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Chasing Phosphohistidine, an Elusive Sibling in the Phosphoamino Acid Family

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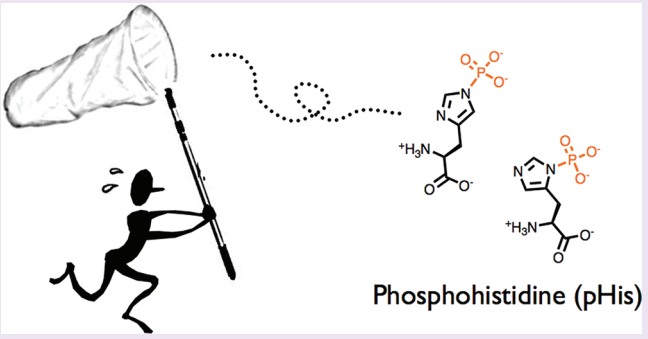
ABSTRACT: This year (2012) marks the 50th anniversary of the discovery of protein histidine phosphorylation. Phosphorylation of histidine (pHis) is now widely recognized as being critical to signaling processes in prokaryotes and lower eukaryotes. However, the modification is also becoming more widely reported in mammalian cellular processes and implicated in certain human disease states such as cancer and inflammation. Nonetheless, much remains to be understood about the role and extent of the modification in mammalian cell biology. Studying the functional role of pHis in signaling, either *in vitro* or *in vivo*, has proven devilishly hard, largely due to the chemical instability of the modification. As a consequence, we are currently handicapped by a chronic lack of chemical and biochemical tools with which to study histidine phosphorylation. Here, we discuss the challenges associated with studying the chemical biology of pHis and review recent progress that offers some hope that long-awaited biochemical reagents for studying this elusive posttranslational modification (PTM) might soon be available.



Protein phosphorylation is one of the most common and extensively studied posttranslational modifications (PTMs).[1] Protein kinases and phosphatases play critical roles in controlling most cellular signal transduction pathways, and their misregulation has been linked to many diseases including cancer. Accordingly, these enzyme classes have emerged as important drug targets, boosted by the prominent success of imatinib, a protein tyrosine kinase inhibitor used to treat leukemia and other malignancies.[2,3] While protein phosphorylation is known to occur on nine amino acids (Ser, Thr, Tyr, His, Lys, Arg, Asp, Glu and Cys), it is fair to say that phosphoserine, phosphothreonine, and phosphotyrosine capture most of the attention in the literature, at least as it pertains to cellular signaling in higher eukaryotes. This review turns the spotlight away from this group toward pHis, a modification first discovered by Paul Boyer and co-workers in the early 1960s[4] (some 20 years before tyrosine phosphorylation was discovered [5]).

Histidine phosphorylation is best known in the context of the two-component and multicomponent phosphorelay signaling pathways found in bacteria, fungi, and plants.[6,7] By contrast, the modification is largely off the radar when considering the cellular signaling processes in higher eukaryotes.[8−11] This could, of course, be because higher organisms prefer to use other PTMs to convey biochemical information, or alternatively it might be because there exists a biochemical "blind spot" arising from the significant technical challenges associated with the detection of this PTM (see below). The literature does offer some tantalizing glimpses into the hidden world of pHis that suggest the latter scenario might be more accurate. In the case of the slime mold *Physarum polycephalum*, for instance, pHis has been reported to account for 6% of the total phosphoamino acids in its basic nuclear proteins.[12] The prevalence of pHis is strikingly high among these proteins, considering that phosphotyrosine (pTyr) is found in less than 1% of eukaryotic cellular phosphoproteins.[13,14] Further building the case for an underappreciated role for pHis in the cell biology of higher eukaryotes, the modification has been implicated in a growing number of cellular processes, including G-protein signaling,[15−18] ion conduction,[19,20] central metabolism,[21] and chromatin biology.[22−24] In many of these cases, pHis residues are found as autophosphorylated enzymatic intermediates used in phosphotransfer reactions to secondary metabolites (Table 1). However, some proteins are phosphorylated at histidine residues by protein histidine kinases. For example, nucleoside diphosphate kinase (NDPK, also known as nm23) is capable of phosphorylating other proteins such as the potassium channel, KCa3.1, and the metabolic enzyme, ATP citrate lyase. Unfortunately, for many histidine-phosphorylated eukaryotic proteins, the corresponding kinases are still unknown. Indeed, much remains to be understood about the role and extent of the modification in eukaryotic cell biology. In this regard, researchers in this area must contend with a serious lack of chemical and biochemical tools with which to study histidine phosphorylation. In this review, we discuss the many challenges specific to studying the chemical biology of pHis and highlight some recent progress that suggests we might be on the cusp of some major advances in this area.

