A Chemically Designed Enzymatic Cleavage Site for Phosphoproteome Analysis

Jianhe Peng*[a]

Protein phosphorylation is one of the most frequent and important post-translational modifications of proteins. Approximately one third of all mammalian cellular proteins are phosphorylated. Several amino acid residues could be phosphorylated, including serine, threonine, tyrosine, histidine, arginine, lysine, cysteine, glutamic acid, and aspartic acid. However, the phosphorylation of the hydroxyl group of serine, threonine, and tyrosine is the most prevalent, with phosphorylated serine (pS) accounting for roughly 90% of these modifications, and phosphorylated threonine (pT) and tyrosine (pY) accounting for roughly 10% and 0.1% of the total, respectively. Reversible protein phosphorylation plays a very important role in the regulation of cellular events and processes, including signal transduction, gene expression, and cell growth, division, differentiation, and apoptosis.

The analysis strategy of a phosphoproteome is to break down the proteins into peptides and analyze the phosphopeptides.[1] To achieve high-throughput analysis of the phosphoproteome and increase the analysis sensitivity, enrichment of the phosphorylated proteins or their proteolytic phosphopeptides out of the nonphosphorylated proteins and peptides is necessary. Antibodies against pY and even antibodies against pS and pT could precipitate phosphoproteins.[2] Immobilized metal-affinity chromatography (IMAC) is a cheap choice and has improved much in recent years.[3] Special chemistries that target the phosphate group were also utilized. Carbodiimide-catalyzed condensation of cysteamine with a phosphate group formed phosphoramidate with a free sulfhydryl group that could be used for purification;[4] while β-elimination of pS/pT followed by Michael addition to generate a free sulfhydryl group was the base for several affinity enrichments and quantification.[5–8]

Mass spectrometry (MS) has evolved to be an indispensable tool for protein-phosphorylation analysis.[1,9,10] Tandem mass spectrometry (MS/MS) has proved to be very useful in highly sensitive detection and sequencing of phosphopeptides. Under low-energy collision-induced dissociation (CID), phosphopeptides are fragmented to give characteristic marker ions at *m/z* 216.043 (immonium ion of pY in the positive ion mode), and *m/z* 96.97 (HPO_3^-/z) and 78.96 (PO_3^-) (universal for all phosphorylated phosphopeptides in negative mode). pS/pT prefer to lose H_3PO_4 (β-elimination) and give a prominent fragment ion with a shift of $-97.97/z$ relative to the precursor ions, while pY loses HPO_3, with a $-79.97/z$ shift. All these characteristic marker ions and specific neutral losses are utilized in tandem mass spectrometry for high sensitivity detection of phosphopeptides. LC/MS/MS separation followed by nanospray electrospray ionization, ESI/MS/MS, sequencing is a very sensitive approach.[10]

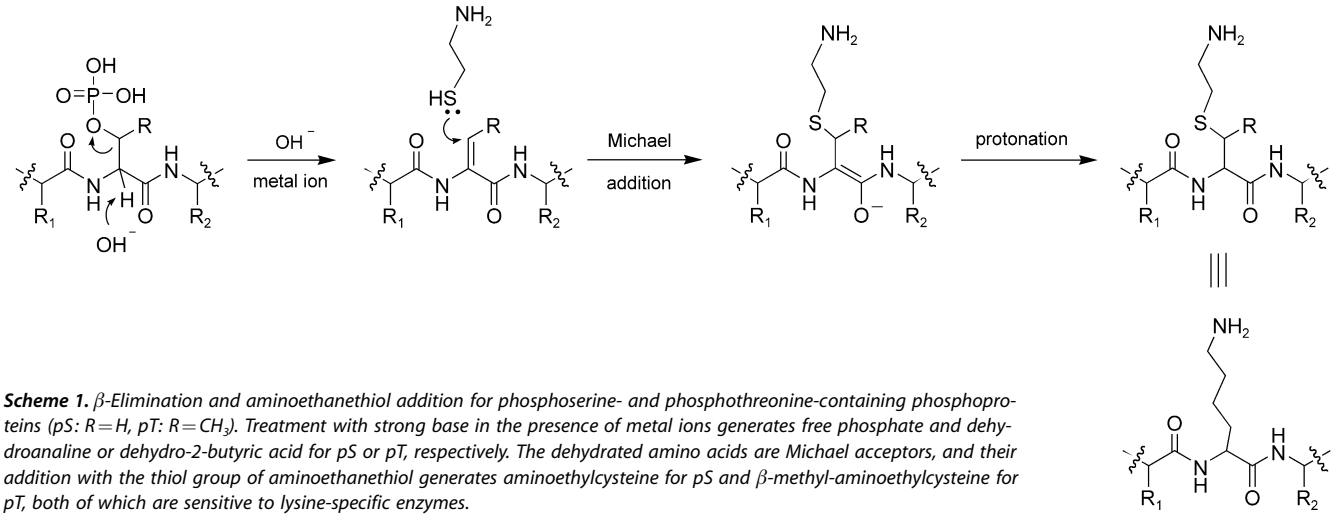
A combination of phosphoprotein-enrichment methods and mass spectrometry is the main stream of phosphoproteome analysis strategies.[1,9,10] Even so, only prominent phosphoproteins are found at the proteome level;[3–5] this indicates that additional fractionation steps will be needed for phosphoproteins in low abundance.

