[27] Identification of Histidine Phosphorylations in Proteins Using Mass Spectrometry and Affinity-Based Techniques

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Abstract

Histidine phosphorylation plays a key role in prokaryotic signaling and accounts for approximately 6% of the protein phosphorylation events in eukaryotics. Phosphohistidines generally act as intermediates in the transfer of phosphate groups from donor to acceptor molecules. Examples include the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS) and the histidine kinases found in two-component signal transduction pathways. The latter are utilized by bacteria and plants to sense and adapt to changing environmental conditions. Despite the importance of histidine phosphorylation in two-component signaling systems, relatively few proteins have so far been identified as containing phosphorylated histidine residues. This is largely due to the instability of phosphohistidines, which, unlike the phosphoesters formed by serine, threonine, and tyrosine, are labile and susceptible to acid hydrolysis. Nevertheless, it is possible to preserve and identify phosphorylated histidine residues in target proteins using appropriate sample preparation, affinity purification, and mass spectrometric techniques. This chapter provides a brief overview of such techniques, describes their use in confirming histidine phosphorylation of a known PTS protein (HPr), and suggests how this approach might be adapted for large-scale identification of histidine-phosphorylated proteins in two-component systems.

Introduction

Reversible phosphorylation is one of the most common and most important mechanisms by which the structure and function of a protein can become post-translationally modified. It is estimated that up to 30% of all proteins may be phosphorylated at any given time (Cohen, 2000). Site-specific phosphorylation of proteins affects localization, turnover, and enzymatic activity, as well as interactions with other proteins and DNA (Patel and Gelfand, 1996; Wolschin *et al.*, 2005). Phosphorylation occurs on the side chains of certain amino acid residues. The resulting phosphoamino acids fall into three categories: O-phosphates, which are formed by serine (Ser), threonine (Thr), and tyrosine (Tyr) and contain phosphoester linkages; N-phosphates formed by

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histidine (His), lysine (Lys), and arginine (Arg), which contain phosphoamidate bonds; and the acyl-phosphate formed by aspartic acid (Asp). The addition and removal of phosphate groups are usually mediated by specific classes of enzymes, such as histidine kinases (phosphorylation of His) and serine/threonine phosphatases (dephosphorylation of Ser and Thr). Phosphohistidines, which are the least stable of the phosphoamino acids, may or may not require histidine phosphatases, depending on the protein and, in particular, the residues adjacent to phosphohistidine (Klumpp and Krieglstein, 2002).

In terms of physiological function, there are two main classes of protein phosphorylation. The first of these encompasses phosphorylation for the purpose of regulating enzymatic activity, and usually involves modification of Ser, Thr, and Tyr residues. The resulting phosphoesters are stable entities, and generally serve to regulate enzymatic catalysis without direct involvement in the catalytic mechanism. Reversible, multisite phosphorylation of Ser, Thr, and Tyr mediates numerous signal transduction pathways in eukaryotic cells (Cohen, 2000). The second class encompasses phosphorylation for the purpose of phosphate group transfer, and is generally restricted to phosphorylation of His residues. Phosphohistidines act as high-energy intermediates in the transfer of phosphate from phosphodonor to phosphoacceptor molecules (Stock *et al.*, 1989), a role for which these labile modifications are well suited. Examples include the bacterial phosphoenolpyruvate:sugar phosphotransferase system (Meadow *et al.*, 1990) and the histidine kinase enzymes found in two-component signal transduction pathways (Parkinson and Kofoid, 1992). The latter are utilized by bacteria, plants, and lower eukaryotes to sense and adapt to changing environmental conditions (Klumpp and Krieglstein, 2002). In bacteria, such adaptations may include changes in motility, cell morphology, and gene expression, as well as the establishment of virulence and antibiotic resistance.

Due to the importance of protein phosphorylation in regulating key biological processes, considerable effort has been put into developing procedures for identifying and mapping sites of phosphorylation, both for individual proteins and on a proteome-wide scale (Beausoleil *et al*., 2004; de la Fuente van Bentem *et al*., 2006; Nühse *et al*., 2003). However, the substoichiometric nature of this modification continues to present major challenges; indeed, the phosphorylated form of a particular protein may represent only a small fraction of its total abundance. Furthermore, many proteins can undergo phosphorylation at different sites (Cohen, 2000), resulting in a number of potential phosphorylated isoforms of which several may be present at any given time. As a result, the full complement of phosphorylated proteins in a cell, tissue, or organism (the phosphoproteome) can be extremely complex.

For His-phosphorylated proteins, the situation is further complicated by the fact that phosphohistidines are susceptible to hydrolysis under the acidic conditions normally used in phosphoprotein and phosphopeptide analysis (Hess *et al.*, 1988; Matthews, 1995). In contrast, the phosphoesters formed by Ser, Thr, and Tyr residues resist acid treatment and so remain intact during extraction, purification, and analytical procedures. Consequently, phosphohistidines generally go undetected in conventional studies of protein phosphorylation (Klumpp and Krieglstein, 2002). Before deciding upon a strategy for large-scale identification of histidine phosphorylations, it is therefore necessary to undertake a critical review of existing techniques for targeting and analyzing phosphoproteins and sites of protein phosphorylation. Such techniques include sample fractionation, affinity purification, gel separation, and mass spectrometry (MS)-based analytical procedures.

Sample Fractionation

As a first step in addressing the low relative abundance of phosphorylated proteins, it is advisable to use some form of fractionation to reduce sample complexity during subsequent analytical steps (Gruhler *et al.*, 2005). Differential centrifugation is a popular technique for fractionation of biological samples and has been applied successfully to phosphoproteomic studies (Nühse *et al.*, 2003). Proteins and organelles differ in size, shape, and density, and can therefore be resolved from cell lysates or tissue homogenates by varying the speed of centrifugation. The forces created at low speeds are small (e.g., $600\times g$) and only very large or dense particles will precipitate, forming a pellet from which proteins can be extracted and/or further purified. At high speed, most particles will precipitate and only soluble proteins will remain in solution. Figure 1 shows a stepwise fractionation protocol for eukaryotes based upon differential centrifugation. This fractionation technique causes minimal disruption to the sample and is, therefore, likely to preserve labile modifications, such as phosphohistidines. However, it should be borne in mind that the denaturing buffers and phosphatase inhibitors normally used to suppress enzyme activity during protein extraction will not guard against hydrolysis of phosphohistidines.