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Table 1. Proteins Linked to Histidine Phosphorylation

proteins	references
pHis as an enzymatic intermediate in phosphotransfer to small molecules	
Nucleoside diphosphate kinase (NDPK)	25,26
ATP-citrate lyase	27
Glucose-6-phosphatase	28
6-Phosphofructo-2-kinase	29
Phosphoglycerate mutase	30,31
Phospholipase D	32
Prostatic acid phosphatase	33
Succinyl-CoA synthetase	4
Proteins phosphorylated by protein histidine kinases	
Annexin I	34
ATP-citrate lyase[a,b]	21
Heterotrimeric G protein[a,b]	15−18
Histone H4	22−24
KCa3.1 potassium channel[a,b]	19,20
Thymidylate synthase	35
Proteins with protein histidine kinase activity	
Histidine kinases in two-component signaling pathways (bacteria, fungi, plants)	6,36−38
NDPK	16,19,21
Histone H4 histidine kinase[c]	39−43
Protein pHis phosphatases	
Protein phosphohistidine phosphatase 1 (PHPT1)	44−46
Protein phosphatase 1A, 2A, 2C	47,48

[a]Phosphorylated by NDPK. [b]Dephosphorylated by PHPT1. [c]Not fully characterized.

CHEMISTRY OF PHOSPHOHISTIDINE

Nomenclature. Unlike any other phosphoamino acid, pHis can exist in two isomeric forms, with regard to the position of phosphorylation on the imidazole ring (Figure 1). As discussed below, both pHis isomers have been found *in vivo*. Unfortunately, a survey of the pHis literature reveals ambiguity in the nomenclature of these isomers. Most of the published reports on pHis, including one of the first reports on the modification,[49] refer to isomer **2** as 3-phosphohistidine. However, there are also cases where **2** is referred to as 1-phosphohistidine, following the conventional numbering scheme for substituted imidazoles.[50] Moreover, in the crystallography literature, **2** and **3** are sometimes referred to as a histidine phosphorylated at Nε2 position and Nδ1 position, respectively.[51]

To avoid this confusion, the International Union of Pure and Applied Chemistry (IUPAC) and International Union of Biochemistry (IUB) jointly published a recommendation for nomenclature of phosphorus-containing biomolecules.[52] Rather surprisingly, even this recommendation remains ambiguous on the terms "3-phosphohistidine" and "1-phosphohistidine". Instead of defining those terms more clearly, it suggests the usage of "τ(tele)-phosphohistidine" for **2** and "π(pros)-phosphohistidine" for **3**. Therefore, we will follow this recommendation throughout this review.

Chemical Instability and Isomerism of Phosphohistidine. The most distinct chemical feature of pHis compared to other common phosphoamino acids is the high-energy P−N bond of its phosphoramidate. pHis has a higher ΔG of hydrolysis (−12 to −13 kcal/mol) compared to phosphohydroxyamino acids (ΔG = −6.5 to −9.5 kcal/mol).[53] The high-energy nature of pHis facilitates the transfer of its phosphoryl group to downstream target molecules. However, the chemistry of the phosphoramidate also makes it challenging to study pHis in biological systems. Both isomeric forms of pHis undergo facile dephosphorylation under acidic conditions (Figure 1). π-pHis is thermodynamically less stable than τ-pHis and is thus hydrolyzed faster. Moreover, π-pHis slowly converts to τ-pHis under mildly basic conditions.[54] This fact raises the distinct possibility that some of the τ-pHis detected in biological systems might have isomerized from π-pHis during the isolation and sample handling processes. pHis can also be dephosphorylated by other nucleophiles such as pyridine or hydroxylamine.[55] In addition, there exists a dedicated protein histidine phosphatase in mammalian cells, and other protein phosphatases can dephosphorylate pHis in proteins (Table 1). Collectively, these chemical properties and biochemical activities hamper the detection of pHis from biological samples.

