Thiophosphorylation of Histidine

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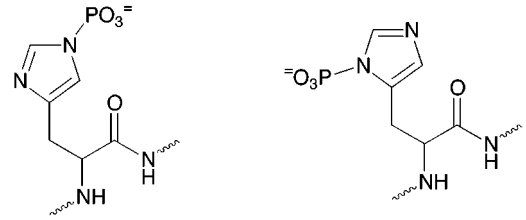
The preparation of a novel phosphorus species, thiophosphoramidate, has enabled the specific thiophosphorylation of histidine at its 3-position. The rates of phosphorylation and thiophosphorylation of histidine are reported, as well as the spectroscopic properties of both thiophosphoramidate and 3-thiophosphohistidine. Structural assignment of the latter was made by analogy to the NMR properties of the known 3-phosphohistidine. The alkylation of 3-thiophosphohistidine by phenacyl bromide serves as a model for the introduction of labeling or probe reagents into histidine phosphorothioate-containing proteins.

Introduction

Phosphorylation of histidine-containing proteins is a key step in the mechanisms of many phosphate transfer enzymes (kinases and phosphatases, inter alia[1−11]) and is the first stage in a wide variety of signal transduction cascades in bacteria, yeast, and higher plants.[12] The phosphate transfer potential ($\Delta G°$ of transfer) of phosphohistidine is very high, estimated to be −12 to −14 kcal/mol.[13]

Initial studies of phosphohistidine were inspired by its isolation as the free amino acid from basic hydrolysates of mitochondria, where phosphohistidine is involved in ATP synthesis by succinyl-CoA synthetase in the citric acid cycle. A simple question of isomerism arises in phosphohistidine as a result of its two imidazole nitrogens and a tautomeric equilibrium that permits either to be phosphorylated. By treatment of free histidine with phosphoramidate, Boyer prepared both isomers,[14] 1-phosphohistidine and 3-phosphohistidine (Chart 1). The latter is thermodynamically more stable. While both are fairly stable to base, they are quite unstable in the presence of acid. They also differ significantly in their hydrolysis rate, with 1-phosphohistidine having a 5 min half-life at pH 2.4 (46 °C), while 3-phosphohistidine has a 25 min half-life. Some studies of phosphohistidines in proteins do not distinguish between these isomers. The hydrolytic reactivity of phosphohistidine has been used to infer its presence in a protein, and even the particular isomer present. Hydrolysis is enhanced by hydroxylamine or pyridine, which has also been used as a diagnostic test for phosphohistidine as compared to other acid-labile protein phosphates. Structures of proteins containing phosphohistidine intermediates[15] have been determined by crystallography.[2] Absent a crystal structure, phosphohistidine structural determination can require full NMR characterization of the phosphoprotein.[16−18] Such work has definitively established the site of phosphorylation, including histidines phosphorylated on either nitrogen. Interestingly, the phosphorylation of histidine seems to have a small effect on overall protein conformation.

Chart 1



3-phosphohistidine 1-phosphohistidine

Preparation of phosphohistidine or phosphohistidine-containing peptides can be accomplished with phosphorylating agents such as phosphoramidate.[19] The phosphorylation of histidine itself with phosphoramidate proceeds first to give the less stable 1-phosphohistidine

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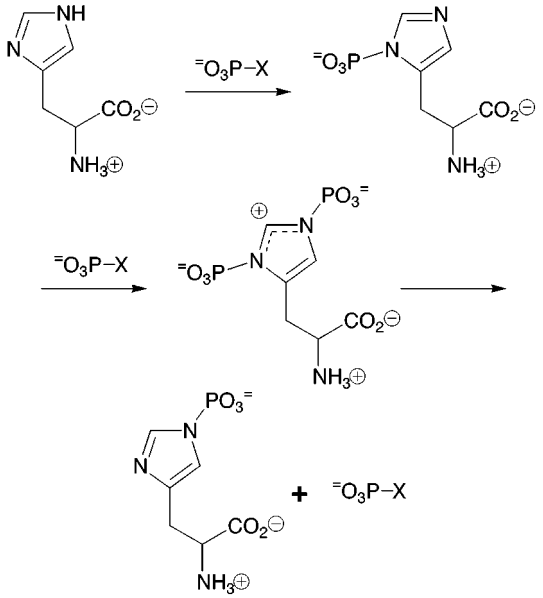
Scheme 1



