A Picomole-Scale Method for Rapid Peptide Sequencing Through Convenient and Efficient N-Terminal Phosphorylation and Electrospray Ionization Mass Spectrometry

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Free or resin-bound peptides were phosphorylated at their N-termini by reacting with dimethyl phosphite in the presence of tetrachloromethane and triethylamine, and some of them were labeled using partial deuterium-labeled dimethyl phosphites (molar ratio of $m + 6$ and $m = 1:1$ or $m + 6$, $m + 3$ and $m = 1:2:1$) as the phosphorylating agents. The monophosphorylation of the lysine-containing peptides selectively occurred on the amino group of the N-terminus, not the side-chain of lysine residue. The resin-bound phosphoramidate peptides were cleaved by TFA before ESI-MS. The modified peptides were determined by electrospray ionization mass spectrometry, the protonated molecules of the unlabeled phosphoramidate peptides showed the singlet peaks, and those of the labeled phosphoramidate peptides displayed the doublet and/or triplet peaks. Tandem mass spectra (MS/MS) of the chosen protonated molecules gave sequential loss of the amino acid residues from the C-termini of the peptides, providing a convenient and rapid method for peptide sequencing. (J Am Soc Mass Spectrom 2006, 17, 995–999) © 2006 American Society for Mass Spectrometry

Rapid and convenient peptide sequencing is highly desirable in proteomics and related bio-science. While the conventional method, Edman sequencing, does not fulfill this task, tandem mass spectrometry (MS/MS) provides a means for rapid identification and characterization of peptides/proteins [1–5]. Unfortunately, a protonated peptide is typically dissociated into a wide variety of N-terminal and C-terminal fragment ions. In general, it is difficult to predict which type of fragment ions will be formed for a single charged peptide, and the MS/MS spectra are often so complicated that de novo sequence determination is impossible. Many efforts have been made to enhance the response sensitivity and simplify mass spectra by modifying peptides. Positive [5–7] and negative [8] ion derivatization of peptides are two usual methods. In the previous research from our laboratory, it was found that the response sensitivity in positive ion FAB-MS [9] could be improved greatly by introducing a

neutral diisopropoxyphosphoryl or diethoxyphosphoryl group to N-termini of peptides. The dissociation efficiency seemed to be higher in this case; unfortunately, a variety of dissociation pathways of the phosphoryl group complicated the mass spectra and sequence interpretation due to successive loss of two alkenes. In this paper, various free or resin-bound peptides were phosphorylated into phosphoramidate peptides using unlabeled and labeled dimethyl phosphites as phosphorylating agents, and the modified peptides were analyzed by ESI-MS, and tandem mass spectra (ESI-MS/MS) of their protonated molecules showed unambiguously amino acid residue sequences of the original peptides.

Experimental

Materials

The Wang and Rink resin-bound protected peptides (~10 mg of peptides on 500 mg of resin) were purchased from Glsynthesis Company (Shanghai, China). They are H-Val-Glu(OtBu)-Gln(trt)-His(trt)-Wang Resin (VEQH, loading 0.6 mmol/g), H-Ala-Lys(Boc)-Asp(OtBu)-Leu-Wang Resin (AKDL, loading 0.6 mmol/g), H-Val-Tyr(tBu)-Lys(Boc)-Asp(OtBu)-His(trt)-Wang Resin (VYKDH, loading 0.56 mmol/g), H-Ala-Arg(Pbf)-Leu-

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Scheme 1. Three phosphorylating reagents and the synthetic route for phosphoramidate peptides.

Val-Glu(OtBu)-Wang resin (ARLVE, loading 0.62 mmol/g), H-Ala-Lys(Boc)-Asp(OtBu)-Trp(Boc)-Val-Rink Amide-AM Resin (AKDWV, loading 0.7 mmol/g), and H-Leu-Pro-Asp(OtBu)-Ser(tBu)-Phe-Ala-Rink Amide-AM Resin (LPDSFA, loading 0.7 mmol/g). Other chemicals were purchased from Aldrich (Shanghai, China).