The acid-lability of pHis can be informative in determining the identity of the phosphoamino acid in a phosphoprotein.[55,56] However, it is noteworthy that the stability of pHis in proteins can differ from that of the free monomer and vary with the protein context. For example, the dephosphorylation rate of the π-pHis containing HPr protein from *E. coli* exhibits a bell-

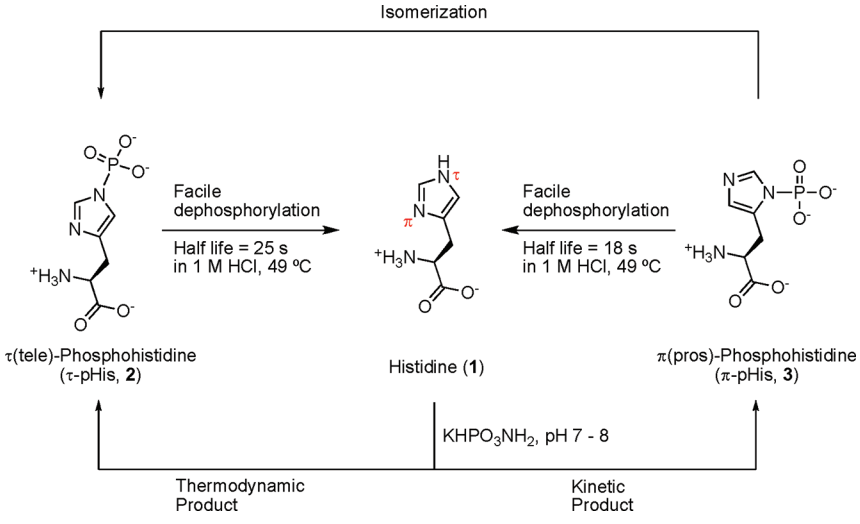

Figure 1. Structure and chemistry of pHis.

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shaped pH profile, with fastest dephosphorylation between pH 5 and 8.[57] Interestingly, the phosphopeptides obtained by V8 protease digestion of the same phosphoprotein show an order of magnitude slower dephosphorylation, with increasing rate at lower pH.

DETECTION AND ANALYSIS OF PHIS

Preparation of pHis-Containing Proteins. There are a number of methods to detect and identify pHis in proteins and peptides.[58] For positive controls to validate such methods, it is necessary to prepare pHis-containing standards. A commonly used technique is chemical phosphorylation. Selective phosphorylation on histidine residues in peptides and proteins can be achieved by treatment with potassium phosphoramidate at pH 7−8. The yield is typically around 80% or higher, and no other amino acid residues are phosphorylated under these conditions.[59] π-pHis is the kinetically favored product in this reaction, but as the reaction proceeds, the thermodynamically more stable τ-pHis becomes the major product (Figure 1). In the case of the pHis monomer, it is possible to isolate each isomer using chromatographic methods. However, it still remains very challenging to prepare pure π-pHis-containing peptides and proteins by this method. Enzymatic phosphorylation selectively yields phosphoproteins phosphorylated on defined histidine residues, but the substrate scope is limited. For example, proteins such as bacterial histidine kinases CheA [60] and NDPK[61] have been autophosphorylated at the histidine and subsequently analyzed. In other cases, histidine phosphorylation of phosphocarrier protein HPr[62] and histone H4[23] have been carried out with protein histidine kinases.

