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Focus on phosphohistidine

Review Article

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Summary. Phosphohistidine has been identified as an enzymic intermediate in numerous biochemical reactions and plays a functional role in many regulatory pathways. Unlike the phosphoester bond of its cousins (phosphoserine, phosphothreonine and phosphotyrosine), the phosphoramidate (P–N) bond of phosphohistidine has a high ΔG° of hydrolysis and is unstable under acidic conditions. This acid-lability has meant that the study of protein histidine phosphorylation and the associated protein kinases has been slower to progress than other protein phosphorylation studies.

Histidine phosphorylation is a crucial component of cell signalling in prokaryotes and lower eukaryotes. It is also now becoming widely reported in mammalian signalling pathways and implicated in certain human disease states. This review covers the chemistry of phosphohistidine in terms of its isomeric forms and chemical derivatives, how they can be synthesized, purified, identified and the relative stabilities of each of these forms. Furthermore, we highlight how this chemistry relates to the role of phosphohistidine in its various biological functions.

Keywords: Phosphohistidine – Histidine kinase – Phosphoramidate

1. Introduction

Reports of protein phosphorylation first appeared in the early twentieth century. Phosphorylation of the protein vitellin was first identified in 1906 by Levene at the Rockefeller Institute for Medical Research (Levene and Alsberg, 1906). Although he had detected phosphate in this protein, it took until 1933 to identify phosphoserine as the phosphoamino acid (Lipmann and Levene, 1932). Since then, the number of amino acids known to be phosphorylated has grown to nine [serine, threonine, tyrosine, histidine, lysine, arginine, aspartate, glutamate, cysteine] (or ten if you include phosphohydroxyproline). Although phosphoserine (**1**), phosphothreonine (**2**) and phosphotyrosine (**3**) (Fig. 1) capture most of the attention in the literature, this review turns the spotlight away from this group and focuses on the chemistry and biological significance of phosphohistidine (**4** and **5**).

Histidine was first synthetically phosphorylated in 1947 (Severin and Yudelovich, 1947) and phosphohistidine was first identified biochemically in 1963 by Paul Boyer's group (Deluca et al., 1963). Boyer subsequently identified phosphohistidine as a phosphoenzyme intermediate of succinate thiokinase in rat liver mitochondria and set the foundation for all subsequent research in this field. Since that time, the phosphorylation of histidine in some enzymes has been recognised as a step in catalysis leading to the formation of a phosphoenzyme intermediate. Histidine phosphorylation is now also widely accepted as being a crucial component of cell signalling in prokaryotes and lower eukaryotes (Klumpp and Krieglstein, 2002). Although reports of such histidine phosphorylation in mammalian cells have been slow to follow, it is also becoming more commonly reported in mammalian signalling pathways and is implicated in certain human disease states (Besant et al., 2003; Tan et al., 2004).

The low frequency of reports of phosphohistidine's role in biological systems compared to that of phosphoester amino acids is mostly due its chemical instability. The P–N (phosphoramidate) bond of phosphohistidine has a higher ΔG° of hydrolysis than the P–O bond of the phosphoester amino acids (**1**–**3**). In addition, phosphohistidine is acid-labile whilst these other phosphoamino acids are acid-stable (Duclos et al., 1991). This acid-lability has meant that the investigation and characterisation of protein histidine phosphorylation and the associated protein kinases has been slower to progress than other protein phosphorylation

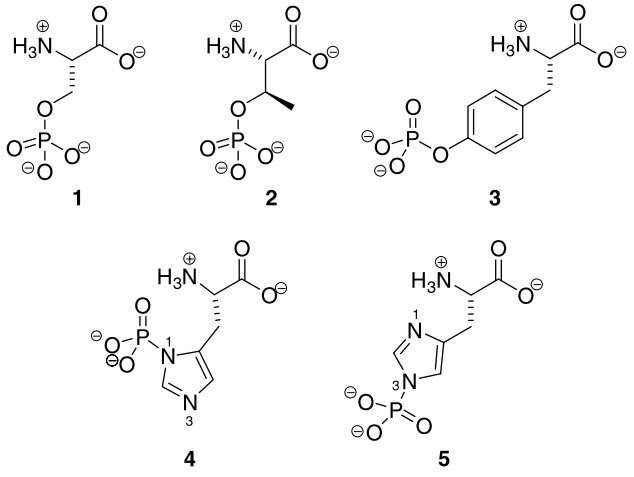


Fig. 1. Common phosphoamino acids. See note on numbering of histidine in the text

studies, since many of the techniques classically used to study protein phosphorylation involve acidic conditions. This is now changing as advances in methodology and instrumentation emerge to overcome these stability issues and are expected to help elucidate the importance of protein histidine phosphorylation in biological systems.

This review will cover the chemistry of phosphohistidine in terms of its isomeric forms and chemical derivatives and the synthesis, purification, identification and relative stabilities of each of these forms. Furthermore, we aim to highlight how this chemical information relates to the role of phosphohistidine in its various biological functions.