Synthesis of the Phosphorylating Reagents

Dimethyl phosphite (DMP-H, 1), deuterium-labeling dimethyl phosphite (D_6 -DMP-D, 2), and partial deuterium-labeling dimethyl phosphite mixture (DMP132-X) (Scheme 1) were prepared according to the known procedure [10]. DMP12-X was obtained by mixing one equivalent each of dimethyl phosphite (DMP-H) and deuterium-labeling dimethyl phosphite (D_6 -DMP-D). DMP132-X was prepared as follows: the mixed solution of methanol (24 mmol) and deuterium-labeling methanol (24 mmol) in 5 mL of dry CH_2Cl_2 was added dropwise to PCl_3 (8 mmol) in 5 mL of dry CH_2Cl_2 at 0 °C under nitrogen atmosphere. The solution was warmed to room temperature and stirred for 30 min, and ^{31}P NMR spectroscopy showed that PCl_3 almost quantitatively transferred into DMP132-X. The crude sample was obtained after distillation of gaseous HCl and CH_2Cl_2 , and it was used without further purification (^1H NMR showed that the purity was more than 98%). ^{31}P NMR of the three phosphorylating reagents: ~12.5 ppm.

Two Methods for Synthesis of Phosphoramidate Peptides

Method A: 1 mg of peptide-bound resin (~2000 beads) was added to a 1.5 mL microcentrifuge tube, 100 μL of

trifluoroacetic acid (TFA) was added, and the peptide was cleaved from the Wang or Rink resin within 30 min. TFA was removed with N_2 , and 10 μL (72 μmol) of triethylamine, 20 μL (0.21 mmol) of tetrachloromethane, 960 μL of methanol and 10 μL (~90 μmol) of phosphorylating reagent were added to the microcentrifuge tube containing the peptide at 0 °C. The microcentrifuge tube was shaken for 2 h at this temperature, the tube was centrifuged, and 10 μL (~0.6 nmol/ μL) of the clear solution was drawn off and diluted with 990 μL of methanol containing 0.05% TFA, and 40 μL (~6 pmol/ μL , ~240 pmol) of the resulting mixture was directly used to analyze by ESI mass spectrometry.

Method B: 1 mg of bound-peptide resin was put to a 1.5 mL microcentrifuge tube, and then 100 μL (720 μmol) of Et_3N , 100 μL (1.04 mmol) of CCl_4 , 400 μL of methanol, 15 μL (~130 μmol) of phosphorylating reagent were added to the microcentrifuge tube at 0 °C. The tube was shaken for 4 h at this temperature, the solution was removed by filtration, and the peptide-bound resin was washed with methanol (3 $\times$ 0.5 mL). About 15 beads (~4.5 nmol of the modified peptide) were picked out and put to a 1.5 mL microcentrifuge tube, and 100 μL of TFA was added to the tube. After 30 min, TFA was removed with N_2 , the residue was dissolved in 1 mL of methanol containing 0.05% TFA, and 40 μL (~4.5 pmol/ μL , ~180 pmol) of the solution containing the phosphoramidate peptide was peptide was analyzed by ESI mass spectrometry.

Mass Spectrometric Conditions

Electrospray ionization mass spectra were acquired on a Bruker ESQUIRE-LC ion trap spectrometer (Billerica, MA) equipped with a gas nebulizer probe. All experiments were carried out in the positive ionization mode. Nitrogen was used as drying gas with flow rate 4 or 8 L/min. The nebulizer pressure was 7 or 11 psi. The applied voltage on the electrospray needle was typically held at 4 kV and the capillary temperature was maintained at 300 °C for all samples. Forty μL of the phosphoramidate peptide solution was injected into the ESI source at a flow rate of 4 $\mu\text{L}/\text{min}$. The number of overlap spectrum was 5. Selected ions were analyzed by multistage tandem mass spectrometry using helium as collision gas.

