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Advances in development of new tools for the study of phosphohistidine

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Short running title: Tools and methods for study of phosphohistidine

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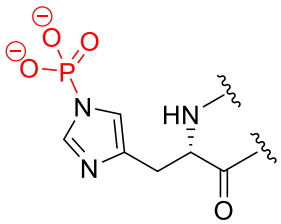
Abstract

Protein phosphorylation is an important post-translational modification that is an integral part of cellular function. The O-phosphorylated amino acid residues such as phosphoserine (pSer), phosphothreonine (pThr) and phosphotyrosine (pTyr), have dominated the literature whilst the acid labile N-linked phosphorylated amino acids, such as phosphohistidine (pHis), have largely been historically overlooked due to the acidic conditions routinely used in amino acid detection and analysis. This review highlights some misinterpretations that have arisen in the existing literature, pinpoints outstanding questions and potential future directions to clarify the role of pHis in mammalian signalling systems. Particular emphasis is placed on pHis isomerisation and the hybrid functionality for both pHis and pTyr of the proposed τ-pHis analogue bearing the triazole residue.

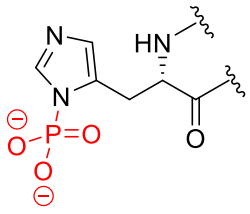
Introduction

Protein phosphorylation is one of the most commonly studied post-translational modifications. In general, the phosphorylation of any amino acid residue results in a change in charge and thus in the protein surface potential. For example, since phosphoryl groups exist mostly as a dianion under physiological conditions,[1] phosphorylation of the amino acids serine (Ser), threonine (Thr), and tyrosine (Tyr), results in a change from neutral to negative (-2). Hence, it should be of no surprise that phosphorylation affects protein conformation, protein-protein interactions, biochemical pathways and its dysregulation is connected to many disease states.[2]

There are nine known phosphorylated amino acid residues Ser, Thr, Tyr, histidine (His), lysine (Lys), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), and cysteine (Cys).[2] The hydroxy O-linked phospho-residues, pSer, pThr, and pTyr have been extensively studied, most probably due to their relative stability in acidic conditions routinely used for analysis. Hence the relatively acid labile N-linked phosphoramidate, carboxy O-linked acyl phosphate and S-linked phosphorothiolate amino acid residues (His, Lys, Arg; Asp, Glu; Cys respectively) have been largely overlooked and less frequently reported. Among the phosphoramidates, phosphohistidine (pHis) is very interesting because of its unique chemical properties: firstly, unlike other phosphorylated residues there are two isomers of pHis; τ- and π-pHis, both of which are found in nature (Figure 1).[3, 4] The τ- and π-pHis are chemically different from each other in both structure, reactivity and stability under certain conditions (*vide infra*). Whether the differences in reactivity and stability are mirrored in proteins is not clear at present. Secondly, pHis serves as a high energy intermediate in the transfer of the phosphoryl group to other amino acid residues which is a characteristic not seen with other phosphorylated amino acid residues. Therefore, not only does pHis change the surface potential of proteins it also possesses transient transferable chemical information that can be potentially tuned depending on the isomer and environment.



τ–pHis residue



π–pHis residue

Figure 1 The two isomers of pHis, τ (tele)- and π(pros)-pHis residues found in nature existing as dianions under physiological conditions. The τ isomer is also known ε/3-pHis, and the π isomer as δ/1-pHis.

His phosphorylation has been found in a number of organisms including bacteria,[5] fungi,[6] and plants[7] and its major role is in cell signalling either via two component or multicomponent phosphorelay systems. Cell signalling via a two component phosphorelay has also been found in yeast,[6] but such processes have not yet been observed in higher eukaryotes (for example mammals, birds and fish).[8] The general role of pHis in prokaryotic and lower eukaryotic cells is shown in Figure 2.

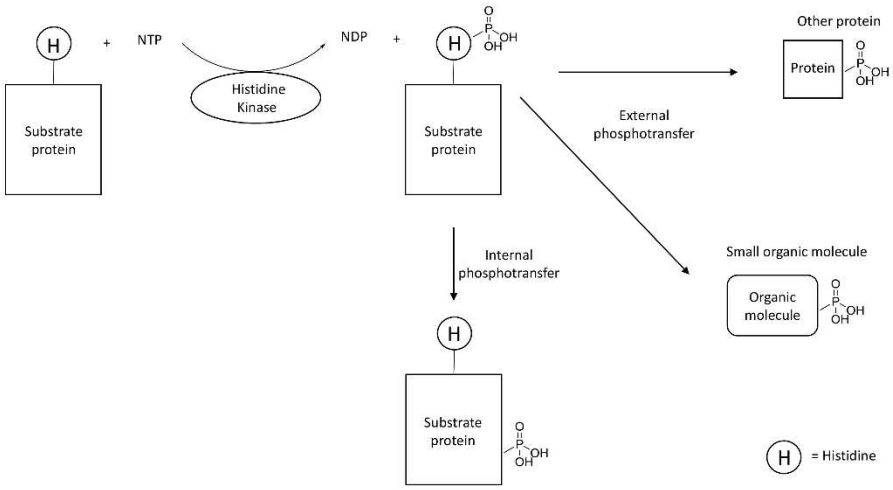


Figure 2 A simplified adopted schematic[9] showing the general role of pHis known thus far[10]

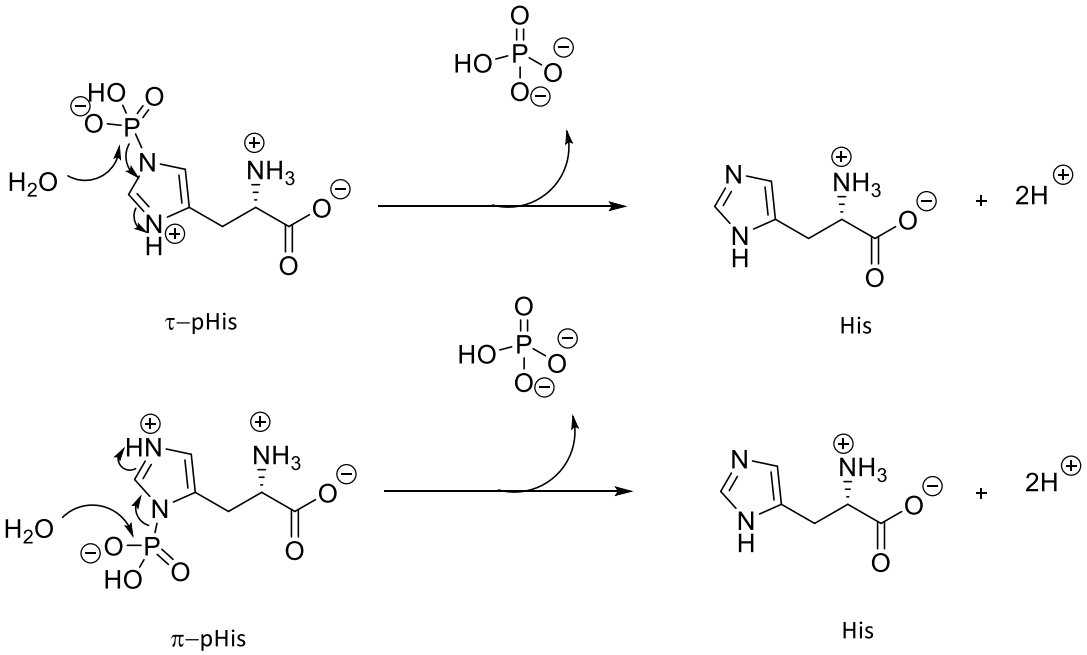
Two isoforms of nucleoside diphosphate kinase (NDPK): Nme1/NDPK-A[11-15] and Nme2/NDPK-B[16-21] have been characterised as mammalian His kinases. Other mammalian His kinases exist. Evidence for Histone H4 His kinases (HHKs) activity has been found in regenerating rat liver,[22-25] foetal rat and human liver,[25] human hepatocarcinoma tissue,[25] pancreatic β cells,[26, 27] thymus[28] and Walker-256 carcinosarcomas,[22, 29] but have not been fully characterised or purified. Interestingly, HHKs from regenerating rat liver[23] and Walker-