Subfractionation methods (e.g., for resolving mitochondrial and microsomal proteins) have also been developed to further simplify protein and proteome analysis (Hanson *et al.*, 2001; Nühse *et al.*, 2003). Linear and discontinuous sucrose density gradient fractionation procedures have traditionally been used to purify plasma membranes and mitochondrial complexes (Hodges *et al.*, 1972; VanPutte and Patterson, 2003). However, these methods are generally time-consuming, result in low yields, and are prone

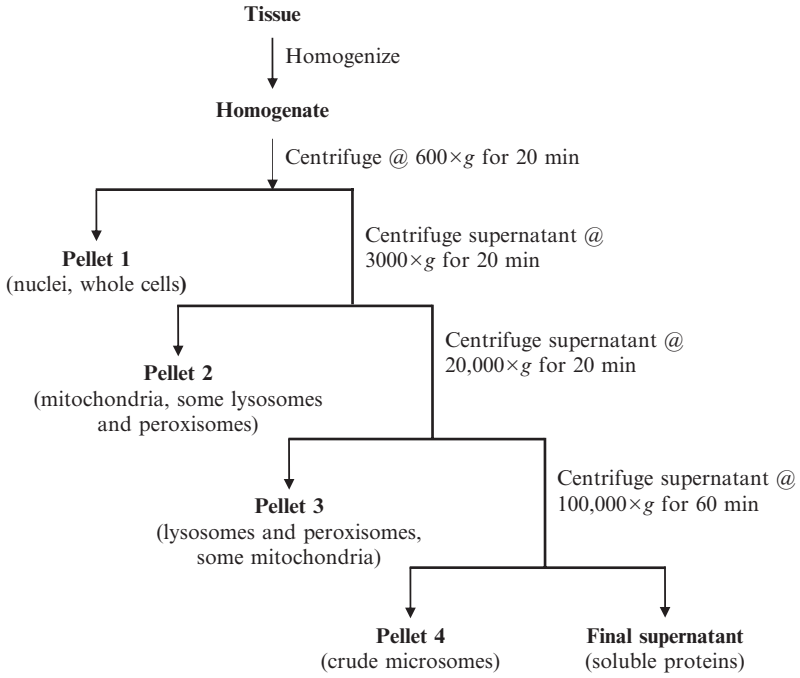

FIG. 1. Sample fractionation by differential centrifugation (courtesy of Dr. Uma K. Aryal, NRC Plant Biotechnology Institute). Plasma membrane proteins in crude microsomal fractions may be further resolved by aqueous two-phase partitioning.

to contamination. Affinity purification of plasma membranes from crude microsomal pellets has been achieved using an aqueous two-phase system comprising polyethylene glycol (PEG) and dextran (Nühse *et al.*, 2003; Schindler *et al.*, 2006). When mixed, these two polymers separate into upper (PEG) and lower (dextran) phases. Subsequent partitioning of anionic species into the lower phase results in the latter's becoming negatively charged. This, in turn, causes plasma membranes to migrate into the upper or interphase regions, effecting separation of subcellular membranes on the basis of charge rather than density (Larsson *et al.*, 1987; Persson and Jergil, 1992). As well as enhancing detection of phosphorylated and other low abundance proteins, fractionation by differential centrifugation and two-phase partitioning are very useful for subcellular localization of proteins, complementing informatics tools such as TargetP (Emanuelsson *et al.*, 2000) and SignalP (Bannai *et al.*, 2002) that are also available for this purpose. Two-phase partitioning may also assist in the detection of His-containing

proteins, which migrate preferentially into the PEG phase (Wuenschell *et al.*, 1990).

Phosphoprotein Enrichment

Regardless of whether or not sample fractionation procedures are employed, the purification and analysis of phosphoproteins can be greatly enhanced using affinity-based techniques. Monoclonal antibodies raised against specific protein modifications (e.g., phosphorylated Ser, Thr, or Tyr residues) can be used to isolate proteins carrying these modifications, either by immunoaffinity chromatography or immunoprecipitation (Liu *et al.*, 2004). Such antibodies can also be used to visualize modified proteins separated on one- or two-dimensional electrophoresis gels (see later). Antibody purification of proteins containing phosphorylated Ser and Thr residues has met with limited success (Grønborg *et al.*, 2002), due, in part, to the relatively small size of these phosphoamino acids. However, enrichment of Tyr-phosphorylated proteins using immunoprecipitation can be very efficient, enabling the identification of hundreds of phosphorylated proteins in biological tissues (Rush *et al.*, 2005). A practical limitation with this approach is that nontarget proteins may be co-purified by interaction with the target proteins or with the antibodies themselves, which, in practice, are rarely (if ever) 100% specific for the target modification. Fortunately, many of the commercially available anti-phosphotyrosine antibodies are reactive toward phosphohistidine (Klumpp and Krieglstein, 2002), which also contains a relatively large aromatic structure. Such antibodies may therefore be effective in recovering both Tyr- and His-phosphorylated proteins, although this hypothesis has yet to be tested.

Immobilized metal-ion affinity chromatography (IMAC) has long been used for targeted enrichment of phosphorylated proteins (Andersson and Porath, 1986). This application exploits the particular affinity of phosphate groups for metal ions such as Fe^{3+} and Ga^{3+}, which arises from the predominantly electrostatic interaction between oxygen atom and trivalent metal ion (neither of which is easily polarized). Figure 2 shows the steps involved in a typical IMAC extraction procedure. Commercial kits based on Fe(III)- and Ga(III)-IMAC are available for selective enrichment of phosphoproteins from protein extracts (Wolschin *et al.*, 2005). Immunoaffinity-based kits for the removal of high abundance proteins (HAP) are also available, and can enhance detection and analysis of phosphorylated and other low abundance proteins (LAP). Most of these commercial kits have been developed and optimized for use with mammalian (e.g., plasma) proteins. Consequently, the protocols developed for these products are not applicable to, or may require extensive modification for use with,

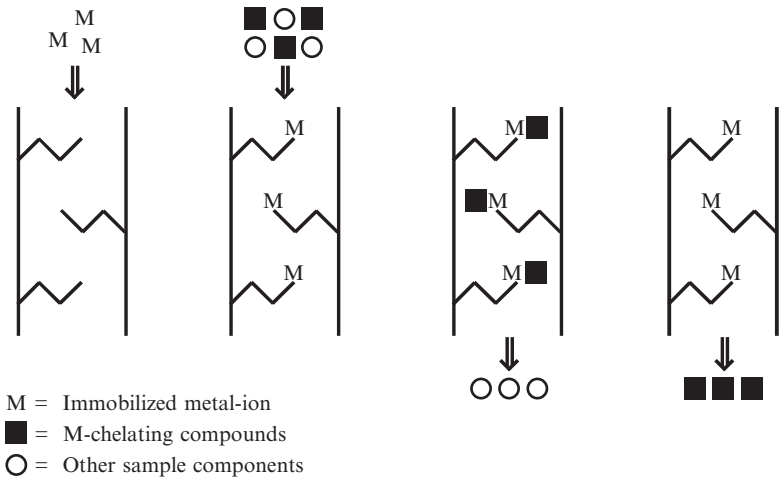

FIG. 2. Immobilized metal-ion affinity chromatography (IMAC). Selected metal ions are loaded onto a chelating column. The sample is then passed through the column, and compounds with an affinity for the immobilized metal ions are selectively retained. Once the column has been washed, the retained compounds can be recovered by displacing them from the immobilized metal ions. This can be achieved by elution with a competing ligand or by raising or lowering pH, depending on the nature of the retained compounds.

plant and/or bacterial systems. Moreover, recovery of intact phosphoproteins using affinity-based methods relies upon the stability, and accessibility, of the target modification. Such methods may not, therefore, be suitable for the recovery of His-phosphorylated proteins unless experimental conditions can be adjusted to prevent hydrolysis of the phosphoamidate bond during extraction.

