Histidine Phosphorylation

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Detection and Identification of Protein-Phosphorylation Sites in Histidines through HNP Correlation Patterns**

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Cellular processes and cell signaling are regulated by posttranslational modifications, such as protein phosphorylation. One of the most important phosphorylations occurs at histidine residues.[1,2] As a result of the acid lability of the N–P bond formed and the two possible sites of phosphorylation of the imidazole ring, this phosphorylation is the most difficult to characterize by biophysical methods despite the advancements in mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. Only recently, improvements in the sample preparation for mass analysis have allowed phosphohistidines to be detected.[3–5] However, mass spectrometry cannot provide information about the regiochemistry of the phosphorylation.

A precise identification of the phosphorylated nitrogen atom ($N^{\delta1}$ or $N^{\varepsilon2}$) within the imidazole of the phosphohistidine is still time consuming, needs a lot of effort, or is not yet established. For mass spectrometry, the β-elimination resulting in a characteristic loss of 80 Da ($-PO_3H^-$) in phosphorylated histidines[4] is identical for both the $N^{\delta1}$ or the $N^{\varepsilon2}$ nitrogen site. For NMR spectroscopy, comparison of ^{31}P chemical shifts from 1D ^{31}P spectra or ^{15}N chemical shifts from 2D ^{1}H–^{15}N correlation spectra[6–10] to reference compounds, such as chemically phosphorylated histidine, were inconclusive even if the protein was unfolded.[11] In principle, the regiochemistry can be determined by comparing the pH-dependent or temperature-dependent hydrolysis rates of the N–P bond to free phosphohistidines.[12,13] However, hydrolysis rates of phosphohistidines in proteins can be different than in free phosphohistidine because of the environment in the protein and they are cumbersome to determine.[14] Further, detection of hydrolyzed phosphate calls for additional experimental work, such as chromatographic separation for quantification of ^{32}P-labeled phosphate.[14] Thus a robust and fast analytical method to determine the regiochemistry of histidine phosphorylation is in need.

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For NMR spectroscopists, it is clear that phosphorylated nitrogen atoms can be directly identified by correlation spectra through one-bond scalar couplings between ^{15}N and ^{31}P ($^1J(^{15}N,^{31}P)$). In this study, we will show that the regiochemistry can be determined through correlations of the signals of phosphorylated ^{15}N atoms with protons in the imidazole ring without having to resort to interpretation of chemical shifts. The assignment of the histidine protons in turn is possible by conventional proton–proton NOE or CC-TOCSY experiments.[15] A HNP experiment for this particular combination of nuclear isotopes has not been reported to date and that might be due to the broad range of $^1J(^{15}N,^{31}P)$ coupling constants depending on the chemical structures of the compounds,[16] or the lack of information about the coupling constants in phosphohistidines. Therefore, we characterized the $^1J(^{15}N,^{31}P)$ couplings in phosphohistidines. Based on this characterization, an NMR experiment was developed and is presented herein for directly determining histidine phosphorylation and further identifying the regiochemistry based on correlation patterns rather than on chemical shifts because chemical shifts could vary depending on the fold of the protein.

Magnetization transfer from ^{1}H to ^{31}P in phosphohistidines can be achieved through scalar couplings between ^{1}H and ^{15}N ($^2J(^1H,^{15}N)$ and ($^3J(^1H,^{15}N)$) and between ^{15}N and ^{31}P ($^1J(^{15}N,^{31}P)$) for all three possibilities of phosphorylated histidine (Figure 1). One, two, and three cross peaks between ^{1}H and ^{31}P for **1**, **2**, and **3**, respectively, can be seen in a spectrum, provided that the efficiency of the transfer through the $^1J(^{15}N,^{31}P)$ couplings is as independent as possible of the phosphorylation pattern. Intensities of cross peaks depend on the size of couplings between ^{1}H and ^{15}N ($^2J(^1H,^{15}N)$ and $^3J(^1H,^{15}N)$). Since $^3J(^1H,^{15}N)$ (−2 Hz) is significantly smaller than $^2J(^1H,^{15}N)$ (−5 to −10 Hz) in histidine, the transfer through $^3J(^1H,^{15}N)$ is inefficient and thus cross peaks arising through $^3J(^1H,^{15}N)$ are smaller by a factor of 6 to 20 than those from the $^2J(^1H,^{15}N)$ couplings.[6,17] Therefore, the phosphorylation states can be determined with individual peak patterns in correlation spectra (HNP experiment). Since the $^1J(^{15}N,^{31}P)$ couplings in phosphohistidine are not well documented and, in proteins, pK_a values may vary, we determined them for a range of pH values under which the different phosphohistidines are stable: from pH 3 to pH 10 for **1** and **2** (Figure 2a), and from pH 6 to 9 for **3** (Figure 2b).