256-carcinosarcomas[29] each phosphorylated histone H4 leading to a τ-pHis residue. In a later study, ^{31}P NMR suggested the presence of τ-pHis on His18 of phosphorylated histone H4 when Walker-256-carcinosarcomas were used as the kinase source.[30] However, ^{31}P NMR studies suggested the presence of π-pHis in phosphorylated histone H4 when using regenerating rat liver as the kinase source.[30] In this particular case it is not clear which isomer of pHis is formed and on which His residue. Mammalian pHis phosphatases which have been characterised include: protein pHis phosphatase 1 (PHPT1);[19, 31-37] Lys/His phosphatase (LHPPase);[38, 39] serine threonine protein phosphatases (PP1/2A/2C); [40, 41] T cell Ubiquitin Ligand-2 (TULA-2);[42, 43] and the recently reported phosphoglycerate mutase-5 (PGAM5).[44] Additionally, pHis phosphatase activity has been reported in rat tissue extracts but these have not been fully characterised.[45-48]

Not only is His phosphorylation predicted to be prevalent in eukaryotic proteins[40] it has also been associated with important mammalian cellular processes. For example, pHis has been shown to be present in heteromeric G proteins (GNB1) which are involved in G protein signalling,[17, 49, 50] KCa3.1 potassium channel which is involved in ion conductance,[20, 51] ATP-citrate lyase (ACLY) which is involved in cell metabolism,[11] Histone H4 which is involved in chromatin biology,[23, 24, 30] transient receptor potential-vanilloid-5 (TRPV5) which regulates urinary Ca^{2+} excretion,[19] and phosphoglycerate mutase 1 (PGAM1) which is involved in glycolysis.[52-55] Other mammalian pHis proteins include P-selectin, which plays an important role in the function of blood platelets,[56] annexin A1 a multi-functional Ca^{2+} dependant phospholipid binding protein found in airway epithelia cells, [57] thymidylate synthase which catalyses N-methylenetetrahydrofolate assisted C(5)-methylation of dUMP required for DNA synthesis,[58] glucose-6-phosphatase involved in glucose homeostasis,[59, 60] nicotinamide phosphotransferase (NAMTP) involved in reforming nicotinamide adenine dinucleotide (NAD^+) from nicotinamide[61] and prostatic acid phosphatase which is found in high levels in prostate cancer cells.[62, 63] However, the functions of many of these pHis proteins, and the specific pHis isomer involved, as well as corresponding kinases and phosphatases remain unknown.

Chemistry of phosphohistidine

The chemistry of pHis amino acid was first studied by Hultquist *et al.*[64, 65] and has been covered extensively in a review by Attwood.[66] In summary, pHis residues contain a weak phosphoramidate bond with the phosphoryl group being most susceptible to hydrolysis when the imidazole nitrogen is protonated (Scheme 1).[64, 65] Hence, the rate of hydrolysis of the pHis is dependent on pH. The respective pKa values of the imidazole nitrogen suggests hydrolysis can occur even under physiological conditions (pKa 7.3 for π-pHis at 46 °C[65] and pKa 6.4 for τ-pHis at 25 °C[64]). It is important to note that pHis pKa values have been found to vary depending on salts in solution. Gassner *et al.*, found π-and τ-pHis to have a pKas of 7.74 and 6.88 respectively from the titration of the reaction mixture of His with potassium phosphoramidate adjusted to pH 7.2 at 25 °C.[67]



Scheme 1 Hydrolysis of the protonated τ- and π- pHis imidazole nitrogen to His and phosphate.

Hultquist studied the decomposition of τ-pHis over the pH range 2-5, by following the decrease in absorbance of τ-pHis, and the decomposition of π-pHis over the pH range 2-11 by following the increase in absorbance of His, each at 46 °C.[65] Both isomers decompose more rapidly at low pH and, at all pHs measured, π-pHis was less stable than τ-pHis. Unlike τ-pHis, the rate constant for dephosphorylation of π-pHis decreases smoothly between pH 2-4, and remains approximately constant over the pH range 4-6; above pH 6 (~ pKa of imidazole nitrogen of π-pHis) to pH 9 the rate constant decrease slowly again before a

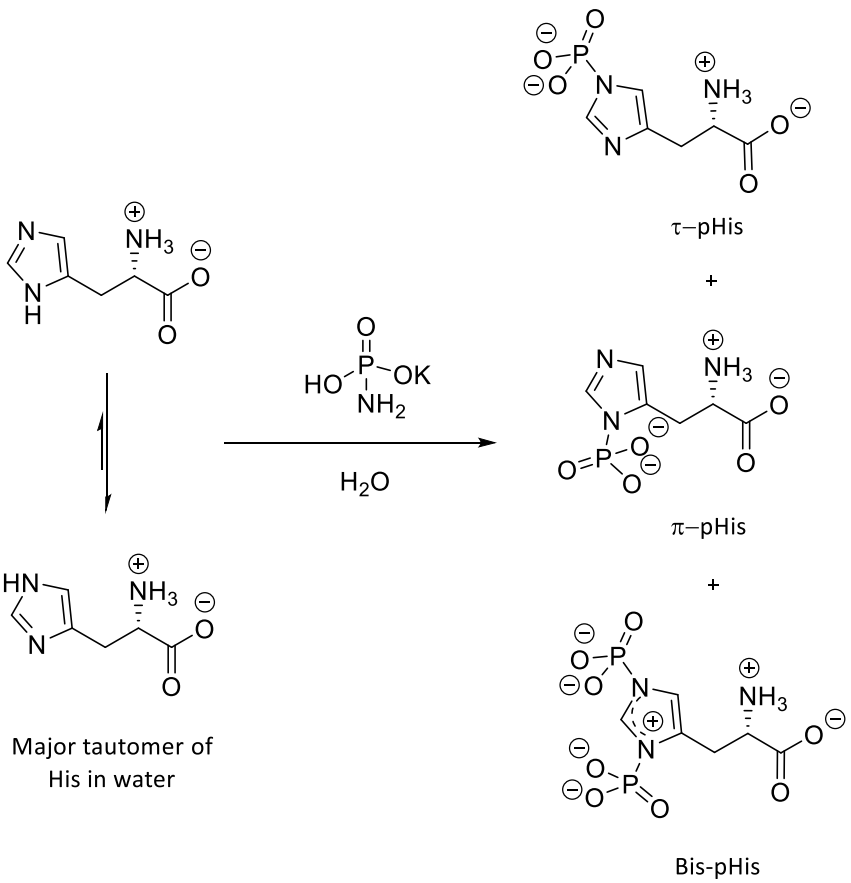
further decrease above pH 9 which is approximately the pKa of the amine (~9.6).[64-66] Nonetheless, the data clearly shows both π-His (pH 9-11, 46 °C)[65] and τ-pHis (pH 8-10, 80.5 °C)[64] are stable in solution for extended periods provided the right conditions are used. Hence, application of pHis standards in experiments such as ELISA, chromatography, and dot blots with protein conjugates, or any other test where the pHis standard is needed, should be possible.