One important point in the phosphoproteome project is the coverage of phosphorylation sites. Phosphopeptides cannot be detected by MS if they are too small or too big. To partially solve this problem, Schlosser et al. used the less specific protease elastase to generate overlapping peptides with a suitable size (500–1500 Da) for MS analysis.[12,13] Furthermore, IMAC was used for enrichment of phosphopeptides. This approach was shown to have increased coverage of phosphorylated sites in protein kinase A, and found three new phosphorylation sites in human transcription-initiation factor TIF-IA. Meanwhile, Han et al. have reported a dual-enzyme digestion method.[14] After trypsin digestion, the bigger tryptic phosphopeptides were further digested by protease K to get a suitable size for MS analysis.

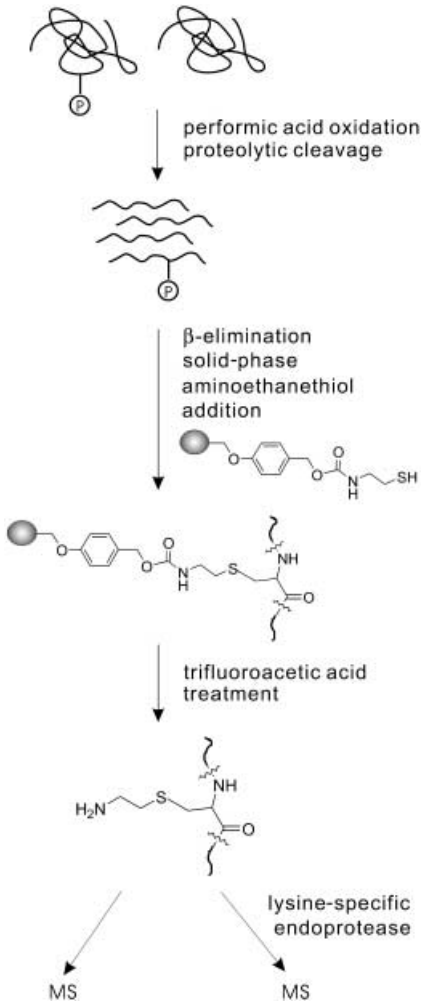
The method published by Rusnak et al. and Knight et al. chemically modified pS/pT to be enzyme sensitive.[15,16] For the first time, pS/pT sites were enzymatically and even exclusively targeted. According to this method, pS and pT were turned into aminoethylcysteine and β-methyl-aminoethylcysteine, respectively, by β elimination followed by Michael addition with 2-aminoethanethiol (Scheme 1). Aminoethylcysteine is isosteric with lysine, and is susceptible to lysine-specific endoproteases.[17] Surprisingly β-(methyl)aminoethylcysteine could also be a substrate for lysine-specific enzymes. Knight et al. revealed that the diastereomeric products of the Michael addition showed different enzymatic susceptibility: only the derivatized peptides with *R* stereoconfiguration at C_β were substrates for lysine-specific enzymes.[16] This gives approximately 50% enzymatic cleavage at the aminoethylcysteine sites and generates a ladder of peptides corresponding to the successive partial cleavage at pS/pT sites. By modifying lysine into homoarginine, exclusive cleavage at the pS/pT sites was achieved. A solid-phase reaction that gave aminoethylcysteine made pS/pT phosphopeptide enrichment possible (Scheme 2). In MS/MS analysis, the y1 ion, *m/z* 165.1,

[a] *Dr. J.-h. Peng*
Department of Cellular Biochemistry
Max-Planck-Institute of Biophysical Chemistry
Am Fassberg 11, 37077 Göttingen (Germany)
Fax: (+49) 551-201-1009
E-mail: jpeng@gwdg.de

  DOI: 10.1002/cbic.200400016



Scheme 1. *β-Elimination and aminoethanethiol addition for phosphoserine- and phosphothreonine-containing phosphoproteins (pS: R = H, pT: R = CH_3). Treatment with strong base in the presence of metal ions generates free phosphate and dehydroanaline or dehydro-2-butyric acid for pS or pT, respectively. The dehydrated amino acids are Michael acceptors, and their addition with the thiol group of aminoethanethiol generates aminoethylcysteine for pS and β-methyl-aminoethylcysteine for pT, both of which are sensitive to lysine-specific enzymes.*



Scheme 2. *Exploiting the solid-phase aminoethanethiol reaction for phosphoproteome analysis. After oxidation of cysteine and methionine, pS/pT-containing peptides undergo β-elimination and are captured by solid-phase aminoethanethiol addition. The eluate can be analyzed directly by mass spectroscopy; or bigger peptides could be first digested by lysine-specific enzyme and then analyzed by mass spectroscopy again.*

was unique for high-sensitivity precursor ion scans.

