in the quaternary ammonium cation. FAB high-energy CID MS/MS using HX110/HX110 was not performed on the fragment ion *m/z* 476. On the other hand, as for compound **1**, the 20-hydroxy-2,2-dimethylicosanoyl side-chain behaves as the leaving group and, indeed, the porphyrin macrocycle acts as a stable charge center, a particular requirement for the observed charge-remote fragmentation. Thus, we have successfully shown (+) FAB mass spectral differences and analogies not only between 20-hydroxy-2,2-dimethyl-icosanoyl picket-fence porphyrinatoiron and the 20-cholinephosphoryl analog but also between the two types of picket-fence porphyrins shown here and previously reported pivaloyl tailed picket-fence porphyrins.

Since about 90% of new porphyrin derivatives have been synthesized from tetraphenylporphyrin (TPP), the importance of studies as stated here, applying MS/MS for structural characterization of functional groups around tetraphenylporphyrin (TPP)-based system, will increase even further in the future.

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