Phosphoamino Acid Analysis. The traditional detection/identification method for pHis is phosphoamino acid analysis.[4,63] Due to its base-stability, pHis survives chemical degradation of a phosphoprotein under strong alkaline conditions, *e.g.*, 3 N KOH at 100 °C. By contrast, pSer and pThr are decomposed under such conditions. Alternatively, the phosphoprotein can be fully digested with the nonspecific protease, Pronase N.[64] Following chemical or enzymatic digestion, pHis can then be identified by reverse-phase thin layer chromatography (RP-TLC), reverse-phase electrophoresis, high-performance liquid chromatography (HPLC), or mass spectrometry.[55,58] Once pHis is known to be present in the phosphoprotein, Nytran filter-based binding assays can be performed to quantify the level of histidine phosphorylation, based on the acid-labile, base-stable nature of pHis.[65] It is important to remember that while these protein chemistry approaches can confirm the presence of pHis in a phosphoprotein or phosphopeptide, they cannot provide direct information on the exact phosphorylation site if there are multiple histidines.

NMR-Based Approaches. NMR spectroscopy is a versatile analytical tool in studying phosphoproteins.[66] ^{31}P is a naturally abundant isotope that gives rise to a strong NMR signal, circumventing the need for any exogenous isotopic labeling schemes. In addition, the paucity of resonances in ^{31}P spectra of phosphoproteins makes it straightforward to interpret the spectra. In 1977, Gassner *et al.* reported the first detailed ^{1}H and ^{31}P NMR characterization of free τ- and π-pHis.[62] They then utilized these chemical shift assignments and their pH dependence to show that pHis in the enzymatically phosphorylated *S. aureus* phosphocarrier protein HPr is the π-pHis isomer. Conversely, chemically phosphorylated HPr was shown to have τ-pHis isomer.[62] Similarly, Smith and co-workers used ^{31}P NMR to detect τ-pHis on histone H4 phosphorylated by a histidine kinase activity derived from Walker 256 carcinoma cells.[24] Other examples where ^{31}P NMR has been used to characterize the nature of the phosphoamino acid include *Dictyostelium discoideum* NDPK (π-pHis)[61] and CheA from *E. coli* (τ-pHis).[60]

Recently, Griesinger and co-workers showed that heteronuclear ^{1}H-^{15}N-^{31}P correlation experiments can be used to unambiguously distinguish the isomeric forms of pHis.[67] The advantage of this method is that it does not require pK_a or chemical shift information on the pHis of interest, which can vary depending on the microenvironment around the pHis. As a drawback, the protein of interest needs to be labeled with ^{15}N-histidine.

Mass Spectrometry. In recent years, mass spectrometry (MS) has become an invaluable research tool in phosphoproteomics by providing information on site-specific phosphorylation of proteins.[68] In "bottom-up" approaches, the phosphoprotein of interest is identified using radiolabeling or phosphoprotein-specific staining, and the protein is digested into peptide fragments. After enrichment for the phosphopeptides, the peptides are separated by chromatographic methods and analyzed by MS or MS/MS. Unfortunately, the acid lability of pHis is a serious liability when using standard proteomic work-flows; typical sample preparation procedures involve the use of acidic environments such as trichloroacetic acid precipitations or the use of acidic eluents in liquid chromatography (LC) separations. To compound matters, pHis readily loses its phosphoryl group during MS analysis, particularly under positive ionization detection mode. Fortunately, as noted below, there has been some progress made recently in overcoming these isolation and detection issues.[58,69]

Due to the low abundance of phosphoproteins, it is common that the biological sample is enriched for the "phosphoproteome" by immunoaffinity chromatography or immobilized metal affinity chromatography (IMAC).[70] While pan anti-pTyr antibodies have been extremely useful in purifying and identifying novel tyrosine-phosphorylated proteins, the lack of such antibodies for pHis has precluded analogous strategies for histidine-phosphorylated proteins. IMAC enrichment at the level of histidine-phosphorylated proteins is yet to be demonstrated. However, Napper and co-workers have reported the enrichment of pHis-containing peptides obtained from proteolytic digestions.[71] They performed *in vitro* enzymatic phosphorylation of the HPr protein from *E. coli* and quickly digested the phosphoprotein using *S. aureus* V8 protease (pHis underwent extensive dephosphorylation during longer incubations with trypsin). Subsequent IMAC using Cu(II) successfully enriched pHis-phosphopeptides, which were analyzed by MALDI-TOF MS. IMAC with Fe(III) or Ga(III), which are commonly used in phosphopeptides enrichment, proved ineffective in this case.