(kinetic product), which is converted to a 1,3-diphosphohistidine salt, which is finally (1−2 days) converted to the 3-phosphohistidine isomer (thermodynamic product) (Scheme 1). The primary amine does not appear to be problematic provided that the reaction is performed under conditions where it is protonated. The 3-isomer is more stable, presumably as a result of reduced steric demands.

The instability of the phosphohistidine isomers has impeded study of their chemistry and biology. One approach to increasing the stability of phosphohistidine, as with other biological phosphate derivatives,[20] is through substitution of sulfur for oxygen. This strategy offers an additional attraction in that a highly nucleophilic atom is introduced that can be used for attachment of probe molecules. This concept has been explored with serine phosphates.[21,22] As a model for the corresponding histidine derivatives, electrostatic potential surfaces of the lithium salts of 4-methylimidazole-1-phosphate and 4-methylimidazole-1-phosphorothioate were calculated using the AM1 semiempirical method. They are closely similar, supporting the idea that introduction of the sulfur atom will make only a small steric and electronic perturbation to phosphohistidine, so that thiophosphohistidine should sustain its chemical and enzymatic reactivity, though to a reduced extent.

While this work was underway, another publication appeared[23] reporting the thiophosphorylation of histidine and histidine-containing peptides (using $PSCl_3$ because these workers were unable to prepare thiophosphoramidate). They assigned the single isomer formed in histidine thiophosphorylation with $PSCl_3$ as the 3-isomer on the basis of a chromatographic mobility comparable to 3-phosphohistidine. They showed that thiophosphohistidine is stable at pH 1 (3 h), whereas phosphohistidine is not.

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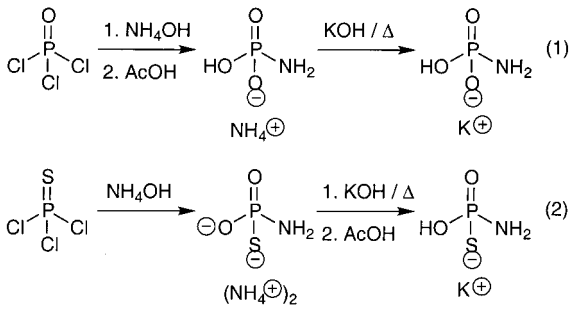
Results

To establish a baseline for studies of histidine thiophosphorylation, histidine phosphorylation with phosphoramidate was first examined. The required phosphorylating and thiophosphorylating reagents are not available commercially and were synthesized as shown (eqs 1 and 2).[24] The preparation of phosphoramidate has



been reported.[25] This literature procedure had never been applied to $PSCl_3$, and surprisingly, the thiophosphoramidate product was an unknown compound. Initial investigations simply substituting $PSCl_3$ into the literature procedure were inadequate, with almost 90% of the product consisting of inorganic salts. The ^{31}P NMR spectrum was very clean, however. Modifications required for a useful preparative thiophosphoramidate synthesis involved reducing the ammonia concentration and stirring for an extended period until a homogeneous solution resulted. The salt that was precipitated with acetone proved to be the analytically pure diammonium salt. After metathesis, potassium thiophosphoramidate is obtained as very white flakes.

