Selective Enrichment of Thiophosphorylated Polypeptides as a Tool for the Analysis of Protein Phosphorylation*

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A chemoselective alkylation method is described for the isolation and subsequent identification of thiophosphorylated peptides/proteins. The method involves thio phosphorylation of proteins using adenosine 5’-O-(thiotriphosphate) (ATP S) followed by selective in situ alkylation of the newly thiophosphorylated proteins resulting in a stable covalent bond. The chemoselective alkylation exploits the relatively high nucleophilicity of the sulfur in thiophosphate residues, whereas the nucleophilicities of phosphates, amines, and other functionality of amino acids are negligible or significantly suppressed. Modified alkylation reagents linked to biotin or solid supports (e.g. glass or Sepharose beads) with or without a photocleavable linker facilitate the isolation of the thiophosphorylated peptide/proteins. This approach is demonstrated through the localization of phosphorylation sites on myosin regulatory light chain. We anticipate that this technique will be useful for isolation and subsequent identification of newly thiophosphorylated proteins, produced either in vivo or in vitro, thus facilitating the dissection of protein phosphorylation networks. Molecular & Cellular Proteomics 2:242–247, 2003.

Protein phosphorylation can profoundly affect the function of proteins and is often associated with cellular regulation, inter alia, such as transcription, replication, apoptosis, and signal transduction (1). Analysis of the human genome reveals that ~2% of human genes code for protein kinases or protein phosphatases (2). While many kinases are known to be involved in various cellular functions and disease progression, their substrates and phosphorylation sites remain largely unknown. Since characterization of protein phosphorylation networks requires a detailed knowledge of kinase/phosphatase substrates and their specificities as well as localization of phosphorylation sites, efficient techniques for the isolation of newly phosphorylated peptides/proteins would expedite progress in these areas.

A variety of methods have been described for the isolation of phosphate-containing peptides/proteins, including metal ion affinity purification (3), tagging phosphorylated residues through chemical reactions (4, 5), and affinity purification by immobilized anti-phosphoryl-residue antibodies (6). Many of the preceding methods have been used for the identification of protein phosphorylation sites, for the isolation of phosphorylated signal proteins, and for global surveys of protein phosphorylations (3–6). While powerful, these methods are not able to distinguish newly phosphorylated proteins from steady-state phosphorylated proteins. Since one-third of proteins in a cell are estimated to be phosphorylated (7, 8), isolation of newly phosphorylated proteins from unphosphorylated and/or steady-state phosphorylated proteins would simplify the identification of newly phosphorylated proteins responding to diverse signal stimulations.

Herein we report a novel method for the isolation of thiophosphorylated peptides/proteins. The method involves phosphorylation of proteins using ATP S and the selective in situ alkylation of the resultant thiophosphorylated proteins resulting in a stable covalent bond. The alkylation reaction exploits the relatively high nucleophilicity of the phosphoryl sulfur at low pH, whereas the nucleophilicity of phosphates, amines, and other functionality of amino acid residues are negligible or significantly suppressed. The thiophosphate-specific alkylating reagent can be linked to biotin or solid support (e.g. glass or Sepharose beads) with or without a photocleavable linker to facilitate convenient, high yield isolation of phosphorylated peptide/proteins. This approach was demonstrated through the localization of phosphorylation sites in myosin regulatory light chain. In addition, the foregoing technique can be refined and extended to the study of protein phosphorylation networks.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were obtained from commercial companies and used directly. Casein kinase I, casein kinase I phosphopeptide substrate (CKI peptide), and casein kinase I buffer were

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1 The abbreviations used are: CKI, casein kinase I; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MS/MS, mass spectrometry/mass spectrometry; RLC, smooth muscle myosin regulatory light chain; TOF, time of flight; ATP S, adenosine 5’-O-(thiotriphosphate); MOPS, 4-morpholinopropanesulfonic acid; PEO iodoacetate, (−)-iodoacetamide-3,6-dioxoactanediimine; GTP S, guanosine 5’-3-O-(thiotriphosphate).

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from New England BioLabs (Beverly, MA); synthetic peptide (KKFFCAIS, C-peptide) was from BioSynthesis Co. (Dallas, TX); ATP, bovine serum albumin (BSA), potassium chloride, sodium chloride, n-octyl β-D-glucopyranoside, L-cysteine, α-cyano-4-hydroxy-cinnamic acid, and dithiothreitol were from Sigma; ammonium bicarbonate, monobasic sodium phosphate, and dibasic sodium phosphate were from Fisher Scientific; (+)-bromothymol blue-iodoacetamide-3,6-dioxo-octanediamine (PEO iodoacetyl biotin), ImmunoPure ω-biotin, and ImmunoPure immobilized monomeric avidin were from Pierce; trifluoroacetic acid was from Fluka (Buchs, Switzerland); acetic acid was from Aldrich; and HPLC grade solvents were from EM Science (Gibbstown, NJ). Smooth muscle myosin regulatory light chain and myosin light chain kinase were kindly provided by Drs. Kris Kamm and Gang Zhi, Department of Physiology, University of Texas Southwestern Medical Center at Dallas.