We find a strong dependence of the $^1J(^{15}N,^{31}P)$ couplings on the pH value for **1** and **2** varying between 5 and 17 Hz. Since the inflection point of the $^1J(^{15}N,^{31}P)$ couplings coincides with the pK_a value of the respective unphosphorylated nitrogen atom[12,18] we conclude that the protonation state of this nitrogen atom strongly influences the size of the coupling

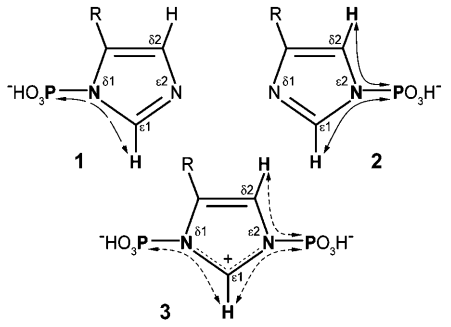

Figure 1. Illustration of phosphorylation states in the imidazole ring of histidine. **1**: δ1-phosphorylation, 1-phosphohistidine; **2**: ε2-phosphorylation, 3-phosphohistidine; **3**: δ1,ε2-diphosphorylation, 1,3-diphosphohistidine. Arrows indicate possible magnetization transfer pathways used by NMR (HNP experiment) to identify individual species **1**, **2**, and **3**. ———: a single $^{1}H^{\varepsilon1}$-$^{15}N^{\delta1}$-$^{31}P^{\delta1}$ correlation; -----: $^{1}H^{\varepsilon1}$-$^{15}N^{\delta1}$-$^{31}P^{\varepsilon2}$, $^{1}H^{\delta2}$-$^{15}N^{\varepsilon2}$-$^{31}P^{\varepsilon2}$, and $^{1}H^{\delta2}$-$^{15}N^{\varepsilon2}$-$^{31}P^{\varepsilon2}$ correlations; ——: $^{1}H^{\varepsilon1}$-$^{15}N^{\varepsilon2}$-$^{31}P^{\varepsilon2}$ and $^{1}H^{\delta2}$-$^{15}N^{\varepsilon2}$-$^{31}P^{\varepsilon2}$ correlations. ^{1}H, ^{15}N, and ^{31}P nuclei involved in the magnetization transfer are in bold. Magnetization is transferred through $^{2}J(^{1}H,^{15}N)$ and $^{1}J(^{15}N,^{31}P)$ scalar couplings. Identification of phosphorylation sites in the imidazole ring of histidine can be achieved by counting the number of cross peaks (which will be the same as the number of arrows).

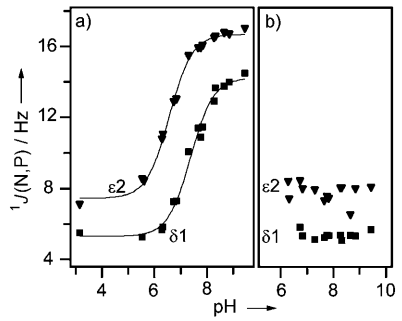

Figure 2. pH dependence for the $^{1}J(^{15}N,^{31}P)$ scalar coupling for a) **1** (■ δ1-phosphorylation) and **2** (▼ ε2-phosphorylation) and b) **3** (■ δ1-phosphorylation and ▼ ε2-phosphorylation). The coupling constants were extracted from 1D ^{31}P NMR experiments of a pH titration series and were fitted with a sigmoidal function shown as solid lines. Inflection points of **1** and **2** were at pH 7.4 and 6.6, respectively, which reflect protonation of the non-phosphorylated nitrogen atom of histidine. Since **3** does not have a non-phosphorylated nitrogen atom no pH dependence is observed in (b). The Figure clearly shows the coupling constants of **1** and **2** at low pH value are almost identical to the corresponding coupling constants of **3**, due to the similarity of the electron environment for the fully protonated imidazole ring. As a result of fast hydrolysis, signals of **3** could not be observed below pH 6.0.

constant. In agreement with this interpretation, for **3**, which does not have a nitrogen atom that can be protonated, the $^{1}J(^{15}N,^{31}P)$ coupling constants are independent of pH value. Owing to the steepness of the titration curves the difference of pK_a values for **1** and **2** of only 0.8 units results in $^{1}J(^{15}N,^{31}P)$ couplings that differ by almost a factor of 2 at pH 6.3. The strong dependence of $^{1}J(^{15}N,^{31}P)$ on the protonation state is not surprising, since similar effects were observed for ^{1}J-($^{13}C,^{15}N$) couplings in histidine upon pH titration without phosphorylation.[17,19]

Similar to the $^{1}J(^{15}N,^{31}P)$ couplings for **1** and **2**, pH-value dependent sigmoidal curves were also observed for ^{15}N chemical shifts (data not shown). Note that the $^{1}J(^{15}N,^{31}P)$ values for **3** are almost identical to the ones for protonated **1** and **2**. We conclude that the electron-withdrawing effect of the phosphate group is similar to that of a proton.