The thiophosphoramidate salts were characterized by several methods. First, the salts precipitated from the reaction mixture were analytically pure. The ^{31}P NMR signal for the ammonium salt is found at δ 41.51 ppm, while that for the potassium salt is at 39−42 ppm, depending on pH. The potassium salt is a monobasic acid with a pK_a of ∼1.5, determined by the pH dependence of this chemical shift. The IR spectrum shows a 614 cm^{-1} absorption, which compares well to known PS stretches at 614 and 618 cm^{-1}.[26]

Preparations of these reagents can include varying amounts of salts (presumably KCl and NH_4Cl). Phosphorus content was assayed by NMR comparison to commercial potassium phosphate. Phosphoramidate synthesis has yielded >90% pure potassium salt. Thiophosphoramidate synthesis has yielded the analytically pure potassium salt. In general, reactions performed on smaller scales tend to yield purer products. While relatively stable, hydrolysis and discoloration may occur during storage if proper precautions, such as storage under argon in a freezer, are not taken. Discoloration signals poor performance in thiophosphorylation reactions. The thiophosphoramidate salts are significantly less soluble in aqueous solution than the phosphoramidates.

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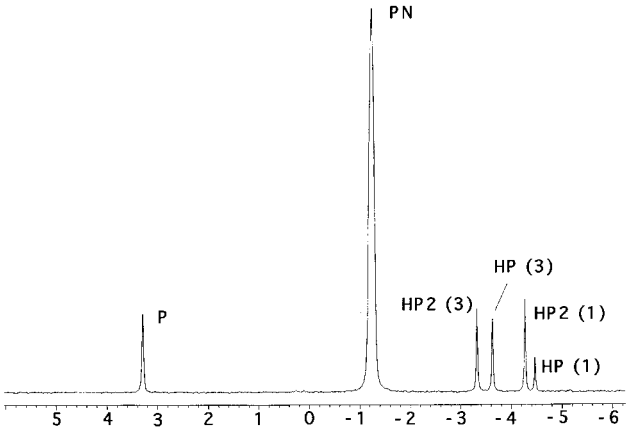


Figure 1. ^{31}P NMR spectrum of histidine phosphorylation. P is inorganic phosphate. PN is potassium phosphoramidate, HP(1) is 1-phosphohistidine. HP2 is 1,3-diphosphohistidine, HP2(1) is the 1-phosphate of the diphosphate, and HP2(3) is the 3-phosphate of the diphosphate. HP(3) is 3-phosphohistidine.

To establish a baseline for thiophosphorylation experiments, phosphorylation of histidine was studied by ^{31}P NMR. A sample spectrum after 2 h is given in Figure 1, showing each phosphorylated histidine species. Assignments are based both on δ values and the reaction course reported in the literature.[14] Semiquantitative studies of reaction kinetics were made using NMR integration.[27] Phosphorus can have a rather long relaxation time, depending on environment. T_1 for inorganic phosphate has been measured at 14 s in H_2O and 50 s in D_2O.[28] It was thus impractical to perform NMR experiments using an acquisition delay sufficiently long to ensure that each phosphorus species is fully relaxed. However, if the environments of all the phosphorus species in solution are equivalent in connectivity to hydrogen atoms and in valency, then their relaxation times should be roughly equivalent. Thus, integration of these nuclei can give reliable values for the abundance of each phosphorus species with respect to one another, even if they are not fully relaxed. These criteria are met for both phosphorylating reagents and their hydrolysis products, but this assumption is not adequate for the phosphohistidine and thiophosphohistidine products. Phosphorus in these compounds has connectivity through three bonds to one or two hydrogens. The effect of this distant connectivity is small but real. Phosphorus nuclei bound to histidine relax slightly faster than those that are not. Thus, their integrations will be slightly enhanced relative to those of other phosphorus nuclei. By varying the acquisition delay, an estimate was made of the enhancement of phosphohistidines compared to phosphates in a histidine phosphorylation reaction. It is 25%, calling for scaling of the integrations of phosphohistidine species by 0.8 to give a valid relative population. This scaling was applied to all rate data presented.