Metal oxide affinity chromatography (MOAC) has been used successfully to enrich intact proteins containing phosphorylated Ser, Thr, and Tyr residues (Laugesen *et al.*, 2006; Wolschin *et al.*, 2005). This technique compares favorably with commercial products for phosphoprotein enrichment. Furthermore, the incubation and elution buffers required for MOAC are nonacidic and may, therefore, preserve phosphohistidines, although this technique has yet to be evaluated for the recovery of His-phosphorylated proteins.

Gel Separation

Following extraction, fractionation, and/or phosphoprotein enrichment, individual proteins can be further resolved using one- or two-dimensional gel electrophoresis (1- or 2-DE), depending on the complexity of the sample

or fraction. Gel electrophoresis allows the expression of individual proteins to be compared among samples and differentially expressed proteins to be targeted for identification and further analysis. This requires visualization of the gel-separated proteins using autoradiography, visible, or fluorescent staining methods. Autoradiography of ^{32}P-labeled proteins, following separation by gel electrophoresis and blotting onto a poly(vinylidine) difluoride (PVDF) membrane, is a well-established method for detecting newly synthesized phosphoproteins in systems that are amenable to metabolic radiolabeling, such as (bacterial) cell cultures. Treatment of the PVDF membrane with base, prior to autoradiography, hydrolyzes phosphoserine and phosphothreonine but leaves phosphotyrosine, phosphohistidine, and phospholysine residues intact (Klumpp and Krieglstein, 2002). Additional treatment with acid cleaves the phosphoamidate bonds, leaving only phosphotyrosine residues unhydrolyzed. Hence, one can discriminate among phosphoproteins containing different types of phosphoamino acids, although His-phosphorylated proteins that also contain phosphotyrosine residues may go undetected using this approach. However, proteins targeted using autoradiography may need to be excised from replicate, nonradiolabeled electrophoresis gels for further (MS) analysis in order to address such issues as sensitivity, recovery, and handling of radioactive samples (Conrads *et al*., 2002).

Monoclonal antibodies can also be used to detect gel-separated phosphoproteins in PVDF membranes. Again, this Western blotting approach provides information about which types of residues (e.g., Ser, Thr, or Tyr) are phosphorylated in each protein, as exemplified in Fig. 3. Unfortunately, the production of antibodies specific for phosphohistidine is hindered by the instability of this modification (Klumpp and Krieglstein, 2002). However, the reactivity of many anti-phosphotyrosine antibodies toward

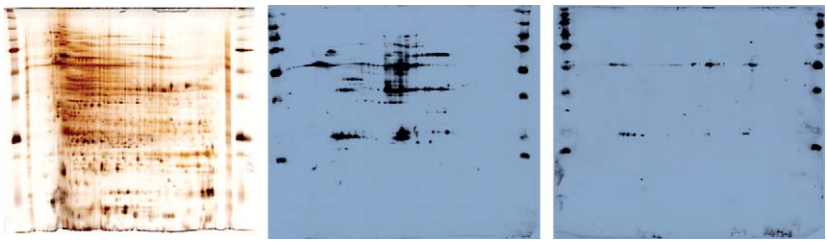

FIG. 3. A silver-stained 2-DE protein gel (left) and Western blots obtained from replicate gels using antibodies against phosphoserine (middle) and phosphothreonine residues (right, courtesy of Dr. Lianglu Wan, NRC Plant Biotechnology Institute). Horizontal spot trains are characteristic of multiple phosphorylation, which has a much greater effect on pI than on molecular weight.

phosphohistidines, which could be 10 to 100 times more abundant that phosphotyrosine (Matthews, 1995), may provide a means of targeting these proteins for subsequent identification and mapping of phosphorylation sites by mass spectrometry (MS) (see later).

Western blotting and autoradiography are very sensitive techniques. Hence, proteins detected using these methods may not be of sufficient abundance for identification and structural analysis using MS, unless a number of replicate gels can be run and corresponding protein spots matched and combined. In contrast, proteins detected by direct in-gel methods such as Coomassie, silver, or fluorescent staining can usually be identified following excision of the individual bands or spots. Furthermore, the development of fluorescent probes for certain modifications (e.g., phosphorylation, glycosylation) means that gels can now be stained once for detection of the target modification, and again to visualize and compare expression levels for every protein in the gel (Vyetrogon *et al.*, 2006). This approach does not provide site-specific information and is, therefore, of limited utility in targeting His-phosphorylated proteins, unless such proteins have been enriched prior to gel electrophoresis. Such information may, however, be obtained by comparing replicate gels run before and after treatment with acid, base, or site-specific phosphatases and observing changes in protein migration and/or visualization that are consistent with dephosphorylation.

Mass Spectrometry

Once the proteins of interest have been isolated using fractionation, affinity, and/or gel-based approaches, one can use mass spectrometry (MS) to identify target proteins and sites of modification (Schweppe *et al.*, 2003). The conventional, ''bottom-up'' approach to protein MS involves the use of a site-specific protease, such as trypsin, to cleave the protein(s) into component peptides. Protein digestion, which normally takes several hours, can be performed in solution (using immobilized trypsin beads, for example) or in-gel, once the spots or bands of interest have been removed and the protein(s) destained, reduced, and alkylated to expose the proteolytic cleavage sites. Automation of this process significantly reduces contamination (especially from human keratin) and, together with automated gel spot excision, enables high-throughput preparation and digestion of protein samples (Ross *et al.*, 2002). The resulting peptides are amenable to exact mass analysis using a time-of-flight (TOF) or Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometer equipped with a matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) source (Aebersold and Mann, 2003). MALDI and ESI are mild

''desorption'' ionization techniques that are capable of generating stable, intact molecular ions from proteins and peptides, from which mass and/or sequence information may be derived (see following text). Using a database search engine such as MS-Fit (http://prospector.ucsf.edu/) or Mascot (http://www.matrixscience.com/), one can compare and match the measured masses of these peptides with theoretical (tryptic) peptide masses generated *in silico* from a protein or gene sequence database such as SwissProt or NCBInr. This technique, known as peptide mass fingerprinting (PMF), is generally performed using a MALDI-TOF mass spectrometer, which provides rapid, simultaneous analysis of protonated $[M + H]^+$ molecular ions generated from the peptides in a protein digest (Yates, 1998). PMF usually works well if the sample contains one or two abundant proteins and the database search is well constrained, for example, by using data of high mass accuracy and/or specifying the organism(s) under investigation. Furthermore, by including phosphorylation of specific residues (e.g., Ser/Thr) as variable modifications in the search, one can identify possible sites of phosphorylation, if peptides carrying this modification appear as additional, significant matches.