**Thiophosphorylation**—The thiophosphorylation reaction for CKI substrate peptide (KRRRALSVASLPG, ROH (where R represents phosphoseryl serine) was carried out in 50 μl of CKI reaction buffer containing 200 μM CKI phosphopeptide substrate, 1000 units of CKI, and 4 mM ATP·S at 30 °C for 5 h. Both CKI and CKI reaction buffers were obtained from New England BioLabs. For kinase reactions for smooth muscle myosin regulatory light chain and myosin light chain kinase were kindly provided by Drs. Kris Kamm and Gang Zhi, Department of Physiology, University of Texas Southwestern Medical Center at Dallas.

**Protein in-solution Digestion**—The protein was dissolved in 50 mM NH₄HCO₃ (pH 8.0) and reduced with 5 mM dithiothreitol at 37 °C for 2 h. The resulting solution was heated at 95 °C for 5 min, cooled at 4 °C for 5 min, and then subjected to prolyteolytic digestion with sequence grade modified trypsin (Promega Co., Madison, WI) at an enzyme to substrate ratio of 1:50 at 37 °C overnight.

**Biotinylation of Thiophosphorylated Peptides and Affinity Purification of Biotinylated Peptides**—One hundred picomoles of PEO iodoacetyl biotin and 30 pmol of peptides (or protein tryptic digest) in 50 μl of reaction buffer at pH 3.5 (0.1 M sodium acetate buffer) or pH 7.0 (0.1 M potassium phosphate buffer) were incubated at room temperature in the dark for a suitable amount of time. The reaction was quenched by adding 100 mM l-cysteine. The buffer pH was adjusted to pH 7.0 by adding 0.5 M phosphate buffer (pH 7.4) when the alkylation reaction was performed at pH 3.5. To the resulting solution was added 3 μl of 5% n-octyl β-D-glucopyranoside and 3 μl of agarose beads immobilized with monomeric avidin, and the mixture was incubated at 4 °C for 30 min. The suspension was collected by centrifugation at 15,000 × g for 3 min, and the supernatant containing unbound peptides was removed. The beads were washed twice with 200 μl of washing buffer A (100 mM KCl, 0.5% n-octyl β-D-glucopyranoside, and 50 mM potassium phosphate buffer, pH 7.4) and briefly with 200 μl of washing buffer B (2 mM NH₄HCO₃). The biotinylated peptides on avidin beads were either eluted by 10 μl of 10 mM D-biotin phosphate-buffered saline (pH 7.4) solution or used directly for MALDI-TOF mass spectrometric analysis.

**Mass Analysis by MALDI-TOF Mass Spectrometry**—One microliter of the avidin beads containing biotinylated peptides was mixed with 0.5 μl of acetonitrile and 1 μl of saturated matrix solution (α-cyano-4-hydroxy-cinnamic acid in water/acetonitrile/trifluoroacetic acid (68: 33.1: 0.8, v/v/v)). The sample was transferred to a MALDI sample plate (Applied Biosystems, Foster City, CA) and dried. Mass spectra were measured on a Voyager DE MALDI-TOF mass spectrometer system (Applied Biosystems) in the positive polarity mode.

**Capillary HPLC/MS/MS Mass Spectrometry**—HPLC/MS/MS analysis were performed in an LCQ DECA ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a nanoelectrospray ionization source. The electrospray source was coupled on line with a Waters CapLC system (Waters, Milford, MA) with MAGIC Variable splitter (Michrom BioResource, Pleasanton, CA). Two microliters of the peptide solution in buffer A (5% acetonitrile, 94.9% water, 0.1% acetic acid (v/v/v)) were manually injected and separated in a capillary HPLC column (50-mm length × 75-μm inner diameter, 5-μm particle size, 300-Å pore diameter) packed in-house with Luna C18 resin (Phenomenex, St. Torrance, CA). The peptides were eluted from the column with a gradient (25–80% buffer B (90% acetonitrile, 9.9% water, 0.1% acetic acid (v/v/v)) in buffer A over 10 min at a flow rate of about 1 μl/min; the eluted peptides were electrosprayed directly into the LCQ mass spectrometer. The MS/MS spectra were acquired in a data-dependent mode, which determined the masses of the parent ions and fragments of the strongest ion.