A reaction progress curve under pseudo-first-order conditions for histidine phosphorylation as determined by ^{31}P NMR with a 10-fold excess of phosphoramidate is

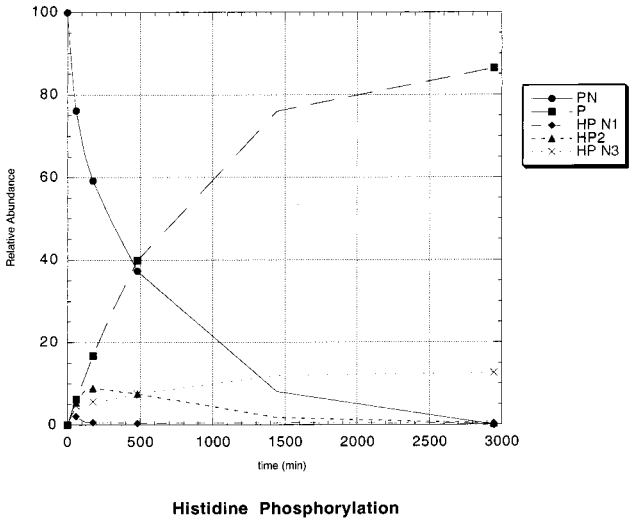
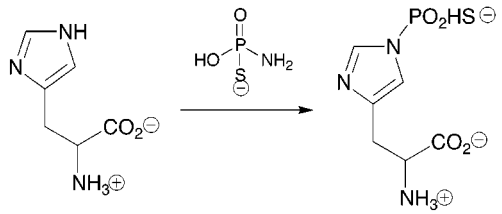


Figure 2. Reaction progress of histidine (97 mM) phosphorylation by potassium phosphoramidate analyzed by ^{31}P NMR, given in mole fraction of phosphorus. PN is phosphoramidate. P is inorganic phosphate. HPN1 is 1-phosphohistidine. HP2 is 1,3-diphosphohistidine, HP2 is the 1-phosphate of the diphosphate, and HP2(3) is the 3-phosphate of the diphosphate. HPN3 is 3-phosphohistidine. Time is in minutes.

Scheme 2



given in Figure 2. These reactions were performed using pure potassium phosphoramidate, as measured by NMR and elemental analysis. N1 phosphorylation has a $t_{1/2}$ of 10 min, diphosphorylation has a $t_{1/2}$ of 70 min, and production of the N3 phosphate by hydrolysis of the former has a $t_{1/2}$ of 100 min. These results for histidine phosphorylation are in good agreement with those reported in the literature.[14] Specifically, the 1-phosphohistidine and the 1,3-diphosphohistidine are the major products at early reaction times. These forms of phosphohistidine are eventually replaced by 3-phosphohistidine. This reaction proceeds essentially to completion, during which turnover of phosphoramidate to inorganic phosphate occurs. When just the hydrolysis step is examined in the presence and in the absence of histidine (Figure 1, Supporting Information), it is evident that histidine catalyzes the hydrolysis of phosphoramidate to phosphate. Histidine thus has "phosphoramidate-ase" activity. Two mechanisms are at work in generating phosphate in the phosphorylation of histidine: direct hydrolysis of phosphoramidate and hydrolysis of the product phosphohistidines.

These experiments provide the background for the thiophosphorylation of histidine with thiophosphoramidate (Scheme 2). Initial reactions were performed using 10% potassium thiophosphoramidate, as measured by NMR, in only a 2-fold excess. The spectrum shows the accumulation of only a single thiophosphohistidine in the reaction, as reflected in the progress curve given in Figure 3. Thiophosphorylation has a $t_{1/2}$ of 500 min at

(27) These are not quantitative because of the rate variation with variation in ionic strength as a consequence of the different salt content of different phosphoramidate preparations, and incomplete phosphorus relaxation.