Within a small neutral peptide (Gly-pHis-Gly) Lecroisey *et al*. found the π-pHis residue to be less stable than the τ-pHis residue in the same position, consistent with Hultquist's findings.[3] Whether the relative stability of π- and τ-pHis residues in this case could be explained by pKa alone is questionable (due to the absence of the primary amine) because it is not known to what extent the peptide backbone affects pHis stability. Furthermore, a denatured pHis protein where tertiary interactions are absent may have a half-life that differs from that in the tertiary structure.[3, 68] For example, the enzyme NDPK has a His residue within its active site, which interacts with a nearby Glu residue assisting in the isomer selective phosphorylation to form π-pHis, but this interaction is absent in the denatured state.[69, 70] Hence, procedures where denaturants are used such as Western blots must take this into account. Another important factor to consider in any procedure involving pHis is the type/concentration of salts present in solution. For example, the calcium salt of π-pHis was found to be very unstable relative to the sodium salt of π-pHis.[65] Thus, each protein phosphorylated on a His residue will likely have a unique half-life under a defined set of conditions and as such, meaningful half-life comparisons can only be made between pHis proteins using normalised conditions.

An important question to be addressed is: does π-pHis isomerise to τ-pHis or vice versa under any conditions because this will have implications for methods used to study pHis? A good place to start to address the question of isomerisation is from the work by Hultquist *et al*., who reported the synthesis, purification and subsequent experimental work on each pHis isomer.[64, 65]

Reaction of His with potassium phosphoramidate in water gives both isomers of pHis as well as bis-pHis (Scheme 2). The pHis isomers were separated using anion exchange

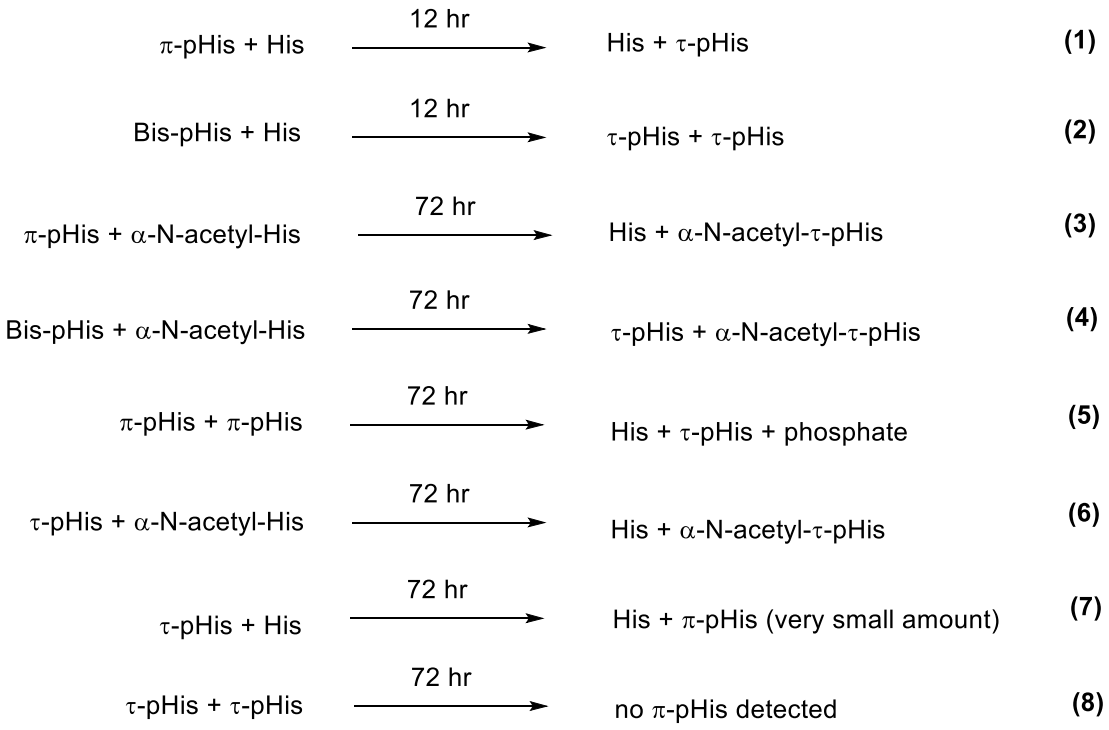
chromatography as either lithium, potassium[64] or sodium salts[65] after desalting. By following the progress of the reaction by electrophoresis it was established that π-pHis formed rapidly before gradually decreasing, accompanied by the formation of τ-pHis and bis-pHis. Similar, observations were made in ^{1}H NMR studies by Gassner *et al.*, where phosphorylation with potassium phosphoramidate (pH 7.2, 25°C) resulted in initial rapid formation of π-pHis (maximum at ~ 10 mins), then bis-pHis (maximum at ~ 60 mins), both of which subsequently decomposed, accompanied by a gradual increase in τ-pHis.[67]



Scheme 2 Synthesis of π-pHis, τ-pHis and bis-pHis by reaction of His with potassium phosphoramidate in water.