However, to confirm sites of phosphorylation and identify phosphorylated and other proteins in complex mixtures (for example, in unresolved protein fractions or gel bands), one must resort to tandem mass spectrometry (MS/MS). This approach typically involves liquid chromatographic (LC) separation and on-line electrospray ionization (ESI), which generates multiply protonated $[M + nH]^{n+}$ molecular ions from the LC-separated peptides. Individual peptide (precursor) ions are then selected and fragmented using a tandem quadrupole, quadrupole-time of flight (Q-TOF), ion trapping, or FT-ICR mass spectrometer (Aebersold and Mann, 2003). The fragment (or product) ion spectra generated by these instruments encodes information about the amino acid sequences of the selected peptides, including modifications to any of the residues. These spectra can be decoded manually, or by using *de novo* sequencing software such as PEAKS (http://www.bioinformaticssolutions.com/), to determine the sequences of the peptides and any sites of phosphorylation, since this modification increases the mass of an amino acid residue by 80 Daltons (Da). They can also be used to identify the parent protein by matching the experimental MS/MS spectra with theoretical (tryptic) peptide fragment ions generated *in silico* from a protein or gene sequence database, using programs such as Mascot or Sequest (Yates, 1998).

The process of collision-induced dissociation (CID), commonly used to generated peptide fragments during MS/MS, may result in loss of phosphate, depending on the residue and the collision energy used. For example, loss of H_3PO_4 (98 Da), which results from β-elimination of the phosphate

group, is diagnostic for peptides containing phosphorylated Ser and Thr residues (Schweppe *et al.*, 2003). In contrast, extensive CID results in the elimination of phosphotyrosine as a stable phosphorylated immonium ion with a mass-to-charge ratio (*m/z)* of 216, which is characteristic of peptides carrying this modification (Steen *et al.*, 2003). Figure 4 shows examples of product-ion MS/MS spectra obtained for standard peptides containing phosphorylated Ser, Thr, and Tyr residues using a Q-TOF mass spectrometer. As is apparent from these spectra, facile loss of phosphate from Ser and Thr tends to inhibit further fragmentation of the peptide, which, in turn, may prevent localization of residues carrying this modification. The replacement of phosphate groups with stable marker molecules has been used to enhance MS and MS/MS analysis, and to enable relative quantification of phosphorylated peptides and proteins (Conrads *et al.*, 2002; Wolschin *et al.*, 2005). Unfortunately, such techniques are currently applicable only to Ser and Thr phosphorylations. Nevertheless, loss of phosphate from Ser or Thr during MS/MS can be used to trigger selection and further fragmentation of the dephosphorylated peptide when performed on an ion trapping or FT-ICR instrument (Beausoleil *et al.*, 2004; Gruhler *et al.*, 2005). This technique, known as data-dependent MS/MS/MS (or MS^3), could

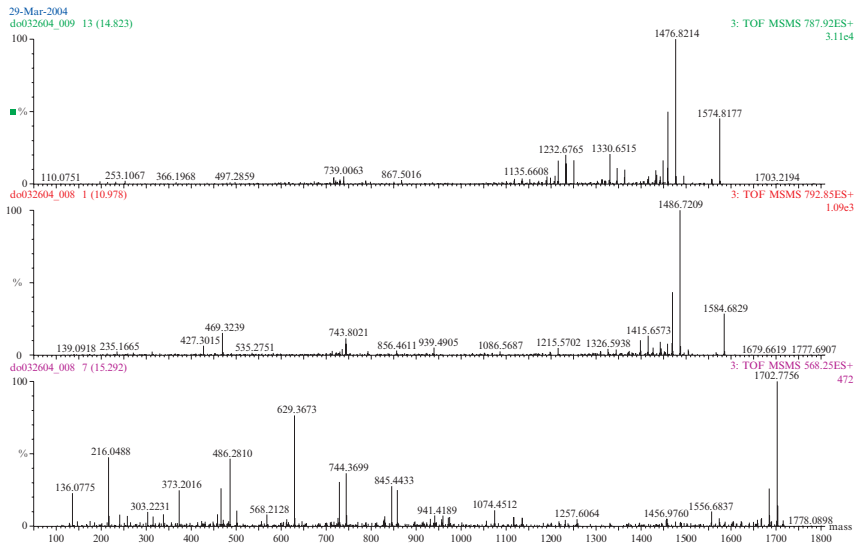

FIG. 4. Product-ion MS/MS spectra for Ser- and Thr-phosphorylated peptides (upper and middle panels), in which the most abundant fragment ion corresponds to neutral loss of phosphoric acid (98 Da) from the intact peptide, and for a Tyr-phosphorylated peptide (lower panel), which gives the diagnostic phosphotyrosine immonium ion (*m/z* 216).

be used to detect and map sites of phosphorylation in His-phosphorylated peptides, provided that intact phosphopeptide precursor ions can be generated.

FT-ICR mass spectrometers are also capable of performing exact mass MS and MS/MS analysis on intact proteins (Cooper *et al.*, 2005), obviating the need for proteolysis. This ''top-down'' approach allows for a comparison of the actual mass of a modified protein with the theoretical mass of the matching, unmodified protein found in the database. When combined with the MS/MS data generated for the same protein, this approach provides a powerful method for identifying and locating known and novel modifications in target proteins. Furthermore, FT-ICR instruments are compatible with electron capture dissociation (ECD), a technique that preserves labile modifications while promoting fragmentation and sequencing of the peptide backbone (Emmett, 2003). Hence, FT-ICR MS and ECD appear well suited to the identification and structural analysis of proteins containing phosphorylated histidine residues, although the required instrumentation is very expensive and not yet widely available.