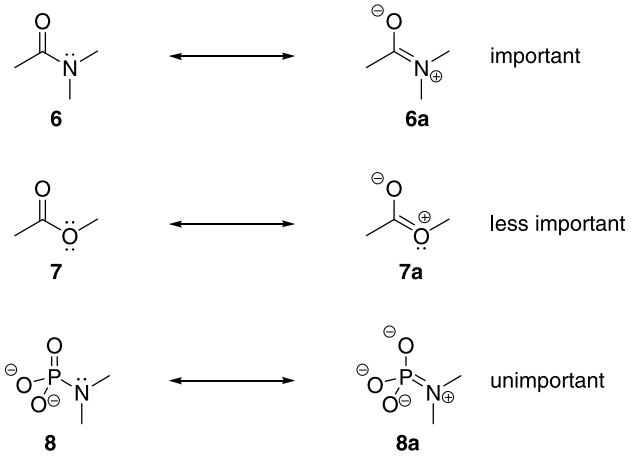
2. Stability of phosphohistidine

Unlike the phosphate esters, phosphoserine (**1**), phosphothreonine (**2**) and phosphotyrosine (**3**) (Fig. 1), the phosphoryl group in phosphohistidine is attached to an imidazole nitrogen, making it a phosphoramidate. As indicated in Fig. 1, there are two biologically relevant phosphohistidine isomers, **4** and **5** (Walinder, 1969; Edlund, 1982; Waygood et al., 1988; Huang et al., 1991; Gross et al., 1996; Spronk et al., 1976; Narindrasorasak and Bridger, 1977; El-Maghrabi and Pilkis, 1984; Huebner and Matthews, 1985; Tauler et al., 1987; Kumble et al., 1996). There seems to be an error, perpetuated in the literature, regarding the numbering of the imidazole ring in the phosphohistidines. IUPAC and Chemical Abstracts designate isomer **5** as 1-phosphohistidine, since the alkyl substituent on the imidazole ring is thereby given the lowest possible number. Historically, however, the vast majority of authors have assigned 1-phosphohistidine as isomer **4** (Hultquist et al., 1966; Pirrung, 1999, Besant and Attwood, 2005). To avoid further confusion, we have decided to stick with the latter convention in this review, that is, 1-phosphohistidine is **4** and 3-phosphohistidine is **5**. Others have more recently used the IUPAC numbering system (Schenkels et al., 1999). Whatever numbering system is used, the confusion highlights the importance of including structural diagrams where regiosiomeric ambiguities are possible.

The chemical stability of the phosphoramidate bond of phosphohistidine in a protein is a key element of its biological function. Thermodynamically, the phosphoester bonds in free phosphohydroxyamino acids and in proteins are more stable (ΔG° of hydrolysis $= -6.5$ to -9.5 kcal mol^{-1}) than the phosphoramidate bond in phosphohistidine (ΔG° of hydrolysis ~ -12 to -14 kcal mol^{-1} (Stock et al., 1990). This is in stark contrast to carboxylic amides (**6**) which are much more stable than esters (**7**). For example, the $\Delta G^{\circ\prime}$s of hydrolysis of acetyl choline and glycine methyl ester are -6.1 and 8.4 kcal mol^{-1} respectively. The internal peptide bonds of a protein, have a $\Delta G^{\circ\prime}$ of hydrolysis of ~ -0.5 kcal mol^{-1} (Mahler and Cordes, 1971), and much of that energy is associated with ionisation of the amine product (Carpenter, 1960).

The increased hydrolytic stability of amides is due to significant delocalisation of the nitrogen lone pair electrons onto the carbonyl oxygen, which strengthens the N–C bond and makes the carbonyl carbon less electrophilic and therefore less susceptible to attack by nucleophiles such as water. Thus, the charge-separated canonical form **6a** is a significant contributor to the electronic structure of amides (Scheme 1). This familiar explanation is also used to rationalise the lack of basicity of the amide nitrogen and rigidity of (restricted rotation about) the amide bond.

Because of the greater electronegativity of oxygen, the ester oxygen lone pair is lower in energy and not delo-



Scheme 1

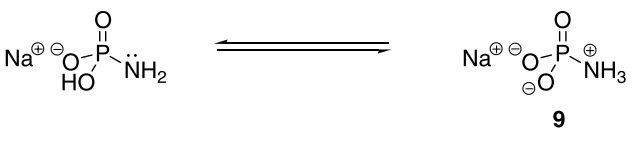
calised as readily. Accordingly, **7a** does not contribute as significantly to an ester's electronic structure and the C–O bond in esters is weaker than the C–N bond in amides. Furthermore, the stronger inductive effect of the more electronegative oxygen atom makes the carbonyl carbon of esters more electrophilic and thus more susceptible to attack by water and other nucleophiles.

Despite the structural similarities between amides and phosphoramidates (e.g. **8**) the contribution of canonical form **8a** is rather insignificant (Fig. 1) (Modro, 1981; Emsley and Hall, 1976). There is poor overlap of the nitrogen lone pair orbital with the phosphoryl π-bond because the phosphorus d-orbital involved in π-bonding is in a higher energy shell and hence further from the nucleus than the lone pair orbital on nitrogen (Modro, 1981). Accordingly, the phosphoramidate N–P bond does not benefit from any substantial stabilisation due to delocalisation of electrons.

One important consequence of the localisation of the nitrogen lone pair is that phosphoramidate nitrogens are basic. Indeed, monosodium phosphoramidate exists as the zwitterion **9** in the solid state (Scheme 2) (Hobbs et al., 1953) and, presumably, this form is favoured in neutral solution. Once protonated, the leaving ability of the amino group is markedly enhanced and this is a key factor affecting rate of hydrolysis in phosphoramidates and monophosphate esters (Kirby, 1978).

In the specific case of phosphoimidazoles like phosphohistidine, the lone pair formally provided by the phosphoramidate nitrogen, is delocalised in a molecular orbital associated with the aromaticity of the imidazole ring, as illustrated for phosphoimidazole (**10**) in Scheme 3. Hence, delocalisation of this lone pair onto the phosphoryl group, as illustrated at the extreme by canonical form **10a**, would be negligible. *N*-Phosphoimidazoles are thus analogous to carbonyl imidazoles which have reactivity more akin to acid chlorides than amides.

For the same reason as described above, the phosphoramidate nitrogen in **10** and the phosphohistidines **4** and **5** is also non-basic. Protonation on the phosphoramidate nitrogen as in **11** would disrupt the aromaticity of the imidazole ring and hence does not occur (Scheme 4). However, the other imidazole nitrogen is quite basic with a pK_{aH} of ∼7.0 in phosphoimidazole (**10**) (Jencks and Gilchrist, 1965), 7.3 in 1-phosphohistidine (**4**) (Hultquist, 1968) and 6.4 in 3-phosphohistidine (**5**) (Hultquist et al., 1966). Even at pH 7, the non-phosphorylated imidazole nitrogen would be significantly protonated as in **12**. The leaving group ability of the protonated imidazole ring in **12** is increased as the electron density on the phosphoramidate nitrogen is greatly reduced and the ejected leaving group is neutral. Indeed, the rates of hydrolysis of phosphoimidazole (Jencks and Gilchrist, 1965), 1-phosphohistidine (Hultquist, 1968) and 3-phosphohistidine (Hultquist et al., 1966) increase significantly over the pH range 9–6, corresponding to increasing proportions of protonated histidine. Hydrolysis is slow at higher pH, as discussed below.