With the knowledge of the values of the $^{1}J(^{15}N,^{31}P)$ couplings in phosphohistidines we designed the HNP experiment (Figure 3) for magnetization transfer from ^{1}H through

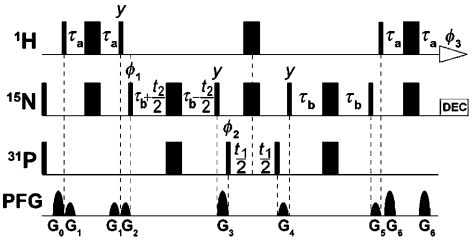

Figure 3. NMR pulse sequence of the HNP experiment for identification of phosphorylated histidines. Thin and thick bars represent 90° and 180° non-selective pulses, respectively. Default phases are *x* unless indicated otherwise. Delay times are set to $\tau_a = 11$ ms, $\tau_b = 25\text{ ms} = (4^{1}J(^{15}N,\ ^{31}P))^{-1}$ and phases are cycled in the following manner: $\phi_1 = \{x, -x\}$, $\phi_2 = \{x, x, -x, -x\}$, and $\phi_3 = \{-x, x, x, -x\}$. ^{1}H and ^{31}P carrier frequencies were set to $\delta = 4.7$ ppm and -3 ppm, respectively. ^{15}N carrier frequencies were set to $\delta = 210$ (for histidine), 200 (for HPr), and 205 ppm (for PRDI), respectively. ^{15}N decoupling was achieved by the GARP pulse sequence with field strength of 1.3 kHz. PFG (pulsed field gradients) indicate the magnetic field gradient strength applied along the *z* axis: G_0 and G_6: duration = 1 ms; G_{1-5}: = 0.5 ms; G_0: amplitude = 45 G cm^{-1}, G_1: 11 G cm^{-1}, G_2: 17 G cm^{-1}, G_3: 36 G cm^{-1}, G_4: 6 G cm^{-1}, G_5: 15 G cm^{-1}, and G_6: 30 G cm^{-1}. States-TPPI quadrature detection methods were applied for frequency discrimination in both indirect dimensions by alternating phases of ϕ_1 and ϕ_2. Experiments were carried out on a Bruker Avance I spectrometer equipped with a QCI probe head operating at the ^{1}H frequency of 600 MHz.

^{15}N to ^{31}P and back. It is a regular long-range $^{1}H,^{15}N$-HSQC[6] combined with an additional $^{15}N,^{31}P$-HSQC. The $^{1}H,^{15}N$-HSQC is preferred over the HMBC since there are no homonuclear couplings of ^{1}H that could reduce the transfer function in the HSQC. The dephasing and rephasing delay for the $^{1}J(^{15}N,^{31}P)$ is set to $\tau_b = 25$ ms based on an average coupling (10 Hz; Figure 2) from the free phosphohistidine to achieve sufficient magnetization transfer for all phosphohistidine species. To test the performance of the HNP experiment, we applied it to a mixture of **1**, **2**, and **3** which are readily obtained by phosphorylation of histidine by phosphoramidate (PA; Figure 4).[12] In agreement with Figure 1, compound **1** gave one $^{1}H,^{31}P$ cross peak, compound **2** shows two $^{1}H,^{31}P$ cross peaks with identical ^{31}P chemical shift, and compound **3** shows three $^{1}H,^{31}P$ cross peaks at three of the four corners of a rectangle. The additional minor peaks for **1** and **3** originate from non-zero $^{3}J(^{1}H,^{15}N)$ couplings between $^{1}H^{\delta2}$ and $^{15}N^{\delta1}$ as described earlier.[6]

To show the applicability of the sequence to a protein, we chose the phosphocarrier protein HPr which is a central