Results and Discussion

Preparation of Dimethyl Phosphite and the Phosphoramidate Peptides

Reaction of PCl_3 with three equivalents of CH_3OH in CH_2Cl_2 gave dimethyl phosphite (1, DMP-H), the corresponding deuterium-labeling dimethyl phosphite (2) was obtained when three equivalents of CD_3OD was used instead of CH_3OH in the reaction and a mixture of 1 and 2 (1:1) gave DMP12-X. Reaction of PCl_3 with 1.5 equivalents of CH_3OH and 1.5 equivalents of CD_3OD

Figure 1. (a) ESI-MS spectrum of DMP-VYKDH-OH. (b) ESI-MS² spectrum of the protonated molecule [M + H]⁺ at *m/z* 769 in Figure 1a. (c) ESI-MS³ spectrum of a₂-ion at *m/z* 343 in Figure 1b. (d) ESI-MS² spectrum of the protonated molecule [M + H]⁺ at *m/z* 725 of DMP-AKDWV-NH₂.

yielded DMP132-X (Scheme 1). Reaction of the three reagents with peptides gave the corresponding phosphoramidate peptides.

Phosphorylation of the Free Peptides and Peptide Sequencing

Reaction mixtures obtained by method A (see the Experimental section) were analyzed directly. For example, the primary ESI-MS spectrum (Figure 1a) of phosphoramidate peptide DMP-VYKDH-OH cleaved from Wang-resin shows the protonated molecule [M + H]⁺ at *m/z* 769, the protonated diphosphorylated product at *m/z* 877 was also observed due to the presence of a Lys residue. The protonated monophosphorylated peptide at *m/z* 769 was selected for MS/MS analysis (Figure 1b). The b_n-type ions (*m/z* 614, 499, 371) were observed together with a₂-ion at *m/z* 343. ESI-MS³ of the a₂-ion produced the b₁-ion at *m/z* 208 (Figure 1c), allowing the phosphoramidate peptide to be easily sequenced as DMP-VYKDH-OH. Interestingly, the intense fragment ions in Figure 1b all contain a phosphoryl group on the amino group of the N-terminus and

not the lysine side-chain, showing that monophosphorylation selectively occurred on the N-terminal amino group of the original peptide under the phosphorylating conditions described above. In fact, similar examples of selective N-terminal modification for peptides containing lysine residue have been found using S-pentafluorophenyl [tris(2,4,6-trimethoxyphenyl)phosphonium]acetate bromide [6] and 5-bromonicotinic acid N-hydroxysuccinimide ester [11] as the modifying reagents by Watson et al. and Tsunasawa and coworkers, respectively.

We also sequenced the phosphoramidate peptides containing a C-terminal amide group using a similar procedure. For example, the primary ESI-MS spectrum of DMP-AKDWV-NH₂ showed the protonated molecule [M + H]⁺ at *m/z* 725 and the protonated diphosphorylated peptide at *m/z* 833. ESI-MS² (Figure 1d) of the protonated molecule [M + H]⁺ at *m/z* 725 produced the b_n-type ions at *m/z* 609, 423, 308, and ESI-MS³ of b₂-ion at *m/z* 308 in Figure 1d yielded the b₁-ion at *m/z* 180. Therefore, the complete amino acid sequence of the phosphoramidate peptide was determined as DMP-AKDWV-NH₂. It was, however, difficult to identify quickly the protonated phosphoramidate peptides due to the presence of impurities in the spectra.

Labeling of Free Peptides via Phosphorylation and Peptide Sequencing

We overcame this problem with a labeling method involving phosphorylation of the free peptides with partial deuterium-labeled dimethyl phosphite (DMP12-X or DMP132-X) (Scheme 1). The protonated molecules of the monophosphorylated peptides showed doublet or triplet peaks due to isotopic labeling, and the diphosphorylated peptides on the amino groups of both the N-terminus and side-chain of Lys residue displayed multiple peaks. The non-peptide impurities showed singlet peaks because they lacked amino group and could not be phosphorylated. For example, in the

Figure 2. (a) ESI-MS spectrum of DMP12-AKDWV-NH₂. (b) ESI-MS² spectrum of the protonated molecule [M + H]⁺ at *m/z* 725 in Figure 2a.

spectrum of the phosphoramidate peptide DMP12-AK-DWV-NH₂, we could easily identify the double peaks at m/z 725, 731 in Figure 2a corresponding to the protonated molecules of DMP12-AKDWV-NH₂, ESI-MS² of the protonated molecule $[M + H]^+$ at m/z 725 gave b_4 (m/z 609), b_3 (m/z 423), and b_2 (m/z 308) ions (Figure 2b), and further fragmentation of the b_2 -ion yielded the b_1 -ion at m/z 180. The results gave clear sequence information for the phosphoramidate peptide.