**RESULTS**

**A Strategy for Chemoselective Tagging of Thiophosphorylated Residues**—Phosphorothioic acid has three apparent proton dissociation constants: pK₁ < 2; pK₂ = 5.75; pK₃ = 10.4 (9). We reasoned that pK₁ for the phosphorothioic acid in the thiophosphorylated residue is similar to that of the free phosphorothioic acid, i.e. ≤3.0. Therefore, when the solution pH is adjusted to below 4.0, a significant proportion of the phosphorothioic acid on thiophosphoresidues still exists in ionized form, while the side chains of all the basic amino residues (e.g. Arg and Lys) and the amine at the N terminus are protonated, while that of cysteine residue is neutralized. The protonated N-terminal amine and protonated side chains of basic residues are non-nucleophilic. Neutral cysteine thiol still retain some nucleophilicity; their reactivity is significantly lower than that of the anionic sulfur on thiophosphorylated residues. These differences allow chemoselective alkylation of thiophosphorylated residues in the presence of other amino acid residues in natural state (Fig. 1).

**Chemoselective Alkylation of Thiophosphorylated Residues in Low pH Buffer**—To test the feasibility of chemoselective alkylation of thiophosphorylated peptides and subsequent enrichment, two model peptides were alkylated: the first peptide (KKFFCAIS, or C-peptide) containing cysteine and lysine...
residues and the second one (the thiophosphorylated CKI peptide, KRRRAL(pS)VA(p-SS)LPGL, or T-peptide) including thiophosphorylated serine (p-SS), phosphorylated serine (pS), arginine, and lysine residues. The T-peptide was produced by thiophosphorylation of the CKI peptide in the presence of ATP/H9253.

Iodoacetamide was used as the alkylation agent to survey the effect of pH on selectivity. At pH 7.0, both peptides were 100% alkylated after 1 h of alkylation reaction (Fig. 2, C and D). The alkylated peptide has a mass increase of 57 Da. However, when the pH was decreased to 3.5, a negligible amount of C-peptide was alkylated, while 70% of the T-peptide was modified (Fig. 2). MS/MS experiments showed that the alkylation reaction occurred at the sulfur atom in the cysteine residue for C-peptide or in the thiophosphorylated residue for T-peptide (data not shown). These results confirm that at low pH, the alkylation favors the thiophosphorylated residue.

**Affinity Enrichment of Biotinylated Peptides**—Next we used PEO iodoacetyl biotin to alkylate and simultaneously tag the thiophosphorylated peptide. PEO iodoacetyl biotin contains an iodoacetamidyl moiety that has an electrophilic property similar to that of iodoacetamide. Alkylation using this reagent linked a water-soluble biotin to the thiophosphorylated residue, allowing affinity enrichment by solid beads containing immobilized monomeric avidin. Non-alkylated peptides present in solution were washed away. The enriched biotinylated peptides were mixed with d-cyano-4-hydroxycinnamic acid matrix solution and loaded onto the sample plate for MALDI-TOF mass spectrometric analysis. Alternatively the enriched peptides were eluted by 10 mM d-biotin phosphate-buffered saline solution, and the masses of the eluted peptides were determined.

When the alkylation reaction was carried out at pH 7.0, both the C-peptide and T-peptide (Fig. 3A) were biotinylated and subsequently affinity-enriched (Fig. 3B). On the other hand, when the reaction was carried out at pH 3.5, only the T-peptide was biotinylated and affinity-enriched afterward, while the C-peptide was not (Fig. 3C). Thus, chemoselective biotinylation of the thiophosphorylated peptide allows selective enrichment of the thiophosphorylated peptides.

**Enrichment of the Thiophosphorylated Peptide in a Complicated Peptide Mixture**—To further demonstrate the utility of
the approach for the isolation of thiophosphorylated peptides, we mixed the T-peptide with BSA tryptic digest. BSA was selected due to its high number of cysteine residues (total, 25), which have low nucleophilicity and might potentially interfere with the chemoselective reaction. We mixed 30 pmol of T-peptide with 30 pmol of BSA tryptic peptides (Fig. 4A), and the resulting peptide mixture was subjected to biotinylation reaction at pH 3.5 for 1 h. Only thiophosphoresidue containing C-peptide (MH, 2116) was alkylated and subsequently enriched.