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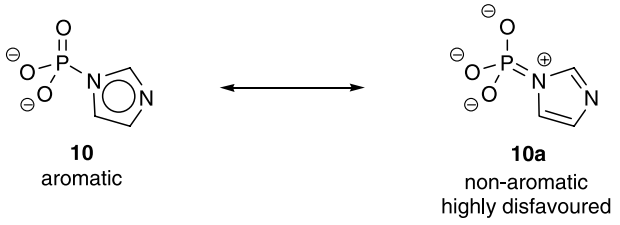
Scheme 2

One might look for support for the reasoning above in the hydrolysis of *N*-phosphorylpryrrole (**13**) (Scheme 5). The strength of the N–P bond in **13** should be very similar to that in *N*-phosphorylimidazole (**10**), but it should be more resistant to hydrolysis than *N*-phosphorylimidazoles or phosphoramidates derived from aliphatic amines because it lacks a basic nitrogen. Unfortunately, *N*-phosphorylpryrrole (**13**) has never been reported, however, the expected reactivity is borne out in the analogous cyclic phosphoramidates **14** and **16** (Scheme 5). In reactions of **14** with alcohols, the pyrrole ring is retained in the product **15**, but with **16**, imidazole (**18**) is lost too rapidly for the reaction rate to be observed (Ramirez et al., 1977). This contrasting reactivity was attributed to the ''apicophobic'' nature of the pyrrole nitrogen, but a simpler explanation is that general acid catalysis in the case of the imidazole **16** stabilises the transition state en route to the triester product **17**.

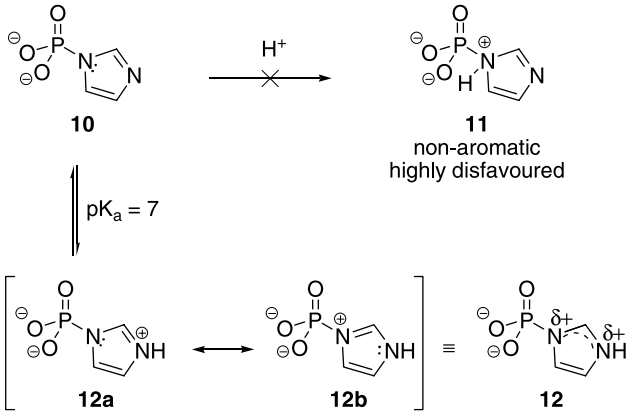
It should be noted that previous studies of the hydrolysis of phosphoimidazole have shown that it is rather insensitive to general acid or general base catalysis (Lloyd et al., 1971) but these were conducted at slightly acidic pHs, under which conditions the imidazole nitrogen would already be protonated. In other words, it is the monoanion **12** that is insensitive to general catalysis, but protonation of the distal imidazole nitrogen is necessary before P–N cleavage can occur.

Phosphohistidines, therefore, possess a high-energy phosphoramidate bond and have a greater propensity (than phosphate esters) to transfer the phosphoryl group to other molecules. Two-component and multi-component phospho-relay signalling systems found in bacteria, and lower eukaryotes make full advantage of this characteristic of phosphohistidine (see details below).

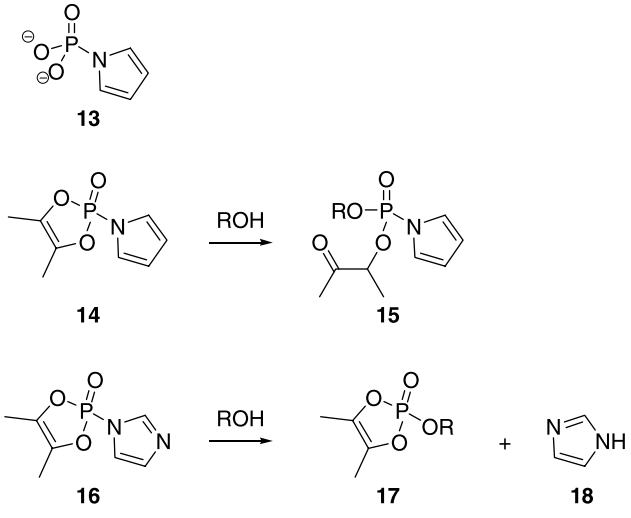
In addition to this thermodynamic property of phosphohistidine, its kinetic stability under acidic and basic conditions also differs from the phosphohydroxy amino acids.



Scheme 3



Scheme 4



Scheme 5

The phosphohydroxy amino acids are stable even in a reasonably harsh acidic environment. In the presence of 1 M HCl at 100 °C the half-lives of free phosphoserine and phosphothreonine are about 18 h, whilst that of phosphotyrosine is about 5 h (Duclos et al., 1991). For the reasons given above, free phosphohistidine is unstable in acid such that in 1 M HCl at 49 °C, 1-phosphohistidine and 3-phosphohistidine have half-lives of 18 and 25 s respectively (Hultquist, 1968).

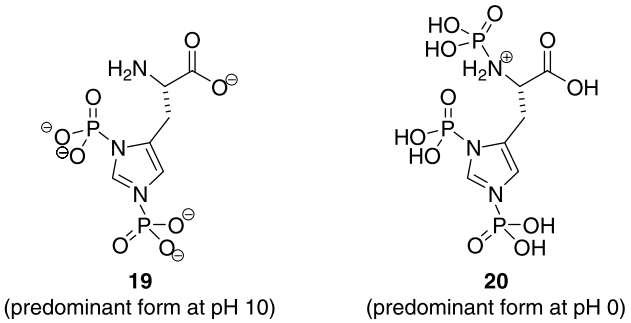


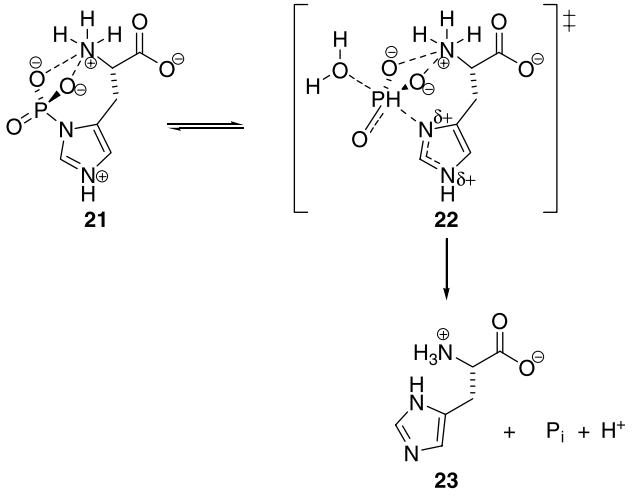
Fig. 2. 1,3-Diphosphohistidine. See text for details

The difference in kinetic stability between 1- and 3-phosphohistidine is accentuated at milder pHs, for example, the rate of hydrolysis of the 1-isomer is ~10 times that of the 3-isomer at pH 5 (Hultquist, 1968). This has been attributed to the proximity of the *N*1-phosphoryl group (Hultquist 1968; Matthews, 1995) to the protonated α-amino (aminium) group and is supported by the observations that:

1. In 1,3-diphosphohistidine (**19**, Fig. 2) there is a marked decrease in rate of hydrolysis above pH 9, corresponding to loss of a proton with a pK_a of ~9.6.
2. There is a lag in the hydrolysis of α-*N*,1,3-triphosphohistidine (**20**) under acidic conditions, suggesting that phosphorylation of the α-amino group stabilises the *N*1-phosphoramidate (Hultquist, 1968), although steric hindrance may also play a role in this case.