Phosphopeptide Enrichment

Despite the inherent sensitivity and specificity of mass spectrometry, the identification and structural analysis of phosphorylated peptides by MS and MS/MS can still be problematic, since phosphorylation is usually substoichiometric and tends to inhibit positive ionization by MALDI or ESI (a prerequisite for peptide fragmentation and sequencing). For this reason, researchers continue to investigate ways of enriching phosphorylated peptides from protein digests prior to MS analysis. Strong cation exchange (SCX) chromatography is often used as the first step in multidimensional liquid chromatography (MDLC), an alternative approach to gel-based protein analysis (Wolters *et al.*, 2001). Entire protein extracts or fractions are digested, usually with trypsin, and the resulting peptides loaded onto a SCX column. Solutions of increasing salt concentration are used to elute peptide fractions from the column, which are then separated by reversed-phase high performance liquid chromatography (RP-HPLC) and analyzed on-line by ESI-MS/MS, or off-line by MALDI-MS/MS. As with proteins, fractionation of peptides can assist in the detection of phosphorylation sites by reducing sample complexity during subsequent analytical steps (Gruhler *et al.*, 2005). Furthermore, whereas unmodified tryptic peptides (which have basic C-terminal residues) usually carry a net charge or 2+ or greater in solution, the presence of a negatively charged phosphate group generally limits this charge to 1+. Consequently, most phosphopeptides tend to elute from the SCX column before unphosphorylated peptides. This approach

has been used successfully to enrich phosphorylated peptides for on-line MS analysis, greatly reducing background interference/ion suppression by unphosphorylated peptides (Beausoleil *et al.*, 2004). When combined with stable isotope labeling techniques such as SILAC (Gruhler *et al.*, 2005), this gel-free approach can also be used for relative quantification of phosphoproteins in different samples. Unfortunately, His-phosphorylated peptides are unlikely to survive the acidic conditions necessary for SCX or reversed-phase liquid chromatography.

Strong anion exchange (SAX) chromatography has also been evaluated for peptide fractionation (Nühse *et al.*, 2003) prior to enrichment of phosphopeptides using IMAC. The latter, which is based upon the affinity of oxygen-containing groups (e.g., phosphate, carboxylate) for trivalent metals ions, can be made more specific for phosphopeptides by converting carboxylic acid groups to methyl esters (Ficarro *et al.*, 2002). However, this process biases against IMAC recovery of singly phosphorylated peptides and may lead to unwanted peptide modifications (Wolschin *et al.*, 2005), making it unsuitable for phosphohistidines. SAX helps to compensate for this bias by permitting differential elution of singly and multiply phosphorylated peptides using different salt concentrations (Nühse *et al.*, 2003). Unfortunately, the recovery of His-phosphorylated peptides from SAX fractions is also compromised by the acidic conditions required for desalting by solid-phase extraction (SPE) prior to IMAC and/or MS analysis.

IMAC has been used extensively for selective recovery and enrichment of peptides containing phosphorylated Ser, Thr, and Tyr residues from protein digests. As with intact phosphoproteins, the metal ions most commonly used for this purpose are Fe^{3+} and Ga^{3+} (Nühse *et al.*, 2003; Posewitz and Tempst, 1999). IMAC media consist of acidic, metal-chelating functional groups bound to a solid support such as Sepharose, agarose, cellulose, polystyrene resin, or silica (Liu *et al.*, 2004). The most commonly used functional group is iminodiacetate (IDA), which is small, hydrophilic, and binds metal ions tightly while leaving coordination sites available for peptide or protein binding (Arnold, 1991). The most popular alternative, nitrilotriacetate (NTA), surrounds and binds metals more tightly that IDA, allowing strong metal chelators (e.g., His-tagged proteins) to be recovered by IMAC without stripping the metal from the column. Several products are commercially available for peptide purification by IMAC, including column packing materials, prepacked analytical or extraction columns, and preloaded extraction beads. Disposable pipette tips packed with IMAC media have also been developed, which allow rapid enrichment of phosphopeptides from small volumes of protein digests. These tips make it possible to extract His-phosphorylated peptides with sufficient speed to preserve the intact phosphohistidine residue for subsequent identification

by MS (Napper *et al.*, 2003). Furthermore, it is possible to discriminate between peptides containing phosphohistidine and phosphoesters using IMAC (see following text), something that cannot be achieved using SCX, SAX, or MOAC, although the latter is effective in enriching phosphorylated peptides as well as intact phosphoproteins (Wolschin *et al.*, 2005).

Identification of Phosphohistidine in a Model Protein

Having reviewed the techniques currently available for targeting and analyzing phosphorylated proteins and peptides, it is apparent that only rapid and/or noninvasive methods are likely to be effective in recovering intact His-phosphorylated proteins, and identifying phosphohistidines within these proteins. The following describes a preliminary investigation into the use of two such methods, IMAC and MALDI-TOF MS, for selective recovery and identification of a His-phosphorylated peptide derived from the phosphocarrier protein HPr in *Escherichia coli*. HPr occupies a central role in the bacterial PTS, which mediates phosphorylation-dependent sugar uptake (Meadow *et al.*, 1990) as well as numerous regulatory roles in bacterial metabolism (Postma *et al.*, 1993; Titgemeyer, 1993). HPr undergoes phosphorylation at a conserved histidine residue (His-15) and functions as a phosphotransfer protein between Enzyme I, the initiating enzyme of the PTS, and a sugar-specific Enzyme IIA protein (Anderson *et al.*, 1971; Waygood *et al.*, 1985). The kinetics of HPr phosphorylation and the phosphohydrolysis properties of this protein are well established.

Phosphorylation and Digestion of HPr

Enzyme I, Enzyme IIA^{glc}, and HPr proteins from *E. coli* were purified to homogeneity using previously published protocols (Anderson *et al.*, 1991; Brokx *et al.*, 2000; Napper *et al.*, 2001), as was the CheY protein from *Salmonella typhimurium* (Stock *et al.*, 1985). Phosphorylation of HPr was achieved by combining 20 ng of Enzyme I with 10 μg (1.1 nmol) of HPr in 20 μl volumes of a reaction mixture containing 2 m*M* $MgCl_2$, 5 m*M* phosphoenolpyruvate, and 20 m*M* HEPES buffer (pH 7.0). Complete phosphorylation of HPr was achieved after incubation at 37° for 15 min. For unphosphorylated controls, the phosphodonor (phosphoenolpyruvate) was omitted from the mixture.

Rapid proteolysis was achieved by adding 2.5 μl of 0.4 mg/ml *Staphlococcus aureus* V8 protease (endoproteinase C) in 0.1 *M* bicine (pH 8.6) and 1 m*M* EDTA to a 20 μl volume of phosphorylation reaction mixture. Digests were incubated at 37° for just 30 min in an attempt to preserve the phosphohistidine residues, which undergo extensive hydrolysis

during longer digestion periods (Anderson *et al.*, 1993) and are therefore incompatible with conventional trypsin digestion procedures. To estimate the final peptide concentration, aliquots of the V8 digest were separated by SDS-PAGE and the intensities of digested (peptide) and undigested (protein) bands quantified using a phosphoimager. The intensity ratio of digested to undigested bands was 10:1, corresponding to a maximum theoretical concentration of 44 pmol/μl for the phosphorylated HPr peptide VTITAPNGL(pH)TRPAAQFVKE (residues 6–25).