Digested peptides from phosphoproteins are often separated by HPLC using an acidic mobile phase before being analyzed by mass spectrometry. To prevent pHis hydrolysis, Kleinnijenhuis *et al.*[72] employed neutral or basic buffers as the eluent but observed poor resolution and decreased MS sensitivity. However, by employing a short 10-min gradient, an acidic mobile phase (0.5% acetic acid) could be used without extensive pHis hydrolysis.[72] Hohenester *et al.* also reported successfully using 0.1% formic acid solutions for HPLC separation of pHis-peptides.[73] It is noteworthy that these studies were performed using *in vitro* phosphorylated peptides

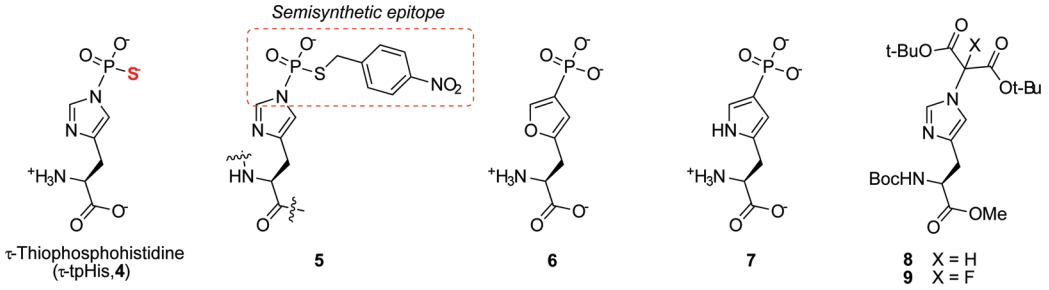

Figure 2. Stable pHis analogues.

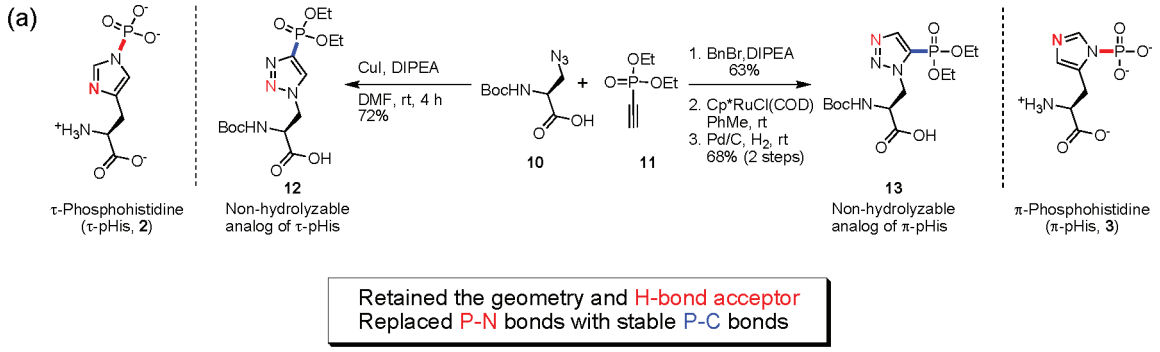

Figure 3. (a) Design and synthesis of pTza as pHis analogues. (b) Peptide dot blots using anti-τ-pHis18 antibody. (c) Western blots of chemically histidine-phosphorylated histone H4. Adapted with permission from ref 80.

and proteins with >90% phosphorylation. Biological samples with lower degrees of phosphorylation might be more difficult to detect using these procedures.