The observation of π-pHis forming more rapidly is consistent with the major tautomer (τ protonated nitrogen) of His present in water (Scheme 2).[71] Hultquist's phosphoryl transfer experiments can be used as a plausible explanation for the accumulation of τ-pHis with the decrease in π- and bis-pHis over time (Scheme 3). Phosphoryl transfer reactions (1)-(8)

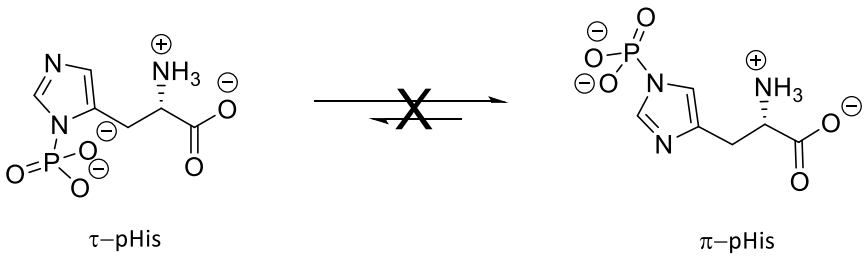
suggest that the phosphoryl group of pHis can be donated to the imidazole nitrogen of His or α-N-acetyl-His. Comparison of (1), and (5), with (7) and (8) suggests that π-pHis has the more labile phosphorus nitrogen (P-N) bond compared to τ-pHis. Reactions (2) and (4) also show there is a preferential transfer of the π-phosphoryl group of bis-pHis to form the τ-pHis. These observations are dependent upon the reaction conditions used (0.1 M Tris buffer, pH 8.5, at 4 °C)[64] but provide a credible explanation as to how τ-pHis accumulates over time from the reaction of His and potassium phosphoramidate.



Scheme 3. Phosphoryl transfer reactions of π-pHis, τ-pHis and bis-pHis as donors and His and α-N-acetyl-His as acceptors in 0.1 M Tris buffer, pH 8.5, at 4 °C.[65]

Importantly, reactions (1), (5) and (7) show that one isomer of pHis can convert to the other isomer, but only via a bimolecular reaction with His. Though this can be viewed as isomerisation, it is in fact a bimolecular process and strictly not a unimolecular isomerisation as has been proposed to occur under certain conditions, both for pHis and pHis residues (Scheme 4).[72-76] Indeed, Hultquist himself avoided the term isomerisation and defined reactions (1), (5), and (7) as conversion of one isomer to the other.[65] It is unlikely unimolecular isomerisation is occurring under acidic conditions because the hydrolysis of

the calcium salt of π-pHis (1 M HCl, 49 °C)[65] and the lithium salt of τ-pHis (0.5 M HCl, 48.5 °C)[64] followed first order rate kinetics.



Scheme 4 Inferred unimolecular isomerisation of π-pHis to τ-pHis and *vice versa* that does not occur.

Concerning the question of isomerisation of pHis residues in proteins, Lecroisey *et al*., found using ^{31}P NMR only the π-pHis residue both in chemically phosphorylated *Dictyostelium* NDPK (25 °C, 50 mM Tris-HCl, 10% D_2O, pH 8.1) and in the denatured state after treatment with 9 M urea (*vide supra*).[3] Similarly, Williams *et al*., observed only τ-pHis by ^{31}P NMR in rat liver ACLY (25 mg/ml, 50 mM Tris-HCl, 14% D_2O, pH 8.4) both for the functioning enzyme and when denatured with 1 % SDS and 1% β-mercaptoethanol.[77] Wagner *et al*., found that upon base hydrolysis (3 M KOH, 120 °C, 100 mins) of [γ-^{32}P]ATP phosphorylated rat liver NDPK, only π-pHis was detected in the hydrolysate by TLC analysis against the reference compound.[11] In the same study only τ-pHis was detected by TLC from the base hydrolysate of (^{32}P) phosphorylated rat adrenal ACLY.[11] These results demonstrate that in these specific examples, isomerisation of the pHis residue does not occur.

By contrast, in two early studies of phosphorylated human erythrocytic NDPK[78] by Walinder *et al*., and of rat liver ACLY[79] by Mardh *et al*., phospholysine (pLys) and both isomers of pHis were found in the base hydrolysate after chromatographic separation of the phospho amino acids. However, ^{31}P NMR,[3] X-ray crystallography (only the π imidazole nitrogen is available for phosphorylation),[69, 70] and base hydrolysate data,[11] have shown that NDPK is autophosphorylated by ATP on a specific His residue to form the π-pHis residue exclusively. Likewise ACLY has been found to be phosphorylated by NDPK or ATP to form τ-pHis only, both by ^{31}P NMR and in the base hydrolysate.[11] So why the discrepancy with the results from Walinder *et al*. and Mardh *et al*.? It may be that the phosphoryl group of one isomer of

pHis was transferred either directly to another His residue giving the other pHis isomer (Scheme 3, eq. 1 and eq. 7), or to a Lys residue (nitrogen lone pairs are known to accept the phosphoryl group from phosphoramidate containing compounds),[80, 81] which then subsequently phosphorylated His under the particular experimental and analysis conditions used.

Although, in this review we have focused the topic of pHis isomerisation mainly on the ACLY and NDPK, there are other examples of pHis containing proteins which have been characterised by ^{31}P NMR, or X-ray crystallography and show no evidence of isomerisation. For examples see: Histone H4,[30] Succinyl -CoA synthase,[68] and phosphocarrier protein HPr.[67]

Detection of phosphohistidine

There are many ways to detect pHis and extensive reviews on the subject exist.[72, 75, 82] However, in the context of this review, some of the most important methods need to be discussed briefly. Detection of pHis residues using antibodies will be discussed in a later section. ^{31}P NMR spectroscopy is a useful spectroscopic method to detect pHis residues but requires large amounts of purified sample. Nonetheless, it is one of the methods that allows pHis isomer distinction against pHis standard chemical shifts. In some cases, the local peptide sequence of the pHis residue is required to generate reference chemical shifts of π- and τ-pHis residues within a sequence and this is not always known. For example, Lecroisey *et al.,* found that the ^{31}P NMR chemical shifts of phosphorylated NDPK did not match any known τ-pHis or π-pHis ^{31}P NMR chemical shifts.[3] The chemical shifts of the pHis residue also differed by a large amount in the native (-2.72 ppm) and denatured state (-4.20 ppm) (*vide supra* for conditions).[3] However, π-His phosphorylated peptide (Glu-pHis-Gly, known phosphorylated sequence of NDPK) matched the denatured state chemical shift which was used as a reference.[3] Another consideration that must be taken into account when characterising pHis by ^{31}P NMR is pH because there are three states the phosphoryl group can exist in; as the phosphonic acid, monoanion, and dianion which will effect chemical shifts.[67] Usually basic conditions (pH > 8) which stabilise pHis are used, where the phosphoryl groups exists as a dianion.[1]

Wagner *et al*., approach of detecting either π- or τ-pHis from the base hydrolysate of pHis proteins by TLC analysis against pHis standards seems encouraging due to the simplicity of the method.[11] However, Wagner *et al*. used [γ-^{32}P]ATP to phosphorylate the protein samples and the TLC was analysed by autoradiography. On the other hand detection of τ-pHis in the base hydrolysate of enzymatically phosphorylated histone H4 using HPLC against pHis standards has been reported and could be used as an alternative.[83] The method of base hydrolysis, though useful, cannot give any direct information about the site of His phosphorylation or the presence of multiple pHis residues in the protein.