Mapping the Phosphorylation Site in Myosin Light Chain—Next we tested the utility of this technique for mapping protein phosphorylation sites. Bacteria-expressed RLC was thio-phosphorylated by in vitro kinase reaction in the presence of ATPγS and myosin light chain kinase. Proteolytic peptides of RLC were produced by digestion of the protein with trypsin (Fig. 5A). Alkylation and subsequent affinity enrichment resulted in the isolation of a biotinylated peptide with a mass of 2603 (m/z, Fig. 5B), which matched the mass of a monobioti-
nylated peptide (ATSNVFAMFDQSIQEFK) from residue 18 to residue 35 of RLC. The non-alkylated thiophosphopeptide instead of biotinylated thiophosphopeptide was used to perform HPLC/MS/MS analysis for the convenience of spectrum interpretation. Identification of unphosphorylated \( y_{13} \) and thiophosphorylated \( b_7 \) suggested the thiophosphorylation occurred at either threonine 19 or serine residue 20 (residue numbers in RLC sequence, Fig. 5C). Observation of \( b_{16} \) with a mass of \( b_{16} - 114 \) (\( \text{H}_3\text{PO}_3\text{S} \)) suggested the thiophosphorylation occurred at serine 20 (17). Precursor ion scanning quadrupole TOF mass spectrometric analysis of the tryptic digest detected only one monothiophosphorylated peptide (residues 18–35, data not shown). Our data are consistent with the previous report that showed that serine 20 in RLC was phosphorylated by myosin light chain kinase (18, 19).

**DISCUSSION**

There are a few salient features in the described method for the isolation of thiophosphorylated proteins. Our technique is able to distinguish newly thiophosphorylated proteins versus steady-state phosphorylated proteins. While isolation of thiophosphorylated peptides was described in this work, our method can be extended to the isolation of thiophosphorylated proteins and subsequent identification of the proteins. It is estimated that about one-third of proteins in the cell are phosphorylated (7, 8). Isolation of newly phosphorylated proteins from the steady-state phosphorylated proteins and other non-phosphorylated proteins in cells will dramatically reduce the complexity of the protein mixture, making subsequent protein identification and proteomic analysis much simpler. In addition, the technique is reasonably unbiased. The isolation of thiophosphorylated proteins depends on the alkylation reaction between thiophosphorylated residues and an alkylation reagent. As long as the thiophosphate group is exposed to aqueous solution, it can be alkylated. Due to their highly hydrophilic and anionic nature, the phosphorylated residues are usually exposed outside of proteins and therefore accessible for alkylation.

The described technique also provides a convenient method for mapping protein phosphorylation sites. Localization of protein phosphorylation sites is usually carried out in a mass spectrometry laboratory and requires extensive skills in analytical chemistry. The described method allows the isolation of thiophosphorylated peptides even if only a small percentage of the proteins are thiophosphorylated. Enrichment of thiophosphopeptides will make subsequent mass spectrometric analysis and data interpretation much simpler. In addition, the inconvenience of \(^{32}\text{P} \) radioisotope is also avoided.

Due to its irreversible nature, the thiophosphorylation has been widely used in biology to study protein phosphorylation when the phosphorylated state is required to be maintained. Since the substrate-binding pocket is separated from the ATP-binding pocket in kinases, a subtle structural change in ATP is unlikely to change the substrate specificities of most kinases. Indeed ATP analogs other than ATP have been used to study protein phosphorylation and identify kinase substrates (10). We realized that some kinases might use ATP less efficiently than ATP. However, previous works showed that slight modification of the conditions for in vitro kinase reactions (e.g. adding metal ions) could enhance phosphorylation efficiency (11).
Our technique can potentially be used to isolate in vivo thiophosphorylated proteins. The availability of such a technique permits the isolation of newly phosphorylated proteins responding to changes in cellular environments. The isolated proteins, arising under diverse signal challenges, will be compared by a proteomic approach and further identified by mass spectrometry. Indeed protein thiophosphorylation was demonstrated in live cells by Alffrey and his colleagues (12, 13). They showed that after HeLa S-3 cells were labeled with sodium [35S]thio-orthophosphate for 3 h ATPγS, GTPγS, and thiophosphorylated histone proteins were produced. While these experiments await further refinement for practical applications, the results suggest the endogenous enzymatic system is able to use thio-orthophosphate to produce ATPγS and subsequently perform thiophosphorylation in vivo. Alternatively ATPγS can be introduced directly into cells that are permeabilized by chemicals (14–16). Many signal transduction systems remain functional after the cells are treated with cell permeabilization agents (15, 16).

In summary, we developed a method for the isolation of newly thiophosphorylated peptides/proteins. The method provides a convenient tool for mapping protein phosphorylation sites as long as a minor percentage of the proteins are thiophosphorylated. The technique can potentially be applied to the isolation of newly thiophosphorylated proteins, produced either in vivo or in vitro, thus facilitating studies of protein phosphorylation network.

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