It seems unlikely that the aminium group enhances the rate of hydrolysis of the proximal phosphoramidate by general acid catalysis, given that phosphoimidazole monoanion (**12**) is insensitive to general acid catalysis (Lloyd et al., 1971), although the intramolecular nature of the interaction in 1-phosphohistidine (**4**) may invalidate this comparison. Perhaps more likely, the aminium group facilitates hydrolysis through an electrostatic or H-bonding interaction with one or two of the phosphoryl oxygens as in **21**, firstly, by enhancing the electrophilicity of the phosphorus and secondly, through anchimeric assistance: stabilisation of the pentavalent S_N2-like transition state **22** en route to the products, histidine (**23**) and hydrogen phosphate (Scheme 6). In creatine kinase, the aminium and guanidinium groups of lysine and arginine residues respectively, are thought to accelerate phosphoryl group transfer from ATP to creatine in the same way (McLaughlin et al., 1976).

An alternative explanation, plausible under less acidic conditions, is that the deprotonated amino group in a species such as **24** is acting as a nucleophilic catalyst (Scheme 8). Phosphoimidazole monoanion (**12**, Scheme 4) is quite reactive toward nucleophilic attack by amines (Lloyd et al.,



Scheme 6

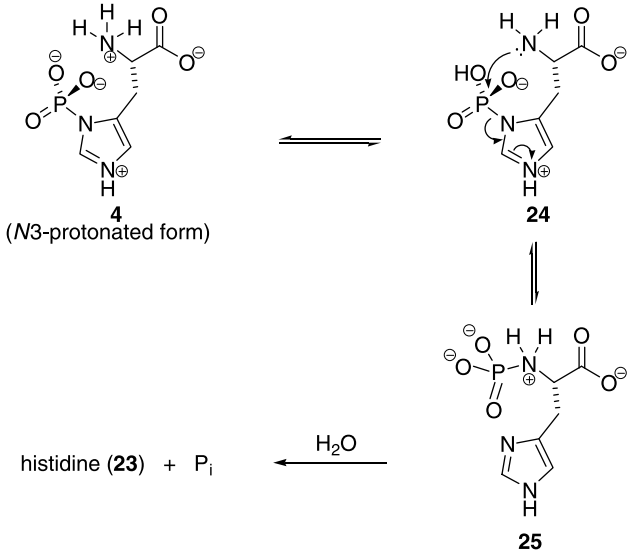
1971) and phosphoramidate zwitterions such as **25** hydrolyse at least an order of magnitude faster than phosphoimidazole monoanion (Lloyd et al., 1971 and references therein). This explanation would seem to be incongruous with the observation that the rate of hydrolysis of 1,3-diphosphohistidine (**19**) drops off markedly beyond pH 9, however in this case, it may be that deprotonation of the phosphoryl groups is also reducing hydrolytic reactivity.

Whatever the mode of catalysis involved, it is not available to the 3-phosphohistidine isomer in an intramolecular sense, since this would involve interaction of the amino and phosphoryl groups through, at most, a highly strained 9-membered ring containing a *trans* double bond. Similarly, in peptides and proteins, 1-phosphohistidine residues would be expected to be more stable than free phosphohistidine since the peptide bond precludes protonation (Matthews, 1995) and dramatically attenuates nucleophilicity relative to a free amino group.

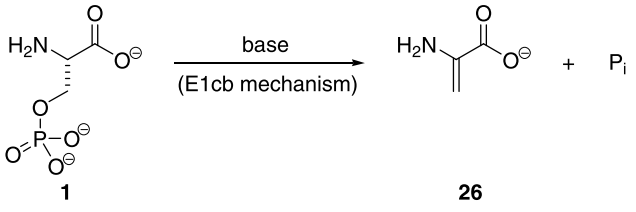
Under alkaline conditions, both free phosphoserine and phosphothreonine are unstable. Treatment of both at 37 °C in 1 M NaOH for 18–20 h results in complete dephosphorylation (Duclos et al., 1991). However, when these phosphoamino acids are present in proteins and peptides, their lability is can vary depending on the relative stabilising influences of neighbouring amino acids (Matthews, 1995). Phosphotyrosine and phosphohistidine are both alkalistable because, under these conditions, the imidazole nitrogen is not protonated and the imidazolate anion is a very poor leaving group. Care does need to be taken when heating them in an alkaline solution that evaporation does not result in increased concentrations of the base (Besant and Attwood, 1998). In instances where alkaline phosphoamino acid analysis of phosphoproteins is performed in 3 M KOH at 100 °C, a mineral oil overlay is recommended to prevent evaporation of water resulting in concentration of KOH. At concentrations of 6 M KOH at 100 °C for phosphohistidine and 9 M KOH at 100 °C for both, phosphohistidine and phosphotyrosine are dephosphorylated (Besant and Attwood, 1998).

The rate of hydrolysis of simple phosphate monoesters is optimal at pH 4, under which conditions the rates of hydrolysis are directly correlated with the ability of the organic moiety to stabilise a negative charge (i.e. leaving group ability) (Corbridge, 1985). Thus, at pH 4 and 100 °C, phenyl phosphate is hydrolysed 43 times more rapidly than ethyl phosphate (Corbridge, 1985) and accordingly, phosphotyrosine would be expected to hydrolyse more rapidly than phosphoserine and phosphothreonine. However, the decreased stability of phosphoserine and phosphothreonine relative to phosphotyrosine under basic conditions is not due to hydrolysis but β-elimination, to give, in the case of phosphoserine (**1**), dehydroalanine (**26**) (Matthews, 1995) (Scheme 8).