Mass spectrometry (MS) has been used in the detection of phosphorylated amino acid residues, including phosphorylated His residues.[72] MS generally uses acidic eluents in chromatographic separation of enzymatically digested peptides prior to analysis, conditions which are not suitable for pHis containing proteins. However, changing the eluent to basic or neutral solution decreases the resolution and sensitivity but mildly acidic conditions (0.5% aqueous acetic acid) have proved successful so long as the contact time is kept to a minimum.[84] Aqueous formic acid (0.1%) has also been used.[85]

One of the challenges with MS analysis is how to eliminate false positives when analysing pHis data. For example, Gonzalez-Sanchez *et al.* found phosphorylation of peptide DAPA**H**DAKD with potassium phosphoramidate resulted in exclusive His phosphorylation which was confirmed by collision-induced dissociation-tandem MS.[72] The same peptide analysed by nano-ultraperformance liquid chromatography nano-electrospray ionisation MS, eluted with 50 mM ammonium acetate and then loaded under acid conditions (pH 2) gave two distinct peaks. One peak was identified as the His phosphorylated peptide (DAPA**pH**DAKD) and the other as the phosphorylated aspartate peptide (DAPAH**pD**AKD).[72] The latter experiment demonstrates false positives could be an issue in pHis proteomic MS analysis using certain techniques. Nevertheless, in a recent study Oslund *et al*. observed a characteristic TRIPLET fingerprint for the decomposition of the pHis residues (neutral losses of 98, 80 and 116 Da) in collision induced dissociation MS analysis of various pHis containing peptides, which could be used to differentiate pHis from other phosphorylated residues.[85]

All the pHis detection methods described so far require an effective enrichment and purification strategy as well as a selective phosphorylation method. This can be quite tricky if the protein of interest requires a pHis kinase for phosphorylation because the pHis kinase

may be unknown or will also have to be isolated and phosphorylated. Lapek *et al*., has developed an MS method for the direct analysis of a whole cell lysate which helps preserve acid labile modifications by using a buffer mixture including ammonium bicarbonate between pH 2.5- 5 (adjusted with formic acid) in the chromatography prior to MS analysis.[86] Twenty pHis phosphopeptides were identified but the authors argue that the buffer system limits the analysis to peptide sequences possessing an intrinsic positive charge. Traditional methods avoid this by using more acidic conditions to positively charge the peptide but this is undesirable with acid labile phosphorylated residues.

Despite MS being a powerful tool for the analysis of post-translational modifications it cannot give any direct information about the pHis isomer present which is an important requirement. Thus, the use of a variety of detection methods which complement each other is needed.

Enrichment of phosphohistidine

The most common ways to enrich a phosphoprotein use an immobilised metal affinity column (IMAC), an immobilised metal oxide columns (IMOC) or an immobilised phospho selective antibody (*vide infra*). The latter has been more successful due to it being generally more selective for a specific amino acid residue.[87] Enrichment of pHis containing proteins using IMAC has had some success but has its limitations. Muimo *et al.* enriched His phosphorylated Annexin A1 with Fe^{3+} and Ca^{2+} affinity columns, but the method was found to be inefficient.[57] Napper *et al*., used Cu^{2+} in what they describe as the selective enrichment of pHis-containing HPr protein from *E.coli*.[88] However, this technique is limited to peptides. These two examples suggest IMAC can be used to enrich pHis samples but requires optimization to avoid the acidic conditions typically used to release the bound pHis proteins from the resin which destroys pHis residues and reduces efficiency.

Phosphohistidine analogues and antibodies

Antibodies have been used extensively in the detection and enrichment of other phosphorylated residues, namely pTyr, pSer and pThr.[87] The development of pTyr antibodies in the late 80's led to a boom in the research field and this arguably led

ultimately to the discovery of protein kinase inhibitor drug Gleevec, which is used to treat leukaemia and other malignancies.[89-91] Among the detection methods which have been used to detect phosphorylated amino acid residues, such as MS, NMR spectroscopy, radiolabelling (^{32}P), and dyes, antibodies hold the most potential. Antibodies allow for rapid non-invasive detection (can be used *in vivo* and *in vitro*), with high sensitivity (up to femtomolar detection[92]) and can be used in enrichment (perhaps most importantly because nearly all the detection methods discussed require a reliable enrichment process prior to analysis) from whole cell lysates.[93] None of the other methods mentioned above has these three characteristics in combination and furthermore antibodies do not have many of the disadvantages associated with other techniques (*vide supra*) so long as they are selective for a particular antigen or target.

There have not been any reports whereby pHis has been used successfully to generate selective antibodies, most likely due to its labile nature. Many authors have reported potential pHis analogues (Figure 3) and one of the first approaches was to substitute one of the oxygens of the phosphoryl group for a sulphur atom to give thiophosphorylhistidine **1** to give a more stable derivative of pHis.[94] There are no reports of analogue **1** in the generation of pHis antibodies. An antibody was raised against the thiophosphorylhistidine derivative **2** but the antibody so raised could not distinguish pHis from other phosphoamino acids. [95, 96] Pirrung *et al*., reported the preparation of malonate **3** and fluoromalonate **4** derivatives of His as τ-pHis analogues.[97] However, no biochemical studies using these analogues have been reported. Schenkels *et al*., reported the synthesis of protected phosphofurylalanine **5** whilst also proposing phosphopyrrole **7** as a potential τ-pHis analogue.[98] Use of the free amino acid phosphofurylalanine **6** allowed the raising of antibodies, but these only detected the antigen, and not natural pHis.[99] The phosphopyrrole **7** was later synthesised by Attwood *et al*.,[66] but the polyclonal antibodies raised against this epitope detected only the analogue and not pHis. Following Schenkels' proposal of phosphofurylalanine **6** as a potential pHis analogue, Lilley *et al*. synthesised phosphothiophene **8**. Polyclonal antibodies generated against this epitope were found to be highly selective for pTyr but did not detect pHis. The results obtained with phosphopyrrole **7** and phosphothiophene **8** suggested that retaining the nitrogen with a lone pair of electrons relative to the phosphoryl group as present in pHis

was necessary. In addition, replacement of the labile phosphorus nitrogen bond (P-N) with a phosphorus carbon (P-C) is probably essential.