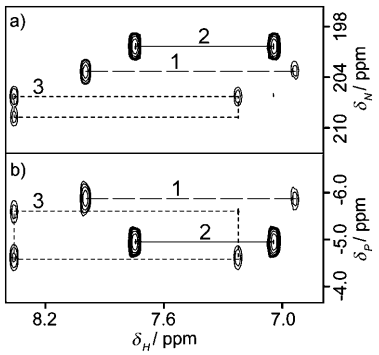

Figure 4. Contour plots of 2D projections from a 3D HNP spectrum of phosphorylated histidines at pH 7.5 and 303 K. a) $^{1}H,^{15}N$ and b) $^{1}H,^{31}P$ projections. The spectrum was recorded with 4 scans in approximately 4 h and 17 (^{31}P), 35 (^{15}N), and 512 (^{1}H) complex points with $t_{1max} = 8.7$ ms, $t_{2max} = 7.2$ ms, and $t_{3max} = 65.8$ ms. Identification of all three possible phosphorylation states in the imidazole ring of histidine is highlighted according to Figure 1: (**– – –**) "one-peak" pattern (pattern 1) characteristic for **1**, (**——**) the "two-peak" pattern (pattern 2) for **2**, and (**-----**) the major "three-peak" pattern (pattern 3) for **3**. The minor peaks in patters 1 and 3 result from a small three-bond coupling ($^{3}J(^{1}H,^{15}N)$) between $^{1}H^{\delta 2}$ and $^{15}N^{\delta 1}$.

enzyme of the phosphotransferase system (PTS) in prokaryotes and regulates the uptake of various carbohydrates.[20] The phosphorylation of histidine 15 is well characterized[21,22] and can be efficiently achieved by enzymatic phosphorylation (see the Supporting Information). We observed a single "one-peak pattern" (Figure 5a and Figure S1 in the Supporting

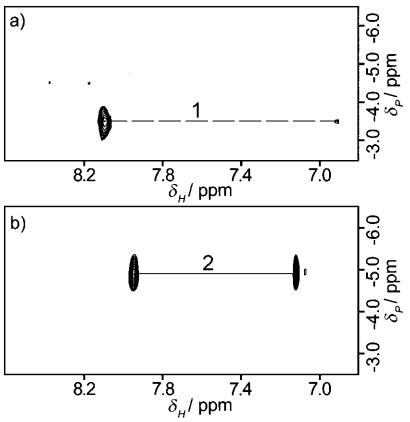

Figure 5. Contour plots of 2D $^{1}H,^{31}P$ projections from 3D HNP spectra of a) phosphocarrier protein (HPr) and b) phosphotransferase system regulation domain I (PRDI) of the antiterminator protein GlcT.

Information) clearly indicating a $N^{\delta 1}$ phosphorylation site corresponding to **1**.[21,22] The additional measurement of the $^{1}J(^{15}N,^{31}P)$ coupling constant (9.5 Hz at pH 7.4) for phosphohistidine in phosphorylated HPr agrees very well with the $^{1}J(^{15}N,^{31}P)$ coupling constant for **1** obtained from the pH titration.

To test the robustness of our method, we measured a 3D HNP spectrum of the phosphotransferase system regulation domain I (PRDI) of the antiterminator protein GlcT from *Bacillus subtilis*. This domain is known to be phosphorylated at a histidine residue but no further information is available, especially on the regiochemistry of the phosphorylation.[23] As shown in Figure 5b, phosphorylation occurs at position $N^{\varepsilon 2}$ of one of the seven histidine residues in PRDI (pattern 2; cf. Figure 4). This result is at variance with the phosphorylation of HPr histidine (pattern 1).

In principle, τ_b can also be adjusted so that all the phosphorylation states of a histidine can be determined based on the individual coupling constants $^{1}J(^{15}N,^{31}P)$ of the free phosphohistidine and the pK_a values of the phosphohistidines in proteins. As mentioned before, pK_a values may vary because of differences in the protein environment. However, since all phosphorylation sites in the free phosphohistidine were easily identified from specific H,P correlation patterns (Figure 4), $\tau_b = 25$ ms is a good starting point. The experiments took 20 and 67 h for HPr and PRDI, respectively. Sensitivity can be improved by a factor of $\sqrt{2}$ by recording the experiment either as a 2D $^{1}H,^{15}N$ or $^{1}H,^{31}P$ correlation spectrum. However, if more than one histidine is phosphorylated in a protein, because of the narrow chemical shift ranges of phosphohistidines (ca. 200 to 210 ppm for ^{15}N and ca. 0 to -10 ppm for ^{31}P), ambiguities arising from possible overlap of ^{15}N or ^{31}P resonances are best avoided using the 3D version. For larger proteins with shorter proton and nitrogen T_2 times the delays τ_a and τ_b can also be reduced.

We have characterized the pH dependence of the $^{1}J(^{15}N,^{31}P)$ coupling constant in phosphohistidines. Based on this coupling, we have proposed an experiment that determines the regiochemistry of histidine phosphorylation and could show its robustness with two proteins, HPr and PRDI. Previous methods to identify the regiochemistry required time-consuming pH titration, hydrolysis, and/or comparison studies with phosphohistidine reference components. We anticipate that the HNP experiment proposed herein will play a significant role in studying histidine phosphorylation which is important in prokaryotic systems. Furthermore, our HNP experiment may give insight into recent findings of phosphohistidines in eukaryotic proteins.[24–26]

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