IMAC Conditions

Disposable metal-chelating pipette tips ($ZipTip_{MC}$), obtained from Millipore Corporation (Bedford, MA), were used to facilitate rapid extraction and enrichment of phosphorylated peptides. The tips were washed 10 times with 10 μl of 0.1% acetic acid in deionized water, then charged with metal ions by aspirating and dispensing 10 μl of a 100 m*M* metal salt solution 15 times. Several metal ions were evaluated for their ability to selectively extract and recover His-phosphorylated peptides by IMAC, including Ga^{3+}, Cu^{2+}, and Fe^{3+}. The charged tips were rinsed 5 times with 10 μl of deionized water, and 5 times with 10 μl of 0.1% acetic acid in 50% acetonitrile. Phosphopeptides were loaded onto the tips by aspirating and dispensing 10 μl of sample 10 times, then washing 10 times with 10 μl of 0.1% acetic acid in 50% acetonitrile. Phosphopeptides were eluted in 2 μl of 1% NH_4OH and immediately neutralized with 1 μl of 2% trifluoroacetic acid (TFA). Samples were then mixed with an equal volume of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (5 mg/ml in 75% acetonitrile with 0.1 % TFA) and applied directly to the MALDI target plate. For comparison, the phosphorylated HPr digest was desalted by SPE using conventional C_{18} ZipTips (Millipore), according to the manufacturer's instructions, then combined with an equal volume of the same MALDI matrix solution, without performing IMAC.

MALDI-TOF MS Conditions

Phosphopeptides were analyzed by MALDI-TOF MS using a Voyager DE-STR instrument (Perseptive Biosystems, Framingham, MA) operating in the linear, reflectron, or post-source decay (PSD) modes with positive or negative ionization (Napper *et al.*, 2003). Reflectron TOF provides the high mass resolution necessary for exact mass measurements, whereas linear TOF provides greater sensitivity at the expense of resolution. PSD is a dissociation process that provides peptide fragment information (albeit at low mass resolution) from which sites of phosphorylation may be inferred.

Full scan spectra were obtained by combining and processing 100 scans, a process that takes less than a minute. For PSD analysis, the reflectron mirror ratio was adjusted incrementally, the resulting spectra combined, and fragment ion peaks assigned using the instrument software (a procedure can be performed automatically, and more rapidly, on newer MALDI-TOF instruments).

Enrichment of His-Phosphorylated Peptides

Positive-ion reflectron MALDI-TOF MS analysis of the V8 digest of phosphorylated HPr generated a spectrum containing most of the expected peptides, as shown in Fig. 5A. The His-containing peptide (6–25) was

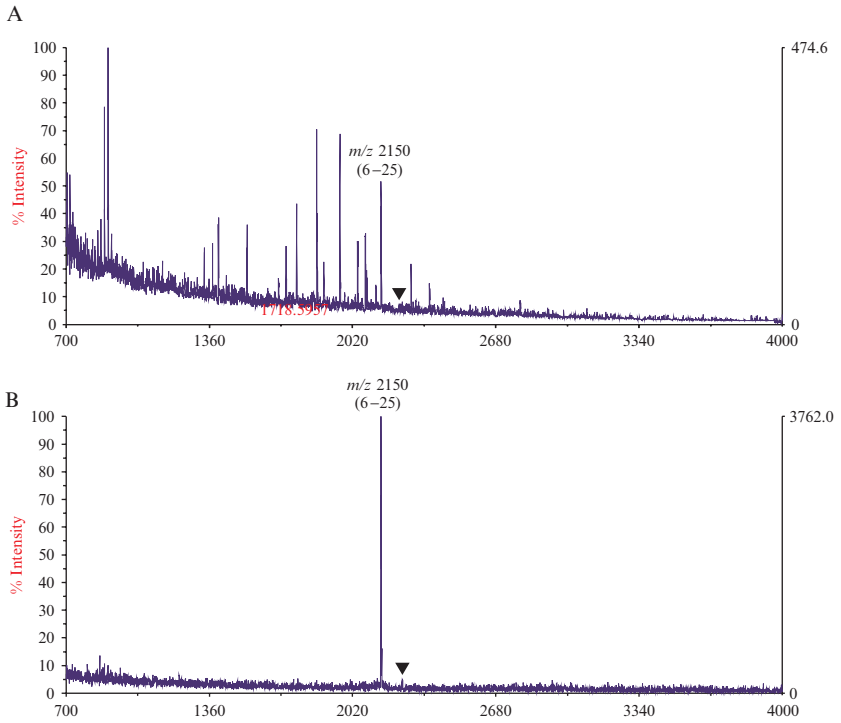

FIG. 5. Positive-ion reflectron MALDI-TOF MS spectra of (A) a complete V8 digest of the His-phosphorylated protein HPr, and (B) a Cu(II)-IMAC extract of the same digest. The His-containing peptide (6–25) is selectively recovered, but detected almost exclusively in nonphosphorylated form (*m/z* 2150). The expected position of the phosphorylated peptide (*m/z* 2230) is indicated by ▼.

detected in unphosphorylated form (*m/z* 2150); however, the phosphorylated form (*m/z* 2230) could not be observed, indicating that histidine phosphorylation is incomplete, labile, and/or inhibits ionization under experimental conditions. Analysis of the Cu(II)-IMAC extract of the phosphorylated HPr digest contained a single intense peak at *m/z* 2150, as shown in Fig. 5B. Again, this corresponds to the predicted mass of the His-phosphorylated peptide without the phosphate group (HPO_3). When the same protocol was applied to a V8 digest of unphosphorylated HPr, no peptide ions were detected, even when operating in the more sensitive linear mode. This suggests that Cu(II)-IMAC extraction is selective for the phosphorylated form of the His-containing peptide, but that the phosphate group is lost before or during MALDI-TOF MS analysis.

Selectivity for Phosphorylated Histidine

Of the metal ions investigated, only Cu^{2+} proved effective in retaining and recovering peptides that contain phosphohistidine residues. To verify that peptides containing unphosphorylated histidine residues are not recovered using this procedure, a mixture containing four purified proteins (Enzyme I, Enzyme IIA^{glc}, and HPr from *E. coli*, and CheY from *Salmonella typhimurium*), each at a concentration of 1 mg/ml, were digested to completion with V8 protease and subjected to the Cu(II)-IMAC extraction protocol. This mixture contains 11 unique peptides bearing His residues; however, none of these was recovered using the Cu(II)-IMAC procedure.