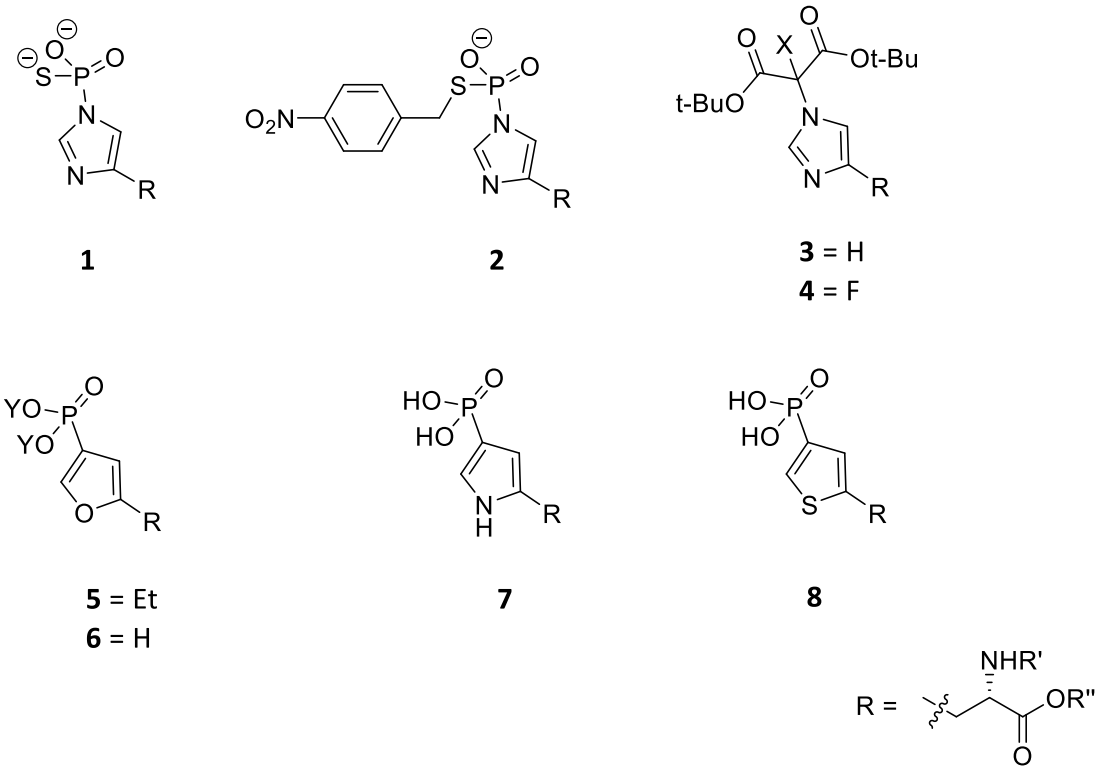


Figure 3 Some of the potential analogues of pHis.

Eerland *et al*., designed and synthesised sulphonamide **9** as a sulphonamide based transition-state analogue of enzymatic pHis dephosphorylation (Figure 4).[100] The analogue was successfully incorporated into peptides, but efforts to use these peptides as pHis phosphatase (PHT) inhibitors, or as baits to pull down pHis-binding proteins, were unfortunately unsuccessful.[101]

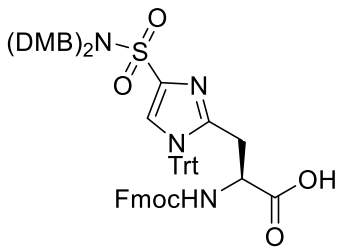


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Figure 4 Sulphonamide based transition state enzymatic pHis dephosphorylation analogue of pHis.

Kee *et al.*, synthesised triazole analogues **10** and **12** as τ- and π-pHis analogues respectively (Figure 5).[102] Density functional theory (DFT) calculations on the residue of triazole analogue **10** suggested a close structural match to τ-pHis but with an observable difference in electrostatic surface potential around the extra nitrogen and lone pair of electrons[103] (see also Supplementary Figure S1[102]). Both analogues were incorporated into peptides via Boc solid phase peptide synthesis (SPPS) and peptide **11** was used to generate antibodies after being conjugated to protein KLH (Figure 5). The polyclonal antibodies were found to cross react with pHis substituted peptide **11** as assessed by dot blots but not in a peptide sequence independent manner (Rb.#3 antibodies showed significant cross reactivity with pTyr containing peptide, see Figure S4[102]).

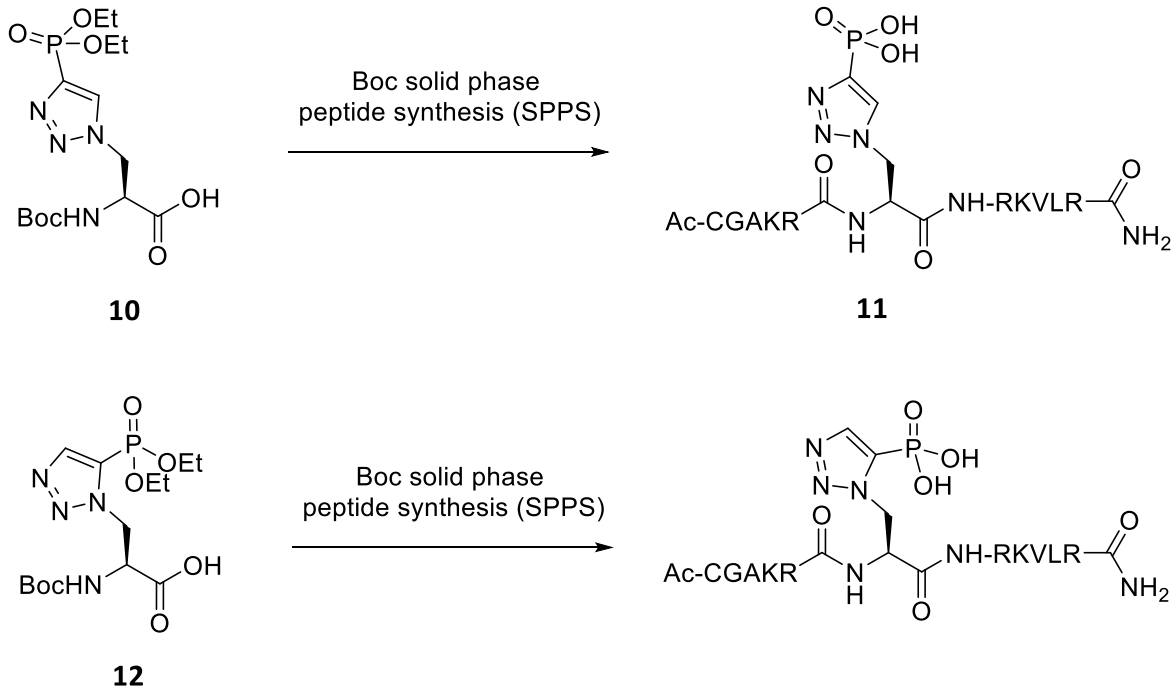
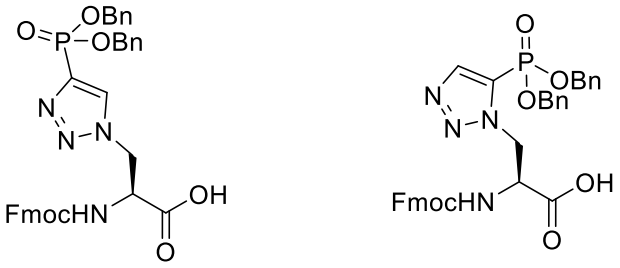


Figure 5 Incorporation of proposed τ- and π-pHis analogues **10** and **12** in to peptides.