Other chemical stability issues arise in solvents such as pyridine or 1 M hydroxylamine (Duclos et al., 1991) where



Scheme 7



Scheme 8

the phosphohydroxy amino acids are stable but phosphohistidine is dephosphorylated. As mentioned above, phosphoimidazoles are quite susceptible to attack by amine nucleophiles (Lloyd et al., 1970) and the resulting phosphoramidates are rapidly hydrolysed. Pyridine is commonly used in thin layer chromatography (TLC) solvents to separate phosphoester amino acids, however, since it can catalyse hydrolysis of phosphohistidine, its use for the separation of the latter is not recommended.

The cleavage of phosphohistidine by pyridine and hydroxylamine has been used to advantage in detecting protein histidine phosphorylation. As an example, the rate of pyridine and hydroxylamine-mediated cleavage of the alkali-stable 3-phosphohistidine residue present in the phosphorylated bacterial factor III lac protein was measured (Hays et al., 1973). They determined second-order rate constants of 0.55 and 0.43 M^{-1} min^{-1} respectively for hydroxylamine and pyridine catalysed dephosphorylation of this protein.

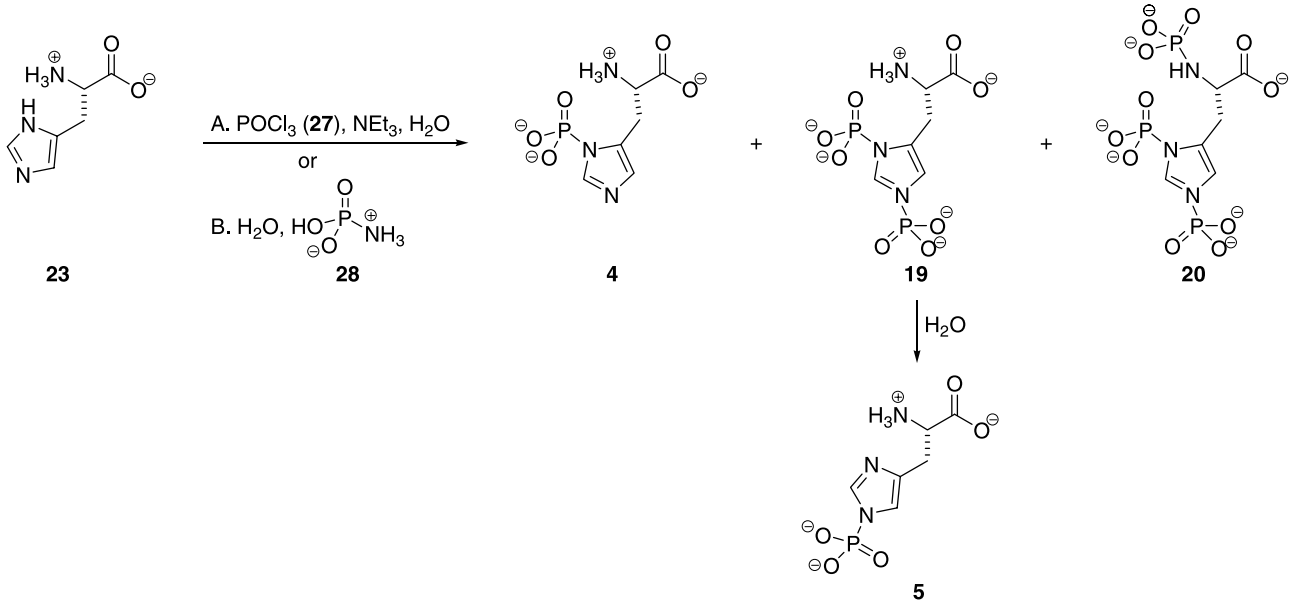
The acid-lability of phosphohistidine compared to the phosphohydroxy amino acids has been a major problem in the study of histidine phosphorylation and the associated protein histidine kinases. It has meant that histidine phosphorylation has often been neglected as a topic of research into post-translational modifications of proteins. Ironically, it is by its very nature that the acid-lability/alkali-stability of phosphohistidine is a basis for distinguishing it from the phosphohydroxyamino acids (and in rare cases, phosphocysteine which has similar stability to phosphotyrosine) (Hultquist, 1968).

3. Synthesis, purification, detection and identification of phosphohistidine

Histidine (**23**) is unique among amino acids in that it has an imidazole ring in its side chain. This imidazole ring has a pK_{aH} of 6.5 in the free amino acid and it is for this reason that histidine often features as an acid-base catalyst in enzyme-catalysed reactions. Histidine can be synthetically phosphorylated to form a phosphoramidate (P–N) bond on either (or both) *N*1 or *N*3 of the imidazole ring. Two different electrophiles have been used for this purpose, phosphoryl chloride (**27**), and phosphoramidate (**28**) (Scheme 9).

Histidine was first phosphorylated by reaction with phosphoryl chloride in alkaline solution (Severin and Yudelovich, 1947) and later with phosphoramidate (Müller et al., 1956). Although it was deduced that the site of phosphorylation was on an imidazole nitrogen, only partial purifications were achieved. Boyer prepared both isomers by reaction with phosphoramidate and isolated them by anion exchange chromatography (Hultquist et al., 1966).

In addition to the biologically relevant phosphohistidines, both 1,3-diphosphohistidine (**19**) and α-1,3-triphosphohistidine (**20**) can be prepared with either phosphoramidate or phosphoryl chloride and isolated under basic conditions (Scheme 9) (Hultquist, 1968). Phosphohistidine has also been synthesised by phosphorylation of polyhistidine with phosphoryl chloride and subsequent alkaline hydrolysis (DiSabato and Jencks, 1961).



Scheme 9

The phosphorylation of histidine by potassium phosphoramidate has complex reaction kinetics. ^{31}P NMR studies have shown that the 1-isomer (**4**) forms most rapidly (t½ = 10 min), followed by the appearance of 1,3-diphosphohistidine (**19**) (t½ = 70 min). Hydrolysis of the 1-phosphoryl group gives the more stable 3-isomer (**5**) (t½ = 100 min) which is the dominant product with prolonged reaction times (Besant et al., 1998). As discussed above, it is thought that both 1-phosphohistidine (**4**) and 1,3-diphosphohistidine (**19**) are less stable than 3-phosphohistidine (**5**) because of the close proximity of the 1-phosphoryl group to the protonated α-amino group (Hultquist, 1968; Matthews, 1995). Given the rapid appearance of the 1-isomer (the kinetic product), it appears as if the α-amino group also has a role in catalysing phosphorylation at *N*1.