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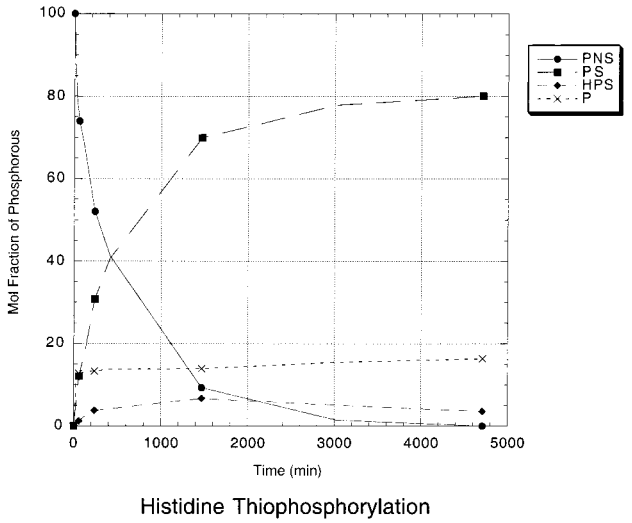


Figure 3. Reaction progress of histidine (97 mM) thiophosphorylation by potassium thiophosphoramidate analyzed by ^{31}P NMR, given in mole fraction of phosphorus. PNS is thiophosphoramidate. PS is inorganic thiophosphate. HPS is 3-thiophosphohistidine. P is inorganic phosphate. Time is in minutes.

pH 8. As with phosphorylation, the dominant process overall is hydrolysis of thiophosphoramidate to thiophosphate, which then hydrolyzes to phosphate (although the $t_{1/2}$ of the latter process appears to be greater than one month). The rate of hydrolysis of thiophosphoramidate in the absence of histidine is illustrated in Figure 2 of the Supporting Information. When hydrolysis is compared in the presence and absence of histidine (Figure 3, Supporting Information), it is evident that histidine is competing with the hydrolysis of thiophosphoramidate. This result suggests that thiophosphohistidine is relatively stable to chemical hydrolysis when compared to thiophosphoramidate. Unlike phosphoramidate hydrolysis, thiophosphoramidate hydrolysis does not accelerate in the presence of histidine. Similar reaction progress curves for histidine thiophosphorylation were obtained using pure potassium thiophosphoramidate in 7-fold excess.

The assignment of structure to the novel thiophosphohistidine was made using NMR spectroscopy. Examination of ^{1}H NMR spectra of phosphorylation reactions containing phosphohistidine or thiophosphohistidine (Chart 2) using single-frequency homonuclear decoupling at the δ and ϵ hydrogens ($J_4 = 1.2$ Hz) enables determination of the coupling between each hydrogen and phosphorus. Four-bond coupling between the β and δ hydrogens was found to be negligible and did not complicate the analysis. The relative abundance of phosphohistidine species in the phosphorylation reaction under the time constraints of this experiment meant that spectral analysis of 1-phosphohistidine was not possible. Analysis of 1,3-diphosphohistidine was performed, but the data were of little assistance in assignment of thiophosphohistidine structure. Data presented following therefore refer only to 3-phosphohistidine.

The comparable coupling constants between the known 3-phosphohistidine and the novel thiophosphohistidine permit assignment of the N3 isomer to the latter. The N1 isomer would have a four-bond coupling between phosphorus and the δ hydrogen, which should lead to a smaller J, when in fact it is larger in the thiophosphohistidine than in phosphohistidine.