Since this first report a number of groups have reported the synthesis of these analogues with different protecting groups to allow for incorporation of triazolylalanine **10** into peptides using Fmoc SPPS.[104-106] In a 2012 paper McAllister *et al.,* reported the synthesis of

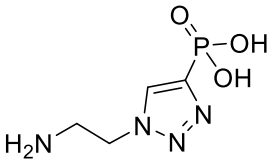
Fmoc dibenzyl protected triazolylalanine **13** (Figure 6), which were incorporated into peptides *via* the Fmoc SPPS strategy.[105] In a later paper, both triazolylalanine analogues **13** and **14** were synthesised by Fuhs *et al*., and incorporated into degenerate peptides again using the Fmoc SPPS strategy (Figure 6 and Figure 8).[74]



13 **14**

Figure 6 Fmoc SPPS compatible triazolylalanine **13** and **14** pHis analogues.

Inspired by the earlier work on the generation of pTyr antibodies using the hapten[107] alone Kee *et al*., used the triazolyl ethylamine **15** conjugated to KLH via a linker to give a much simpler epitope for use in immunisation (Figure 7).[76] The affinity purified antibodies were used to detect pHis in various known peptides where phosphorylation sites vary widely, including histone H4, bacterial His kinase, KinB, and the *E.Coli* metabolic enzyme I (PtsI) and PpSA, illustrating its sequence independence.[76] However, control experiments to assess the selectivity of the antibody against other phosphorylated amino acids/peptides showed significant cross-reactivity with pTyr as observed by ELISA and dot blots (see Supplementary Figure 3, 5 and 6).[85] These results suggest the triazolyl residue **16** has a hybrid functionality for both pHis and pTyr in antibody generation, perhaps similar to the azobenzene reported in the 1980s.[108] Despite the limitations, Kee *et al*., study has demonstrated that pHis analogues alone could be effective as epitopes.



15

Figure 7 Triazolyl ethylamine synthesised by Kee *et al*.[100]

Fuhs *et al*., took both **13** and **14** and incorporated them in neutral peptides libraries (Figure 7).[74] Immunisation with peptides libraries including **16** and **17** conjugated to KLH generated polyclonal antibodies. The antibodies were tested by dotblots/immunoblots against peptide immunogens **16** and **17**, pTyr peptides and two protein targets as standards, His phosphorylated NME1/NME2 and PGAM, which contain a π-His or a τ-pHis residue respectively (Figure 9). Analysis by immunoblots showed the polyclonal antibodies raised against triazolyl peptides **17** recognised phosphorylated PGAM, while the polyclonal antibody raised against triazolyl peptides **16** recognised, in a complementary way, phosphorylated NME1. The triazolyalanine **12** was proposed as a mimic of π-pHis by Kee *et al*., but not used in antibody generation.[102]

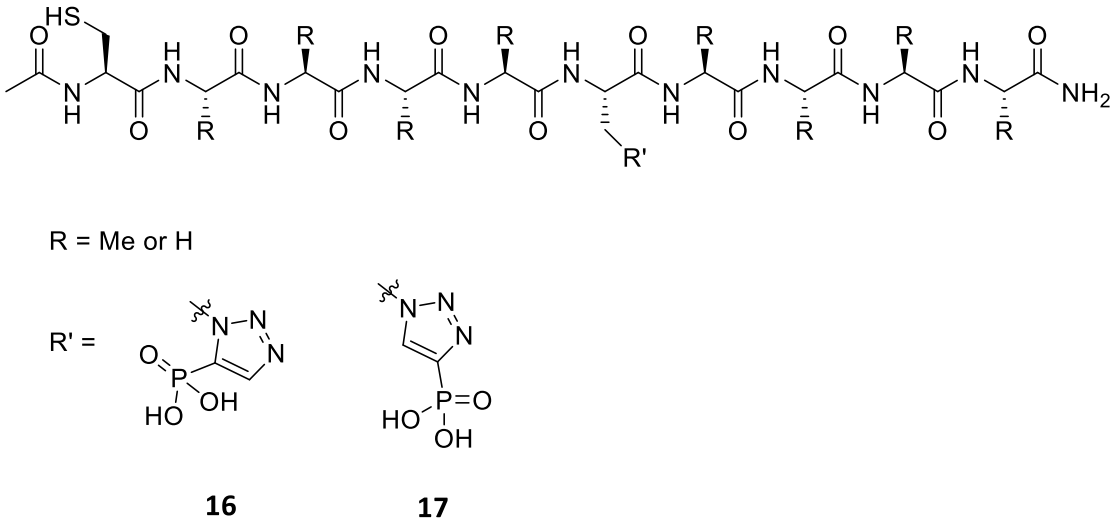


Figure 8 Triazolyl containing peptides **16** and **17** used to generate pHis antibodies.[74]

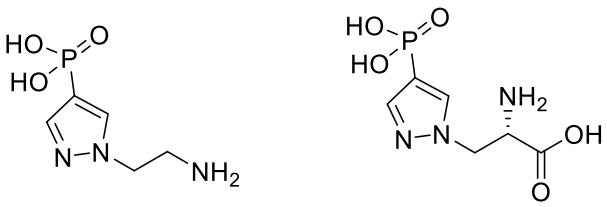
Monoclonal antibodies raised with peptides **16** recognised peptides containing residue **16** but not residue **17** on dot blots and *vice versa* for **17**. The monoclonal antibodies were used in Western blots of many mammalian whole cell lysates and in pHis protein enrichment.[74] Hunter *et al*. found significant overlap of π-and τ-pHis proteins binding to all monoclonals in the enrichment data. It is important to point out that in the initial report by Kee *et al*. the

use of Histone H4 peptide **11** gave peptide sequence dependent polyclonal antibodies and their later method using triazole ethylamine **15** gave cross-reactive polyclonal antibodies for pHis and pTyr.[76, 102]

Evaluation of potential interaction between pHis and pTyr binding domains indicated that peptides containing unprotected triazole residue **16** (as replacement for pTyr) are capable of strongly binding to the prototypical pTyr binding Grb2 SH2 domains.[109] However, this result apparently contradicted a previous study that showed that τ-pHis could not substitute for pTyr in high-affinity binding of peptides to pTyr binding Grb2 SH2 domains.[110] It is worth noting that peptides containing a pHis substitution were not considered in the experimental setup by McAllister *et al*. As such, it is possible that the Grb2 SH2 binding observed most likely resulted from the ability of triazole residue **16** to mimic both pTyr and pHis. Thus, their results are in line with data from antibody generation using the triazole ethylamine **15** (*vide supra*) and supports the notion that this triazole residue has a hybrid functionality for both pHis and pTyr.[76] Furthermore, the peptide sequence dependant antibodies generated to Histone H4 peptide **9** cross-reacted with pTyr substitution (Rb.#3 antibodies showed significant cross reactivity with pTyr, see Figure S4, supplementary information).[102] This indicates that peptides containing this triazole residue may not be entirely suitable as tools to raise pHis peptide sequence dependant antibodies.