We also applied DMP132-X to phosphorylate peptides at their N-termini. The protonated molecules of the monophosphorylated peptides showed triplet peaks, and the diphosphorylated products and impurity produced multiple and singlet peaks, respectively. In the case of a DMP132-AKDL-OH peptide, its primary ESI-MS spectrum (Figure 3a) shows triplet peaks corresponding to the protonated monophosphorylated molecules at m/z 554, 557, and 560 (abundance ratio $\sim 1:2:1$), multiple peaks near m/z 668 from diphosphorylated products, and some singlet peaks including m/z 426, 486, and 588 from impurities. The most intense triplet peak at m/z 557 yielded an ESI-MS² spectrum (Figure 3b) showing b_n -type ions at m/z 436 (b_3), 311 (b_2) and 183 (b_1), allowing straightforward assignment of the sequence.

Phosphorylation of Resin-Bound Peptides and Peptide Sequencing

The resin-bound peptides were also phosphorylated to give the corresponding phosphoramidate peptides using a similar procedure and then cleaved from the resin using TFA (Method B, in the Experimental section). Compared with the free peptides, the resin-bound peptides only provided monophosphorylated products because the ϵ -amino group on the lysine residue was protected. For example, the primary ESI-MS spectrum of DMP-VYKDH-OH showed the base peak at m/z 769 corresponding to the protonated molecule, and ESI-MS² and MS³ spectra (similar to Figure 1b and c) gave

Figure 3. (a) ESI-MS spectrum of DMP132-AKDL-OH. (b) ESI-MS² spectrum of the protonated molecule $[M + H]^+$ at m/z 557 in Figure 3a.

Figure 4. ESI-MS² spectrum of the protonated molecule $[M + H]^+$ at m/z 661 of VYKDH-OH

b_n -type ions at m/z 614 (b_4), 499 (b_3), 371 (b_2), and 208 (b_1), providing the sequence of the phosphoramidate peptide.

Labeling of Resin-Bound Peptides Via Phosphorylation and Peptide Sequencing

We used DMP12-X or DMP132-X to label the resin-bound peptides through N-phosphorylation. The modified peptides were cleaved by TFA and then also unambiguously sequenced using similar procedures to those described above.

Comparison of Unmodified Peptides and Phosphoramidate Peptides in ESI-MS

We compared ESI-MS spectra of the unmodified peptides and the phosphoramidate peptides. Figure 4 shows the ESI-MS² spectrum of the peptide VYKDH-OH. Compared with Figure 1b, Figure 4 exhibits a more complex spectrum, and only characteristic fragment ions b_4 and b_3 were observed. The N-terminal phosphorylating method selectively increased the intensities of b_n -type ions relative to other ion types, and the resulting simplified mass spectra clearly showed sequential loss of amino acid residues from the C-termini of peptides.

Conclusions

The peptides were phosphorylated into the corresponding phosphoramidate peptides, and the electrospray ionization tandem mass spectrometry of modified peptides provided clear sequence information on the original peptides. The protonated partial isotopically-labeled molecules could be quickly identified in their primary mass spectra, which could reduce the consumption of sample during mass spectrometric analysis and improve the veracity of peptide sequencing. The present method has the following advantages: (1) a highly efficient and simple derivatization procedure; (2) selective N-terminal modification during monophosphorylation of peptides containing Lys residues; (3) selective fragmentation on the amide bond to form series of b_n -type ions, so it could be widely applied for peptide sequencing.

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