Divalent transition metal ions are known to interact strongly with unmodified His residues; for example, Ni^{2+}–NTA is used routinely for purifying recombinant proteins in which a terminal poly(histidine) tag has been incorporated. Moreover, Cu^{2+}–IDA has a higher affinity for His-containing proteins than does Ni^{2+}–IDA (Arnold, 1991). Why, then, does Cu(II)-IMAC not recover peptides containing unmodified histidine using the protocol described above? To answer this question, we need to consider the acid/base properties of histidine and the pH of the sample during different steps in the IMAC process. The extraction of His-tagged proteins by IMAC is based on the interaction between divalent metal ions and the imidazole group of histidine. Under basic conditions, the uncharged imidazole ring forms a complex with the immobilized metal ion. Elution of His-tagged proteins and peptides is then achieved by lowering pH and protonating the imidazole nitrogen (pK_a 6.0), which generates a positive charge that is repelled by the immobilized metal cation. During the aforementioned Cu(II)-IMAC procedure, the binding of unphosphorylated histidines is prevented by loading the sample under the acidic conditions

that result from mixing of the weakly buffered sample with residual acetic acid on the IMAC column. This reduces the sample pH from an initial value of 7.0 (20 m*M* HEPES) to around 3.5 (0.1 % acetic acid), at which point protonation of the imidazole ring negates interaction with the immobilized Cu^{2+} ions. Subsequent washing with 0.1% acetic in 50% acetonitrile serves to maintain the imidazole in protonated form, although this may also hydrolyze some of the intact phosphopeptides (see following text).

Detection of His-Phosphorylated Peptides

Previous studies have shown that switching from the positive to the negative ionization mode increases MS detection sensitivity for phosphorylated peptides relative to their unphosphorylated counterparts (Janek

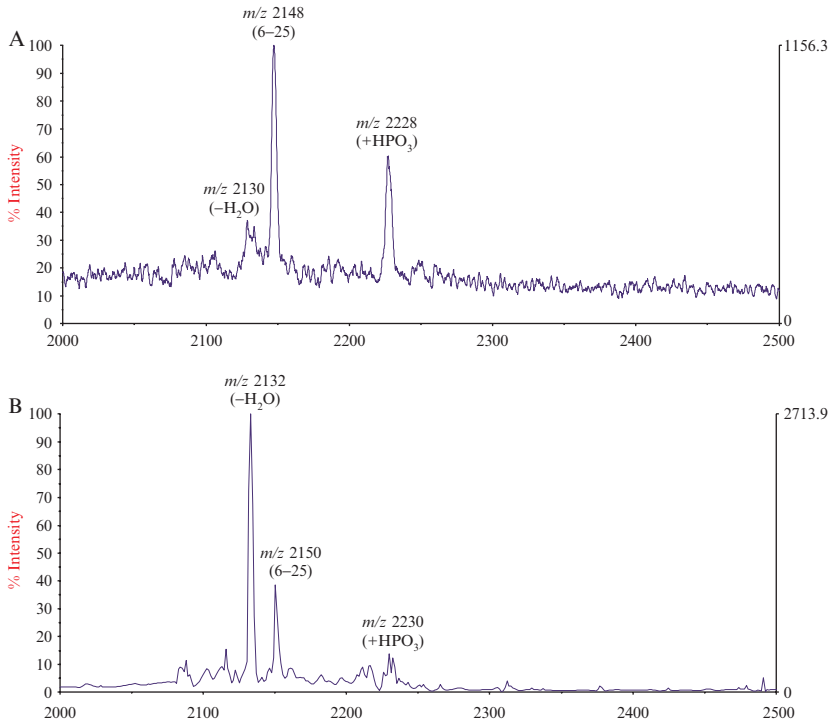

FIG. 6. MALDI mass spectra obtained using (A) negative-ion linear TOF MS analysis of the Cu(II)-IMAC extract from a V8 proteolytic digest of phosphorylated HPr, and (B) post-source decay (PSD) of the His-phosphorylated HPr peptide (*m/z* 2230) generated in positive-ion mode, both of which confirm recovery of the intact phosphopeptide.

et al., 2001). As expected, negative-ion MALDI-TOF MS analysis of the Cu(II)-IMAC extract detected an ion of *m/z* 2228, corresponding to the deprotonated $[M - H]^-$ molecular ion of the intact HPr phosphopeptide, as shown in Fig. 6A. However, the unphosphorylated form of this peptide (*m/z* 2148) was also observed in the spectrum. This further suggests that the intact His-phosphorylated peptide is recovered using the Cu(II)-IMAC procedure but is susceptible to dephosphorylation during IMAC and/or MS analysis.

The same extract was subsequently analyzed in positive-ion mode using PSD with the ion gate set at *m/z* 2230, the expected value for the intact phosphopeptide. The resulting spectrum, shown in Fig. 6B, contains ions that correspond to sequential loss of HPO_3 (80 Da) and H_2O (18 Da) from the His-phosphorylated peptide, which is also observed in the spectrum. Similar results have been reported for collision-induced dissociation (CID) of His-phosphorylated peptide ions generated by MALDI (Janek *et al.*, 2001), confirming that the intact phosphopeptide was recovered by Cu(II)-IMAC. Figure 7 shows the different structures of phosphoester and phosphohistidine residues that give rise to neutral losses of 98 and 80 Da, respectively.

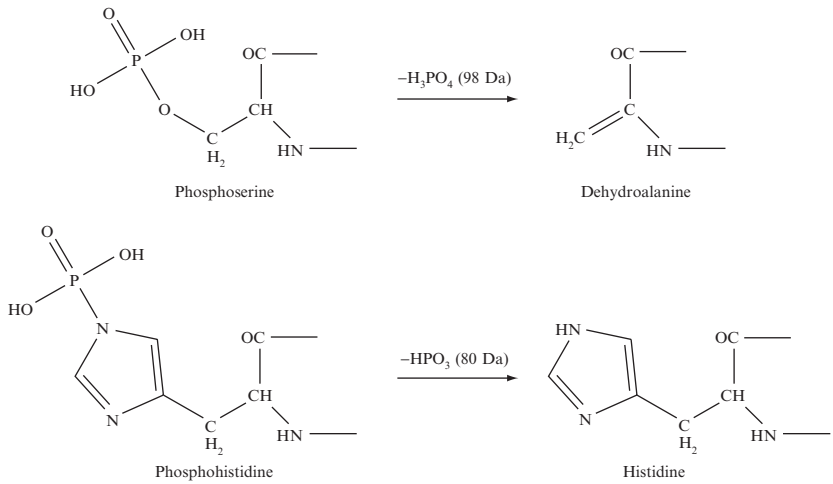

FIG. 7. Characteristic loss of H_3PO_4 and HPO_3 from, respectively, phosphoserine and phosphohistidine residues. The former (which also applies to phosphothreonine) can occur during tandem mass spectrometry, the latter during sample preparation and MS analysis. Neutral loss of HPO_3 can also occur during post-source decay of peptides containing phosphoserine and phosphothreonine residues.