Use of model systems has allowed the MS analysis of pHis in phosphopeptides and phosphoproteins to be explored and optimized. Medzihradszky *et al.* analyzed several synthetic pHis-containing peptides by MALDI-MS and ESI-MS.[59] Under positive ion detection mode, few or no signals from the phosphopeptides were observed, whereas the corresponding unphosphorylated peptides were detected. However, in negative ion mode, the intact phosphopeptides gave the dominant signals.[59] Collectively, these results suggest significant degradation of pHis under positive ion mode. In another study, Kleinnijenhuis *et al.* compared different fragmentation methods for MS/MS and found electron capture dissociation (ECD), electron detachment dissociation (EDD), and electron-transfer dissociation (ETC) were superior than collision-induced dissociation (CID) in suppressing the decay of signal from pHis-peptides.[72] Napper and co-workers analyzed proteolytic digest peptides from phosphorylated HPr using MALDI-TOF MS. They also observed postsource decay of pHis in positive ion mode.[71] In another study, Attwood and co-workers digested and analyzed histidine-phosphorylated histone H4. They observed the expected phosphopeptides by MALDI-TOF and mapped the phosphorylation sites using ESI-MS/MS.[74]

Recently, Cantley and co-workers reported the purification and identification of a pHis-containing phosphoprotein involved in an alternative glycolytic pathway.[31] The radiolabeled phosphoprotein was successfully purified *via* a series of fractionation steps employing a hydroxyapatite (HAP) column, a weak anion exchange (DEAE) column, and a strong anion exchange column (Q). Remarkably, under their optimized conditions, the pHis survived the lengthy purification process. Subsequent SDS-PAGE, in-gel trypsin digestion, and LC−MS/MS analysis identified the protein as phosphoglycerate mutase 1 (PGAM1) phosphorylated at His11. The authors also measured the level of histidine phosphorylation of PGAM by 2D isoelectric focusing (IEF) and SDS-PAGE followed by Western blots using anti-PGAM1 antibodies. This impressive tour de force demonstrates that classical protein fractionation and state-of-the-art MS detection can be successfully employed in the characterization of endogenous pHis-containing proteins.

Antibodies for pHis. Antibodies specific toward phosphorylated amino acid residues have played critical roles in protein phosphorylation research.[75] The advent of the anti-pTyr antibodies in the 1980s led to an explosion of research in this area by revealing the broad occurrence of this PTM in cellular proteomes.[76] Unfortunately, anti-pHis antibodies have

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been much more difficult to obtain compared to other antiphosphoamino acid antibodies. Efforts to utilize pHis itself or pHis-containing peptides as antigens have been unfruitful presumably because they underwent rapid dephosphorylation when injected into animals.[8] Therefore, other strategies have been explored that utilize alternative epitopes to obtain antibodies that can also cross-react with pHis. In fact, some anti-pTyr antibodies have been reported to cross-react with pHis. For example, Frackelton *et al.* immunoprecipitated histidine-phosphorylated ATP citrate lyase from 3T3 mouse fibroblasts using an anti-pTyr monoclonal antibody.[77] However, this antibody cannot distinguish between pHis and pTyr, limiting its applicability.

Shokat, Marletta, and co-workers have developed an elegant semisynthetic method to immuno-detect pHis.[78] When incubated with ATPγS, histidine kinases transferred the γ-thiophosphate to histidine residues *via* autophosphorylation. The thiophosphohistidine (**4**) residue was subsequently alkylated to form a nitrobenzyl thiophosphohistidine (**5**), which was recognized by an antibody specific toward this semisynthetic epitope (Figure 2). This method was initially developed for Ser/Thr kinases,[79] but the same alkylation strategy/antibody combination was successfully employed for a number of bacterial histidine kinases. A limitation for this technology is that the antibody cannot distinguish pHis from other phosphoamino acids, since the semisynthetic epitope is almost identical between them.