Chart 2

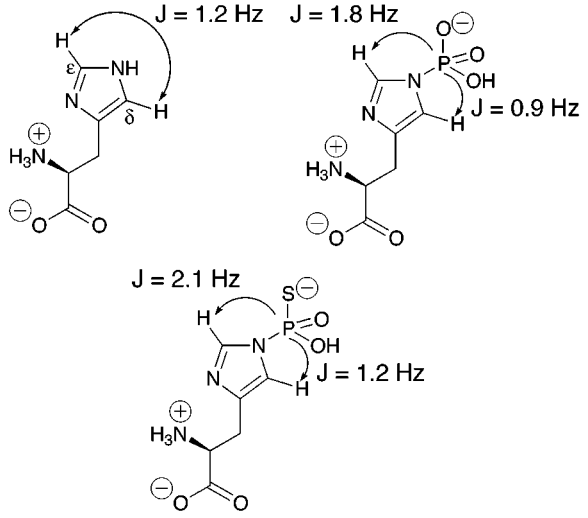
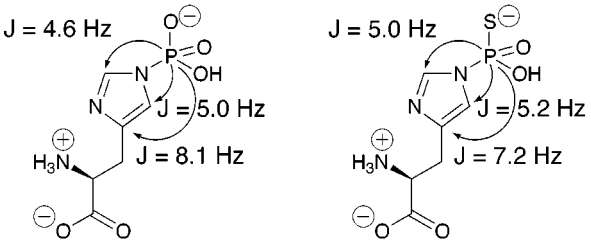
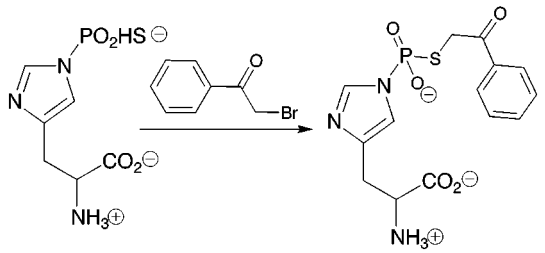


Chart 3



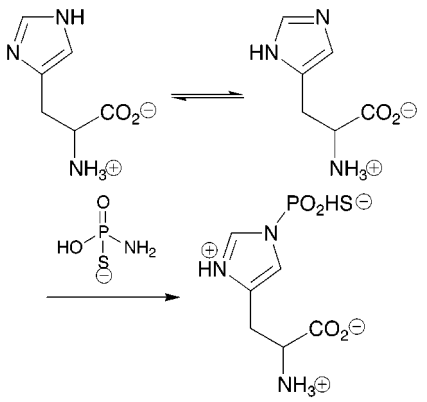
Scheme 3



This structural assignment was further strengthened by heteronuclear decoupling experiments. The most useful information came from [^{1}H]-noise-decoupled ^{13}C analysis (Chart 3). The correspondence of coupling constants between thiophosphohistidine and 3-phosphohistidine further supports the assignment of the thiophosphate as the 3-isomer. The larger C−P coupling to the ipso aromatic carbon in each compound is consistent with the Karplus relationship and well-precedented. If the thiophosphohistidine were the 1-isomer, this large coupling would be to a protonated carbon, not the ipso carbon. Proton decoupling of ^{31}P spectra did not provide useful information. Even when the spectral window was decreased to 20 ppm and the spectral resolution was maximized, the ^{31}P signal for each compound was a broad singlet.

The availability of 3-thiophosphohistidine enabled alkylation by reactive alkylating agents to be investigated. Its reaction with phenacyl bromide was followed by NMR (Scheme 3). The evolution from the starting ^{31}P chemical shift of 41 ppm to that of the product at 16 ppm is observed within 5 min. A comparison reaction of

Scheme 4



thiophosphoramidate with phenacyl bromide likewise produces a 16.5 ppm signal. This chemical shift is seemingly characteristic of $N(PO_2)SR$ functionalities.

Discussion

The outcome of the reaction of histidine with thiophosphoramidate, direct formation of the 3-thiophosphoryl derivative, is divergent from that reported (and recapitulated here) for its reaction with phosphoramidate. Phosphorylation proceeds first by 1-phosphorylation, then diphosphorylation, and finally hydrolysis to the more stable the 3-phosphoryl derivative. Turck's report that thiophosphorylation of histidine with $PSCl_3$ produces exclusively the 3-isomer further supports our structural assignment. It is very unlikely that the 1-thiophosphate derivative was formed as an intermediate in this process but was not observed. Thiophosphates generally show slower rates of reaction than phosphates, so such intermediates would be easy to observe if they were formed. Further, 1,3-bis(thiophospho)histidine would be the intermediate following the 1-thiophosphate, and one would expect to be able to observe this species as well; the 1,3-bis(phosphate) *was* observed in phosphorylation reactions.