Specificity for Phosphohistidines

To determine whether or not the protocol was specific for phosphopeptides containing phosphorylated His residues, the Cu(II)-IMAC procedure was applied to a tryptic digest of bovine β-casein. This protein yields two tryptic peptides that are phosphorylated at one and four Ser residues, respectively, and which produce ions of *m/z* 2062 and 3112 when analyzed by positive ion MALDI-TOF MS (Chalmers *et al.*, 2000; Posewitz and Tempst, 1999). Neither of these peptides was observed in Cu(II)-IMAC extracts, possibly because the strength of the interaction between phosphoserine and the immobilized Cu^{2+} ions prevented elution in 1% NH_4OH (Nühse *et al.*, 2003). Extraction of the β-casein digest using a Ga(III)-IMAC protocol optimized for the recovery of peptides containing phosphoester residues (Posewitz and Tempst, 1999) selectively recovered the singly phosphorylated peptide of *m/z* 2062, as confirmed by positive ion MALDI-TOF and PSD analysis. However, the Ga(III)-IMAC protocol did not recover the His-phosphorylated peptides from the phosphorylated HPr digest, which suggests that it may be possible to use Ga(III)-IMAC and Cu(II)-IMAC sequentially to extract peptides modified with phosphoester and phosphohistidine residues from mixed protein digests. Alternatively, stepwise elution of phosphohistidines and phosphoesters from Cu(II)-IMAC columns may be possible using basic solutions of increasing concentration, assuming that both types of phosphopeptides are indeed retained.

Differential Hydrolysis of Phosphohistidines

Histidine is unique among amino acids in that it can be phosphorylated at two different positions, namely, at atoms NΔ1 and Nε2 of the imidazole ring. For free histidine, phosphorylation at the NΔ1 position is much less stable than that at the Nε2 position due to the interaction of the NΔ1 phosphate group with the positively charged amino group (Hultquist *et al.*, 1966). In peptides and proteins, the amino group of histidine is involved in peptide bonding, and the stability of the phosphate linkage may differ considerably from that of free histidine. Moreover, the local structural and electrostatic environment of the protein may have significant influence on the chemical properties of histidine and may vary in different protein contexts (Anderson *et al.*, 1993; Waygood *et al.*, 1985). For native HPr in *E. coli*, the rates of phosphohydrolysis for NΔ1-phosphohistidine are three times greater than those for free NΔ1-phosphohisitidine (Hultquist *et al.*, 1966), indicating that the protein serves to destabilize the linkage, perhaps to ensure efficient phosphotransfer. The pH-dependence of phosphohydrolysis for phosphorylated HPr is also quite distinctive, exhibiting

a bell-shaped curve with greatest instability of the phosphoamidate bond between pH 5 and 8. The phosphopeptide generated by V8 digestion of phosphorylated HPr has phosphohydrolysis rates an order of magnitude lower than those of the intact protein, showing greatest stability at neutral to basic pH (Hultquist *et al.*, 1966). This allows for the retention of phosphate during base elution of the chelating pipette tips.

Summary and Conclusions

Selective extraction of His-phosphorylated peptides from HPr protein digests can be achieved by immobilized Cu^{2+}-ion affinity chromatography using disposable metal-chelating pipette tips. On-tip acidification of the sample by residual acetic acid apparently inhibits binding of unphosphorylated histidine residues during Cu(II)-IMAC, while recovery of the intact phosphopeptide via base elution is confirmed using negative-ion MALDI-TOF mass spectrometry. Subsequent PSD analysis of the protonated molecular ion shows characteristic loss of the HPO_3 moiety present in His-phosphorylated peptides. Possible refinements to this method include the use of ''cooler'' and/or less acidic matrix compounds, such as 2,4,6-trihydroxyacetophenone (THAP) combined with ammonium citrate (Wolschin *et al.*, 2005) or 2,5-dihydroxybenzoic acid (DHB) combined with phosphoric acid (Kjellstrom and Jensen, 2004), to enhance detection and structural analysis of His-phosphorylated peptides by MALDI-TOF MS, or by MALDI-MS/MS using Q-TOF or TOF-TOF instrumentation.

Although disposable pipette tips are ideal for rapid IMAC, the characteristics of the column packing tend to vary from one tip to another. As a result, there is sometimes a lag in solvent uptake when aspirating solutions, which may result in air being accidentally drawn into the tip. If solutions are aspirated and dispensed with care, however, the packing material will remain properly conditioned, and consistent results should be obtained. An alternative approach is to use open tubular (OT) columns, in which the inner surface of a glass tube is uniformly modified by chemical attachment of a metal chelating group (Liu *et al.*, 2004). OT columns can be used in the same way as disposable pipette tips, the high density and uniformity of the OT-IMAC medium providing enhanced specificity and reproducibility for phosphopeptide enrichment.

While preliminary results are encouraging, there is still much work to be done before routine, proteomewide identification of His-phosphorylated proteins can be achieved. Although fractionation of complex samples would certainly be advantageous, each additional step in the analytical process increases the chance of degrading phosphohistidine residues, unless mild and (preferably) basic conditions can be maintained throughout. This

precludes techniques such as SCX, SPE, and derivatization procedures for enhancing recovery, detection, and/or relative quantification of phosphopeptides. However, differential centrifugation, two-phase partitioning, and/or MOAC may prove suitable for fractionation and co-enrichment of His-phosphorylated and other phosphorylated proteins. Gel electrophoresis combined with phosphate-specific fluorescent staining, or Western blotting for His- and Tyr-phosphorylated proteins, could then be used to resolve and detect phosphoproteins in the enriched fractions. Subsequent identification of His-phosphorylated proteins, and their component phosphohistidines, by mass spectrometry would be facilitated by Cu(II)-IMAC enrichment of His-phosphorylated peptides from in-gel digests. However, this approach requires the development of rapid in-gel digestion and peptide extraction procedures that minimize hydrolysis of phosphoamidate bonds. Alternatively, ''top-down'' analysis by ECD and FT-ICR MS may prove effective in identifying and mapping labile phosphohistidine residues in His-phosphorylated proteins, provided that individual proteins can be isolated or recovered intact from polyacrylamide gels (by electroelution, for example). The latter would also enable the use of rapid microcolumn digestion procedures (Slysz and Schriemer, 2005) for ''bottom-up'' analysis of His-phosphorylated proteins.

Until now, the identification of novel histidine kinases has been performed largely on the basis of sequence similarities among members of this enzyme class. Mild protein fractionation, gel separation, and affinity purification procedures, combined with high-resolution MS and data-dependent MS^n techniques, offer an alternative approach for identifying these and other His-phosphorylated proteins through direct analysis of histidine phosphorylations in proteolytic peptides and/or their parent proteins. Such techniques have the potential to increase significantly our understanding of the role played by histidine phosphorylation in two-component signaling systems. In conclusion, I should like to thank Dr. Scott Napper at the Vaccine and Infectious Disease Organization (VIDO) and Drs. Lianglu Wan and Uma K. Aryal at the NRC Plant Biotechnology Institute in Saskatoon for their assistance in preparing this article, which is contribution 48421 from the National Research Council of Canada.

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