Recently, our laboratory developed synthetic nonhydrolyzable analogs of pHis.[80] This pHis mimetic was successfully utilized to raise antibodies specific toward histone H4 phosphorylated on His18 (see below and Figure 3). We believe this strategy holds great promise for the generation of pHis-specific antibodies for other proteins.

STABLE ANALOGUES OF PHIS

Thiophosphohistidine. In order to overcome the stability and isomerism problems associated with pHis, stable analogues of the phosphoamino acid have been developed as research tools. One such analogue is thiophosphohistidine (tpHis, **4**), in which one of the oxygen atoms of the phosphoramide in pHis is replaced with sulfur. tpHis can be chemically generated from His using $PSCl_3$ or potassium thiophosphoramidate.[81] Since sulfur is less electronegative than oxygen, tpHis is expected to be more stable toward acidic hydrolysis. Indeed, Lasker *et al.*[82] demonstrated that tpHis exhibited enhanced stability under acidic conditions. A tpHis-containing peptide remained intact (>70%) for 3 h even at pH 0, whereas the corresponding pHis-containing peptide was completely dephosphorylated under the same conditions. While thiophosphorylated peptides can be useful tools, chemical thiophosphorylation suffers from lack of chemoselectivity and can lead to modification of other nucleophilic residues such as Lys, Cys, or Ser.[83] Enzymatic thiophosphorylation on histidine residues has been achieved using a number of histidine kinases employing ATPγS.[78,82] In the case of autophosphorylated bovine NDPK, the tpHis was stable enough to survive tryptic digestion and Edman sequencing, which led to the identification of the thiophosphorylation site.[82]

Phosphonate Analogues of pHis. Another approach to address the pHis stability issue is to employ synthetic pHis analogues in which the labile P−N bond is replaced with a stable P−C bond. Analogous strategies have been successfully utilized for generating stable analogues of pTyr, pSer, and pThr.[84] Peptides and proteins incorporating these nonhydrolyzable phosphonate analogues can be readily prepared by solid-phase peptide synthesis (SPPS) and expressed protein ligation (EPL).[84]

In the first such foray in the pHis area, Schenkels *et al.*[50] described the synthesis of a furanyl analogue (**6**) of τ-pHis. However, SPPS employing the analogue is yet to be reported. Similarly, Attwood *et al.*[8] developed a pyrrole analogue (**7**) of τ-pHis, mimicking the protonated imidazole ring of pHis. Polyclonal antibodies were generated against **7**, but unfortunately these failed to cross-react with native pHis. Pirrung and co-workers also reported the preparation of malonate (**8**) and fluoromalonate (**9**) derivatives of histidine as pHis analogues.[85] However, no biochemical studies using these analogues have been published.

Recently, we have designed and synthesized phosphoryltriazolylalanine (pTza) derivatives as stable analogues of pHis.[80] Again, the labile P−N bond was replaced with a stable P−C bond, and a triazole ring was used in place of the imidazole of histidine, preserving the hydrogen bond acceptor (Figure 3, panel a). Our pHis analogue design also took synthetic practicality into consideration since these analogues were to be used in SPPS, which generally requires excess amount of reagents. Accordingly, pTza derivatives **12** and **13**, as analogues of τ- and π-pHis, respectively, were prepared in preparative scale within 4 steps from commercial materials. Of note, both **12** and **13** are available from the same building blocks (**10** and **11**) by simply employing different catalysts in the cycloaddition step. More recently, McAllister and coworkers reported the preparation of an Fmoc-protected version of pTza analogue **12** as well as its application in SPPS.[86]