Due to the limitations of the triazole residue **17** Kee *et al*., reported a second generation pyrazole ethylamine **17** as a τ-pHis analogue (Figure 9).[103] DFT calculations by Kee *et al*., show that the pyrazole analogue **18** residue not only closely matches the τ-pHis residue in structure but also electronically.[103] Affinity purified polyclonal antibodies raised to pyrazole analogue **18** conjugated to KLH via a linker were found to strongly detect pHis over pTyr, as assessed by ELISA against His phophorylated BSA and phosphorylated amino acid conjugates. Concurrently, Lilley *et al*. reported antibodies raised with the pyrazole amino acid **19** conjugated to KLH via a linker (Figure 9).[111] These polyclonal antibodies were also found to have strong selectivity for pHis over pTyr, on dot blots, immunoblots and ELISA against BSA phosphorylated amino acid conjugates as standards. Polyclonal antibodies

reported by Kee *et al*., were used to detect various *in vitro* His phosphorylated proteins including PGAM1, mammalian histone H4 and PtsI, whilst those by Lilley *et al*. antibodies were used to detect immunoprecipitated protein Gβ and both NDPK-A/B from HBE cells.[103, 111]



18 **19**

Figure 9 τ-pHis pyrazole analogues **18** and **19**.

Amino acid substitutions as a phosphomimetic

A useful genetic approach to mimic constitutive phosphorylation of the hydroxy amino acids often involves mutation or substitution of Ser/Thr with Glu or Asp to mimic pSer/pThr or constitutive phosphorylation of these residues. Conversely, substituting Ser or Thr with alanine (Ala) prevents potential phosphorylation and mimics constitutive dephosphorylation. However, Ala is not a perfect mimic for Ser, due to the absence of the hydroxy group. The properties of the phosphoryl and carboxy groups (number of oxygen atoms available for bonding, geometry, size and pKa) differ and these substitutions do not work successfully in every case, highlighting the differences in the structure of the epitope in these substitutions. Interestingly, pSer/pThr antibodies do not recognize substituted Asp or glutamate (Gln). There is no natural mimic for pTyr. Gln is occasionally reported as a pTyr mimic, but it bears very little chemical similarity to pTyr.

Similarly, there are no natural mimics for His or pHis but several reports exist in the literature involving Ala, phenylalanine (Phe), leucine (Leu) and asparagine (Asn) (possessing hydrophobic, aromatic or polar neutral side chains) substitution of His (a charged side chain).[18, 112, 113]

Several unnatural pTyr analogues have been developed and incorporated into synthetic peptides by SPPS and, in some cases, this was followed by ligation of the synthetic peptide

to recombinant proteins of interest. The pTyr analogues, phosphonomethylphenylalanine (Pmp), and difluorophosphono-methylphenylalanine (F2Pmp), have been invaluable tools in understanding the role of pTyr in cellular processes. Peptides containing pTyr analogues p-carboxymethylphenylalanine have also been used as haptens for generation of selective phospho-antibodies, phosphatase inhibitors, affinity ligands and in structural studies.[114]

Advances in genetic code expansion beyond the naturally encoded amino acids have also enabled site-specific incorporation of pTyr analogues and other unnatural amino acids (UAA) into proteins to generate proteins with enhanced properties and probes for function.[115, 116] In order to incorporate the UAA at a genetically defined position in a polypeptide chain, the UAA once added to cells is recognised by an orthogonal synthetase/tRNA pair and used to aminoacylate the orthogonal tRNA which is then decoded by the ribosome in response to a blank codon (inserted into the gene of interest by site-directed mutagenesis at a position of interest) on the mRNA. Thus for incorporation of pHis analogue, this approach would require development of *a)* an aminoacyl-transfer RNA (tRNA) synthetase/tRNA pair that is orthogonal to endogenous synthetases and tRNAs in the host cells; *b)* methods to adapt or convert the aminoacyl-tRNA synthetase amino acid specificity so that it uniquely uses only the pHis analogue and no natural amino acids; *c)* a blank codon to direct insertion of the pHis analogue; *d)* modification of UAA (pHis analogue) charge to allow increased cellular permeability; and *e)* subsequently, a chemo-selective chemical modification reacting only with the incorporated pHis analogue but not the natural amino acids on the protein utilised to modify the UAA. Among several other possibilities and advantages, expression of proteins in which pHis is substituted with a stable pHis analogue will not only help in structural/functional studies but will overcome the complexities associated with identifying the cognate kinases and phosphatases prior to studying the role of pHis phosphorylation at specific sites. Recently, a method has been developed to produce pure tyrosine-phosphorylated proteins by genetic encoding of a stable and neutral phosphotyrosine analogue followed by subsequent deprotection.[117] This approach resulted in site-specific incorporation of native phosphotyrosine into several different proteins including ubiquitin and revealed a potential negative regulatory role of ubiquitin phosphorylation at Tyr59, which exclusively occurs in cancerous tissue but whose significance has hitherto been unknown.

Conclusion

The importance of pHis in mammalian systems continues to be uncovered and suggests pHis function may prove to be much more extensive than presently understood. The data suggest conversion of one isomer of pHis to the other occurs via a bimolecular process under certain conditions but there is no data to support pHis isomerisation via a unimolecular process. Therefore, a specific pHis isomer within a protein (which can be thought of as an isolated system) is unlikely to undergo facile conversion to the isomeric pHis. However, caution must be used when handling pHis and pHis proteins due to the labile/ transferable nature of the pHis phosphoryl group under certain conditions.

The half-life of pHis residues in proteins is an important question to be addressed. It is likely that the half-life will depend on the pHis isomer, solutes in solution and protein structure. A hypothetical example could be an exposed pHis residue in the presence of calcium ions where changes in calcium ion concentrations regulate pHis hydrolysis. The presence of pHis residues which have long life-times could imply a need for regulatory phosphatases, whilst residues with relatively short life times may be self-regulating.

It is likely that mass spectrometry (MS) will be used increasingly to detect pHis and the TRIPLET fingerprint approach described by Kee *et al*. will help in pHis peptide analysis. For many other post-translation modifications if enough is known about the proteins and a pure sample can be obtained, MS analysis alone may suffice. However, due to the acid labile nature of pHis and the presence of two isomers other methods of detection in parallel are needed. Methods such as phosphoamino acid analysis and ^{31}P NMR allow pHis isomers to be distinguished, but require standards.

With the emergence of the long awaited pHis analogues and antibodies, advances are anticipated in the detection and enrichment of existing/unknown pHis proteins and in the understanding of the cellular function of pHis

Conflict of interest

The authors declare that there are no competing interests associated with the manuscript.

Acknowledgements

We apologise that we were unable, due to space constraints, to include all references to work involving pHis.

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