Methods to distinguish the different isoforms of phosphohistidine have been improved over many years of research. Paper electrophoresis (Hultquist et al., 1966), thin-layer electrophoresis (Besant and Attwood, 2000), TLC (Gassner et al., 1977) and reversed-phase thin-layer chromatography (RP-TLC) (Besant et al., 2000) have all been used to separate mixtures of the different phosphohistidine isoforms. Reverse phase (RP) column chromatography has been also used to separate and identify all of the different isoforms of phosphohistidine. RP-column chromatography was performed in conjunction with both ^{1}H and ^{31}P nuclear magnetic resonance spectroscopy (NMR) to verify the identity of **4**, **5** and **19** (Besant et al., 1998). Biologically-derived phosphohistidine has also been detected using ^{31}P NMR. Facilitated by internal phosphohistidine isoform standards, protein ^{31}P NMR in conjunction with HPLC was used to detect 1- and 3-phosphohistidine in histone H4 phosphorylated by histone H4 histidine kinases (HHKs) from regenerating liver cells and Walker 256 carcinosarcoma cells (Fujitaki et al., 1981).

More recently, mass spectrometry has been used to identify phosphohistidine both as the free phosphoamino acid and in proteins. The solvent conditions and the time samples spend in certain solvents are critical for the detection of phosphohistidine. Where possible, acidic solvents including formic acid or trifluoroacetic acid (TFA) should be avoided. Ideally, methanol/ammonia is used as solvent to stabilise the phosphohistidine and is useful in negative ion mode, however, to improve ionisation in positive ion mode a 20 mM ammonium bicarbonate solution in 50% methanol can be used. Purified 3-phosphohistidine was first identified by negative ion electrospray mass spectrometry by Besant et al. (2000) and it has also been identified by various mass spectrometry methods in proteins/peptides by others (see below).

One such mass spectrometric technique used to detect histidine phosphorylation of proteins is combined reverse-phase HPLC-Electrospray Ionisation Mass Spectrometry (ESI-MS) and element mass spectrometry with phosphorus (^{31}P) detection (also known as Inductively Coupled Plasma Mass Spectrometry (ICP-MS)). Using a model of histidine phosphorylation in the bacterial chemotaxis regulatory two-component histidine kinase (CheA), tryptic digests of the phosphoprotein were analysed to successfully identify phosphohistidine by this combination of methods (Wind et al., 2005). Although the solvents used in this study were acidic, the relative degree of phosphorylation was measured over time. The initial degree of phosphorylation was established at 40% by measuring the peak intensities of previously determined histidine phosphorylated peptides. Over a 60-minute period this dropped to 10%. This highlighted: i) the probable underestimation of protein histidine phosphorylation simply due to acid-lability (the same applies to other acid-labile protein phosphoamino acids under standard mass spectrometry sample solvent conditions); ii) the importance of the chemical environment used in preparing and detecting phosphohistidine-containing samples.

A popular enrichment technique currently employed by researchers interested in isolating phosphoproteins or phosphopeptides involves the use of immobilised metal affinity chromatography (IMAC). This type of affinity resin uses various divalent metal cations complexed to a solid support, which are used to bind phosphopeptides. A large array of metal ions (gallium, nickel, iron, zinc, cobalt, copper) have been used for purifying phosphopeptides but one in particular, copper II (Cu^{2+}), has been used for purifying a histidine-phosphorylated peptide. This enrichment technique was used prior to analysis by matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI TOF-MS) (Napper et al., 2003).

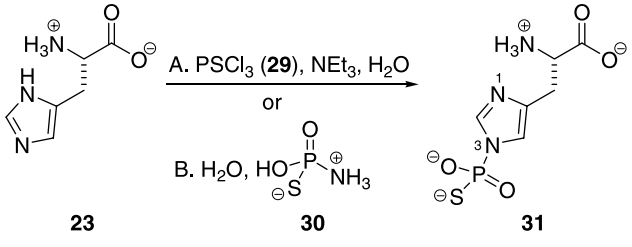
IMAC matrices can also be used to bind proteins or peptides at neutral to alkaline pHs. One common problem faced by all IMAC resins is the non-specific binding from either acidic non-phosphorylated peptides or histidine-rich protein/peptides. These often co-elute with the desired phosphoprotein/phosphopeptide and make analyses of mass spectra difficult. For the copper IMAC resin (Napper et al., 2003), co-purification of false-positive non-phosphorylated peptides was avoided by loading the sample under mildly acidic conditions for only a brief amount of time. As the pKa of the imidazole ring of histidine residues is ~6.5, the ring becomes significantly protonated at a pH lower than 6.5 and will no longer be able to bind to the divalent copper ions. In theory, this means that only the

phosphorylated peptides should bind. (N.B. peptides rich in D and E residues may also bind as the carboxyl side chain has a pKa ~4.4). Elution of phosphohistidine phosphopeptides from the copper IMAC resin was successfully achieved using an alkaline solution of ammonia with subsequent neutralization of the sample with trifluoroacetic acid (Napper et al., 2003).

As shown by Napper et al. (2003) the copper IMAC-enriched phosphopeptides can be analysed by MALDI TOF-MS. Similar to the findings for ESI-MS analysis of phosphohistidine peptides (Wind et al., 2005), it was found that in positive ion mode the relative abundance of the phosphohistidine phosphopeptide to non-phosphorylated peptide was 1:25. Identifying phosphohistidine-containing peptides by MALDI in positive ion mode proved challenging due to the lability of phosphohistidine. Even in negative ion mode, there was still evidence of dephosphorylation of phosphohistidine-containing peptides, illustrating once again the challenges faced in detecting this type of post-translational modification in biological samples.

4. Phosphohistidine analogues

Due to the acid-labile nature of the phosphoramidate bond, researchers have adopted different methods to prepare analogues of phosphohistidine with improved stability. One such method, involves substitution of a phosphoryl oxygen with a sulphur atom to give thiophosphorylhistidine (**31**) (Scheme 10). While the sulfur atom in the thiophosphoryl group is calculated to have only minor effects on the steric and electronic properties relative to the phosphoryl group (Pirrung et al., 2000), it imparts much greater acid stability in the free amino acid, in a thiophosphohistidine-containing synthetic peptide and in the catalytic subunit of the yeast histidine kinase Sln1 (Lasker et al., 1999). One possible explanation for this might be due to the lower electronegativity of the sulfur atom which in turn provides an increase in the stability of the thiophosphoramidate bond and a concomitant decrease in the hydrolysis sensitivity of the thiophosphohistidine.