Both pTza analogues were successfully utilized in the subsequent SPPS of phosphopeptides corresponding to the N-terminal tail of histone H4. The peptide containing **12** was subsequently used as the epitope to generate polyclonal antibodies (**anti-τ-pHis18**) that cross-reacted with histone H4 phosphorylated at His18 but not with the nonphosphorylated histone. Peptide dot blots show that **anti-τ-pHis18** does not recognize other phosphoamino acids or the unmodified histidine (Figure 3, panel b). Histone H4 can be phosphorylated on both of its histidines (His18 and His75), but only the phosphorylation at His18 was recognized, demonstrated by the blots using alanine mutants (Figure 3, panel c). Significantly, this constitutes the first example of pHis-specific antibodies. Development of other pHis-specific antibodies, including sequence-independent pan-pHis antibodies, is in progress in our laboratories. In the same study we generated an α-thioester peptide incorporating pTza by SPPS. This peptide was utilized in the subsequent native chemical ligation with a recombinant fragment of histone H4, providing a full-length histone H4 with pTza in the place of His18. Semisynthetic proteins incorporating nonhydrolyzable analogues of pSer and pTyr have proven to be valuable tools in kinetic studies and pull-down assays to investigate the exact role of the phosphorylation in the parent protein.[84] Analogously, pTza-containing proteins are expected to play important roles in advancing our understanding of histidine phosphorylation.

SUMMARY AND FUTURE PERSPECTIVE

Although protein histidine phosphorylation has been known for 50 years, our understanding of this elusive PTM, particularly in eukaryotic systems, is still limited due to the intrinsic instability of pHis and the technical challenges associated with it. Given

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the strikingly high abundance of pHis in a model eukaryotic organism,[12] there might well exist many unknown biological pathways involving protein histidine phosphorylation, waiting to be discovered and explored with adequate research tools.

In recent years, remarkable advances have been made to address the technical challenges associated with pHis. With improved sample preparation processes and milder ionization/fragmentation methods, mass spectrometry has become increasingly more useful in the identification of histidine-phosphorylated proteins. Still, most studies have used purified proteins or peptides phosphorylated *in vitro*, and identification of novel pHis-containing proteins from *in vivo* samples is still challenging. Mild enrichment methods specific for pHis will be invaluable as this field develops. Recent successes with pTza as stable pHis analogues enabled the development of the first pHis-specific antibodies, which will greatly facilitate the purification and identification of histidine-phosphorylated proteins, just as pTyr-specific antibodies did in the tyrosine phosphorylation research.[76] Semisynthetic proteins incorporating pTza will also be useful in elucidating the function of the histidine phosphorylation in individual proteins.

With the advent of the research tools surveyed in this review, we believe the next few years will be exciting times for histidine phosphorylation research. In the phosphoamino acid family, phosphohistidine remains the less recognized "older" sibling of pTyr. It is worth remembering, however, that 30 years ago pTyr was hardly known to the biomedical community, even less so than phosphohistidine. Ever since, tyrosine phosphorylation research has blossomed, fueled by a plethora of powerful research tools that have ultimately led to the development of clinical drugs such as imatinib. Will phosphohistidine follow the footsteps of phosphotyrosine? Time will tell.

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KEYWORDS

Posttranslational modification (PTM): chemical alterations of protein side chains or backbone connectivity that occur after the protein is translated from RNA; Phosphoproteomics: a global analysis protein phosphorylation states in a proteome; Histidine phosphorylation: a PTM in which a phosphoryl group is added to a histidine residue on a nitrogen of its imidazole side chain; phosphohistidine, the phosphorylation product, is unstable under acidic conditions; Phosphoramidate: a functional group in which an oxygen in a phosphate is substituted with a nitrogen; phosphoramidates are much more easily hydrolyzed than phosphate esters under acidic conditions; Histidine kinase: a protein that phosphorylates histidine residues in itself or in other proteins; Two-component signaling system: a signaling system used in bacteria, fungi, and plants; it consists of a protein histidine kinase and downstream response regulator transcription factors; Phosphohistidine analogue: a molecule that mimics the structure and function of phosphohistidine; stable phosphohistidine analogues that do not undergo facile dephosphorylation have been developed and utilized to overcome the stability issues of phosphohistidine; Protein semisynthesis: a method to prepare proteins by the ligation of a synthetic and a recombinant polypeptide. The synthetic peptide fragment can contain a variety of PTM or nonnatural functional groups to be incorporated into the semisynthetic protein

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