Several phosphorylated proteins have been analyzed by this method. β-Casein serves as a model sample for phosphoprotein analysis. All the phosphorylated sites of β-casein have been mapped, and most of the phosphopeptides have been detected by both matrix-assisted-laser-desorption/ionization (MALDI) MS and LC/ESI/MS/MS. The adjacent multiply phosphorylated site of β-casein was digested into overlapping peptides; this showed the usefulness of the method for this kind of domain phosphorylation analysis. Several other approaches did not show such a capacity.[4,5] The remove of the phosphate group improved the ionization efficiency for MS analysis.

However MaLachlin et al. studied one side reaction in β-elimination quantitatively: under the conditions employed, artifactual elimination of water from unmodified serine and threonine could be as high as 1.7%.[6] The mechanism of β-elimination is not very clear, especially the role of metal ions. Proposals were given to minimize this side reaction. In addition, O-linked oligosaccharides give false-positive results. However O-linked glycopeptides could be separated away by IMAC.[18]

The benefits of using this aminoethylcysteine approach are significant. An aminoethylcysteine, which can be on the C terminus, for example in the case of trypsin and lysine C, reduces the phosphopeptide's size to one that is more suitable for MS analysis. Phosphorylated sites can be indirectly confirmed by adjacent downstream peptides, and the marker fragment ion y1 can be used for highly sensitive detection with a precursor ion scan in LC/MS/MS analysis. Targeting cleavage of pS/pT and generating overlapping peptides, will be useful for dissection of multiple phosphorylated domains. Furthermore, solid-phase aminoethylcysteine reactions could be used for high-throughput phosphoproteome enrichment (Scheme 2). By stable isotope labeling of aminoethanethiol, it is possible to quantify pS/pT.

Keywords: elimination · lysine homologues · mass spectrometry · phosphorylation · proteins

[1] M. Mann, S. E. Ong, M. Gronborg, H Steen, O. N. Jensen, A. Pandey, *Trends Biotechnol.* **2002**, *20*, 261.
[2] M. Gronborg, T. Z. Kristiansen, A. Stensballe, J. S. Andersen, O. Ohara, M. Mann, O. N. Jensen, A. Pandey, *Mol. Cell. Proteomics* **2002**, *1*, 517.
[3] S. B. Ficarro, M. L. McCleland, P. T. Stukenberg, D. J. Bure, M. M. Ross, J. S. Shabanowitz, D. F. Hunt, F. M. White, *Nat. Biotechnol.* **2002**, *20*, 301.
[4] H. Zhou, J. D. Watts, R. Aebersold, *Nat. Biotechnol.* **2001**, *19*, 375.
[5] Y. Oda, T. Nagasu, B. T. Chait, *Nat. Biotechnol.* **2001**, *19*, 379.
[6] D. T. McLachlin, B. T. Chait, *Anal. Chem.* **2003**, *75*, 6826.
[7] F. Thaler, B. Valsasina, R. Baldi, J. Xie, A. Stewart, A. Isacchi, H. M. Kalisz, L. Rusconi, *Anal. Bioanal. Chem.* **2003**, *376*, 366.
[8] W. J. Qian, M. B. Goshe, D. G. Camp II, L. R. Yu, K. Tang, R. D. Smith, *Anal. Chem.* **2003**, *75*, 5441.

[9] T. Koecher, G. Allmaier, M. Wilm, *J. Mass Spectrom.* **2003**, *38*, 131.
[10] D. Arnott, M. A. Gawinowicz, R. A. Grant, T. A. Neubert, L. C. Packman, R. D. Speicher, K. Stone, C. W. Turck, *J. Biomol. Tech.* **2003**, *14*, 205.
[11] F. Zappacosta, M. J. Huddleston, R. L. Karcher, V. I. Gelfand, S. A. Carr, R. S. Ann, *Anal. Chem.* **2002**, 74, 3221.
[12] A. Schlosser, R. Pipkorn, D. Bossemeyer, W. D. Lehmann, *Anal. Chem.* **2001**, *73*, 170.
[13] A. Schlosser, J. Bodem, D. Bossemeyer, I. Grummt, W. D. Lehmann, *Proteomics* **2002**, *2*, 911.
[14] J. Han, M. Pope, C. Borchers, L. M. Graves, *Anal. Biochem.* **2002**, *310*, 215.
[15] F. Rusnak, J. Zhou, G. M. Hatbaway, *J. Biomol. Tech.* **2002**, *13*, 228.
[16] Z. A. Knight, B. Schilling, R. H. Row, D. M. Kenski, B. W. Gibson, K. M. Shokat, *Nat. Biotechnol.* **2003**, *21*, 1047.
[17] T. Masaki, T. Takiya, S. Tsunasawa, S. Kuwahara, F. Sakiyama, M. Soehma, *Biosci. Biotechnol. Biochem.* **1994**, *58*, 215.
[18] A. J. Thompson, S. R. Hart, C. Franz, K. Bamouin, A. Ridley, R. Cramer, *Anal. Chem.* **2003**, *75*, 3232.

Received: January 20, 2004