Scheme 10

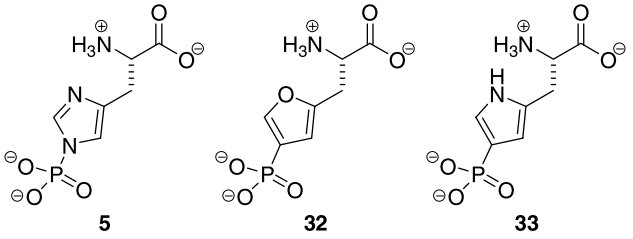


Fig. 3. Stable analogues of phosphohistidine. See text for details

Thiophosphorylation of histidine at *N*3 can be achieved enzymatically by the yeast histidine kinase Sln1 using ATPγS as a substrate for autophosphorylation, or chemically, using methods similar to normal phosphorylation protocols, either with thiophosphoryl chloride (**29**) (Lasker et al., 1999) or thiophosphoramidate (**30**) (Scheme 10) (Pirrung et al., 2000).

Although thiophosphorylation of histidine is a useful tool for in vitro kinase, or synthetic chemical reactions, there are limitations to its use. For example: in vivo chemical histidine thiophosphorylation is unfeasible because it will globally phosphorylate all available histidine, and other nucleophilic residues. Moreover, thiophosphorylation gives only the *N*3-isomer. Biochemically, not all the histidine kinases may be able to use γ-^{35}S-ATP and γ-^{35}S-thio-GTP as substrates (Besant and Attwood, 2005).

The other method of creating a more stable analogue of phosphohistidine is to replace the P–N bond of the phosphoramidate with a hydrolytically stable P–C bond, to give a phosphonate. This has been done by simultaneously substituting a carbon atom for the phosphorylated nitrogen atom of 3-phosphohistidine (**5**) and an oxygen atom for *N*1, giving the furan analogue **32** (Fig. 3) (Schenkels et al., 1999). The authors of this work substituted the oxygen for nitrogen to mimic the H-bond accepting properties of the imidazole nitrogen; the analogous pyrrole derivative **33** would be an H-bond donor.

5. Phosphohistidine-specific antibodies

Anti-phosphotyrosine antibodies have proved extremely useful for the detection of phosphotyrosine residues in phosphoproteins and for immunoaffinity concentration of such phosphoproteins prior to digestion and mass spectrometric peptide analysis. Given the unique structure of the phosphohistidines, it should be possible to raise selective antibodies to this hapten. However, attempts that have been made to generate such antibodies have been unsuccessful. The most likely cause for this is that when phosphohistidine or peptides containing this phosphoamino acid are part of the immunogen, hydrolysis occurs

too quickly to elicit a strong immune response. Future attempts at making phosphohistidine specific antibodies may have more success using non-hydrolysable analogues of phosphohistidine (Schenkels et al., 1999) or more stable thiophosphohistidines. Ideally, two different antibodies will be required to discriminate between the 1- and 3-isomers reported to occur in biological systems.

A peptide containing the pyrrole analogue **33** [CAA**33**] was used in an attempt to generate polyclonal phosphohistidine antibodies. Although antibodies were successful produced, we found that they were selective for only the analogue and not genuine phosphohistidine.

6. How the chemistry of phosphohistidine relates to biology

There are several excellent reviews on two-component histidine kinases that are found in bacteria, fungi and plants (Stock et al., 1990; Dhillon et al., 2003; Grefen and Harter, 2004). Similarly, there are reviews of histidine kinases in mammalian systems (Steeg et al., 2003; Besant et al., 2003; Klumpp and Krieglstein, 2005), although most of these are not of the two-component variety. What all of these studies have in common is the fact that histidine phosphorylation is used in various cell-signalling pathways or is functionally implicated in certain biological processes. As shown by Boyer's group back in 1963 (Deluca et al., 1963), the chemistry of phosphohistidine has certain advantages in biochemical reactions. The propensity for phosphohistidine to transfer its high-energy phosphoryl group to other molecules is used in two-component signalling systems. It has also been postulated that given a favourable acidic pH in the intracellular microenvironment, the labile nature of the phosphoramidate bond of phosphohistidine may be used in a system that requires an on/off switch without the need for additional protein interactions with protein phosphatases.

Many of the two-component systems are involved in signalling systems for sensing the changes of external environment such as temperature, osmolarity, chemo attractants and pH. For example, fruit ripening in plants is a response to changes of ethylene concentration through regulation of the histidine kinase activities of ethylene receptors (Grefen and Harter, 2004).

A typical two-component signalling system found in plants and bacteria is composed of two major functional parts: (1) the histidine kinase and (2) the response regulator protein (Fig. 4). The receptor or sensor protein that has histidine kinase activity exists in the cell membrane as a pre-formed dimer or in some cases, may dimerise in response to the extracellular signal. Each monomer has three domains including a sensing domain, dimerisation domain and kinase domain. When the extracellular stimulus, such as a change in osmolarity is sensed, the kinase

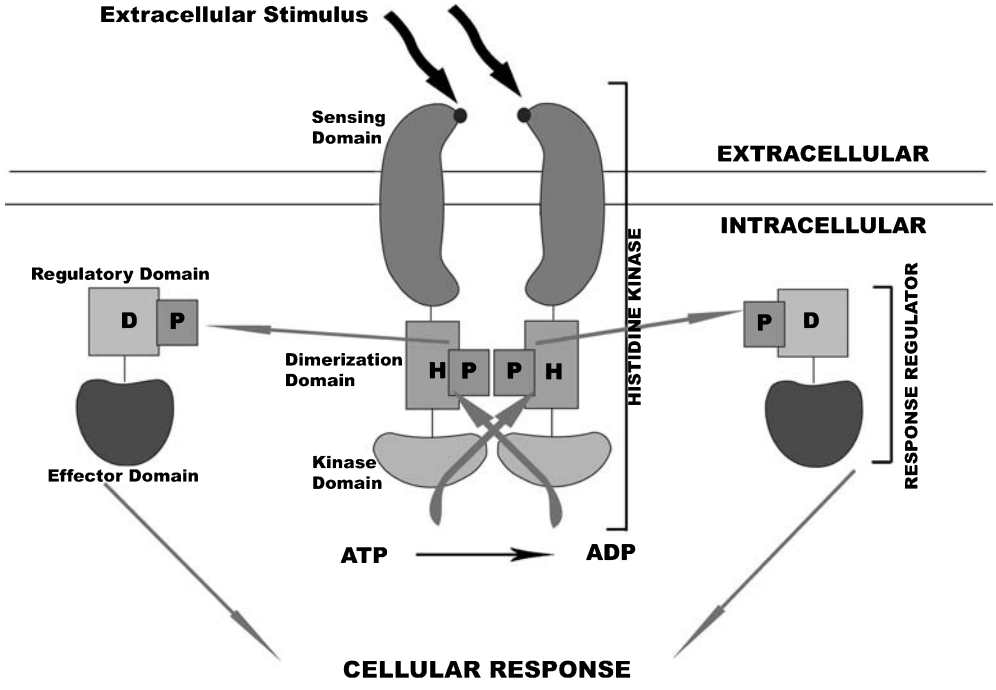


Fig. 4. Diagrammatic representation of a typical two-component histidine kinase signalling reaction. The high-energy phosphate of phosphohistidine is transferred from the transphosphorylating histidine kinase dimer pair to an aspartate residue of the receptor protein

domain is activated and phosphorylates the histidine in the dimerisation domain of its partner monomer using ATP as a phosphoryl donor. The high free energy of hydrolysis of the P–N bond in phosphohistidine facilitates the transfer of the phosphoryl group to an aspartate residue (phosphoaspartate is one of the phosphoanhydride amino acids) of the other component protein, a response regulator, which in turn, triggers the cell response (Fig. 4). There are variations of this transfer system, involving multi-component systems described elsewhere (Waygood et al., 1984) but in each case the biochemistry involved in the signalling pathway stems from the high phosphoryl transfer potential of phosphohistidine.

With the growing evidence of histidine kinases in prokaryotes and lower eukaryotes, there were questions as to whether similar enzymes occur in mammals. By comparison to prokaryotes and lower eukaryotes, little is known about histidine phosphorylation in mammalian cells. It has been estimated that about 6% of total protein phosphorylation in eukaryotes may be accounted for by phosphohistidine (Matthews, 1995). An early study of phosphorylated proteins in rat liver mitochondria showed that there is a greater extent of phosphohistidine formation than phosphoserine (Bieber and Boyer, 1966). A significant amount of phosphohistidine was also found in bovine liver mitochondria and other cellular compartments (Deluca et al., 1963; Zetterqvist and Engstrom, 1966). Since then, phosphohistidine has attracted the attention of researchers and several histidine kinases in mammalian cells have been detected.

One example of a mammalian histidine kinase that takes advantage of the particular chemistry of phosphohistidine is nucleoside diphosphate kinase (NDPK). NDPK is an enzyme that catalyses the interconversion of nucleoside diphosphates and -triphosphates via a phosphohistidyl-enzyme intermediate (Munoz-Dorado et al., 1993). This reaction utilises the transfer of the high-energy phosphoryl group of phosphohistidine as part of its biochemical function. Being a housekeeping enzyme in cellular nucleotide metabolism, NDPK is ubiquitous and is found in plants, bacteria, fungi and mammals as soluble (NDPK A and B) and membrane-bound (NDPK C, D) isoforms. In humans, NDPK is encoded by the *nm23* genes. The Nm23-H1 product of *nm23* is a tumour metastasis suppressor protein, which shares an 80% amino acid sequence identify with NDPK A (Engel et al., 1995).

In accordance with the high ΔG° of hydrolysis of phosphohistidine and analogously to the two-component histidine kinases described previously, it is not surprising that phospho-enzyme form of NDPK is chemically capable of transferring its phosphoryl group to a number of different amino acids on other proteins. In one such protein there was transfer of a phosphoryl group from the active site phosphohistidine of Nm23-H1 to an aspartate residue in the bovine brain aldolase C (Wagner et al., 1997). There is also evidence of Nm23-H1 acting as a histidine-serine phosphotransferase for the kinase suppressor of Ras (KSR) from either transfected 293T cells or MDA-MD-435 breast carcinoma cells. KSR is a scaffold protein that interacts with proteins in the mitogen-activated protein kinase (MAPK) cascade. It was found that both S392 and S434 of KSR could be phosphorylated by phospho-Nm23 in vitro (Hartsough et al., 2002).

There are other reports of histidine phosphorylation in mammalian cells on proteins such as histone H4 (Tan et al., 2004), G-proteins (Kowluru, 2002), p-selectin (Crovello et al., 1995) and many others (Besant and Attwood, 2005). Most of these have been characterised biochemically as phosphohistidine phosphoproteins but their respective kinases have not been identified. Similarly, there are several reports of phosphohistidine phosphatases. More detailed reviews on mammalian histidine kinases and phosphatases can be found elsewhere (Tan et al., 2002; Besant et al., 2003; Steeg et al., 2003; Klumpp and Krieglstein, 2005).

While we have focused on phosphohistidine, the reader should also be aware of the other acid-labile phosphoamino acids, phospholysine, phosphoarginine, phosphoaspartate and phosphoglutamate (Matthews, 1995). Although phosphoaspartate is a common phosphoamino acid in two-component signalling systems and free phosphoarginine is a high-energy phosphate carrier in crustaceans, for all of these acid-labile phosphoamino acids there is little published information regarding their chemistry, biochemistry and biological roles when compared to the acid-stable phosphoester amino acids (phosphoserine, phosphothreonine and phosphotyrosine).

7. Conclusion

Phosphohistidine is one of many phosphoamino acids that are labile under acidic conditions. This has made the study of this class of phosphoamino acid and the respective protein kinases very challenging. Historically, published reports of the occurrence of phosphohistidine in biological systems were sporadic but have become more prevalent recently, particularly in response to interest in bacterial cell signalling. The unique chemistry of phosphohistidine makes it an ideal carrier of a high-energy phosphoryl group that is utilised in numerous biochemical reactions.

In comparison to the phosphoester amino acids, the investigation of histidine phosphorylation in biological systems is still very much in its infancy, especially in mammalian cells. Over the next few years it is likely that there will be a greater interest in this post-translational modification as the development of new methods of investigation of acid-labile protein phosphorylation takes place. Advances and adaptations of existing techniques used to study histidine phosphorylation will certainly have a major impact on the progress of research in this area. The application of stable analogues of phosphohistidine as a research tool and the development of phosphohistidine antibodies will greatly advance research in this field. Without a doubt, the rapidly developing methodology associated with mass spectrometry, with its high sensitivity and mass accuracy, will be at the forefront in these investigations.

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