The direct formation of exclusively the 3-thiophosphohistidine rather than the path followed with phosphoramidate can be explained by application of the Hammond postulate. That is, the more stable thiophosphohistidine isomer is preferentially formed because the transition state for thiophosphorylation is later than for phosphorylation. The inherent chemical instability of the putative 1-thiophosphohistidine, through steric interaction with the adjacent alkyl group, is reflected in the transition state for thiophosphorylation to a greater extent than in the transition state for phosphorylation. This disfavors formation of 1-thiophosphohistidine in this reaction even though it would involve reaction at the more nucleophilic nitrogen in the preferred histidine tautomer. It therefore seems likely that thiophosphorylation proceeds with the disfavored histidine tautomer in order to permit reaction with a more sterically accessible nonaromatic nitrogen (Scheme 4).

Thiophosphohistidine is more stable chemically than phosphohistidine and, given previous studies of thiophosphate derivatives of biologically active phosphates, it is likely that it will also be less reactive in enzyme-catalyzed processes. The results of Turck showed that histidine-thiophosphorylated proteins can be digested with trypsin without loss of the thiophosphate group, whereas histidine-phosphorylated proteins are hydrolyzed under the same conditions. The reactions of thiophosphoramidate and its products proceed more slowly and to a lesser extent than do those of phosphoramidate and its products.

Thiophosphoramidate offers advantages over alternatives, such as $PSCl_3$ or ATP-γ-S, as a reagent for generating thiophosphohistidine. Many biomolecules could not tolerate the high reactivity of the former, and many of their other nucleophilic groups could be thiophosphorylated. The attenuated reactivity of ATPs permits selective (thio)phosphorylation of the active site histidine in proteins but does not permit peptide phosphorylation in the absence of a specific ATP recognition site. Thiophosphoramidate should be useful for histidine thiophosphorylation in amino acids, peptides, and proteins in aqueous and nonaqueous solutions.

The close correspondence of the electrostatic potential surfaces of histidine imidazoles bearing phosphate and thiophosphate should make thiophosphohistidine a useful, more stable substitute and probe for the biological function of 3-phosphohistidine. This method for specifically generating 3-thiophosphohistidine may have advantage in uniquely providing this isomer for study when the site of phosphorylation is not known, ambiguous, or not controllable. However, if a particular enzymatic function *requires* 1-phosphohistidine, this method (which generates the more stable 3-phosphohistidine mimic) will not be able to address such issues.

Experimental Section

NMR Spectroscopy. Single-frequency homonuclear decoupling (acquisition on, delay off) was performed at a power of 27 dB at 25 °C.

Potassium Phosphoramidate (literature protocol). Phosphorus oxychloride is added at 0 °C to 10−30% NH_4OH. After 30 min, the reaction mixture is extracted with acetone. The aqueous layer is collected and adjusted to pH 6 with acetic acid. Ethanol is added to precipitate the monoammonium salt. Cation metathesis is accomplished by dissolving the precipitate in 50% KOH and heating (30 min, 60 °C). The solution is again cooled and adjusted to pH 6, and the salt is precipitated with EtOH.

Potassium Thiophosphoramidate. Thiophosphoryl chloride (98%, 3.3 mL) was added dropwise by syringe to a stirring solution of 10% ammonium hydroxide at 0 °C. The biphasic mixture was stirred vigorously until a homogeneous, clear solution resulted. The contents were transferred to a separatory funnel, and 175 mL of acetone was added. After 5 min, the white precipitate that formed in the bottom layer was collected by filtration. The precipitate (diammonium thiophosphoramidate) was washed with EtOH and ether. It was then dissolved in 2.5 mL of 50% KOH and heated at 60 °C for 30 min. The solution was chilled and brought to pH 6 by the addition of acetic acid. EtOH (100 mL) was added, and the mixture was gently agitated until a homogeneous solution with a white precipitate resulted. After filtration and washing with EtOH and ether, the product (1.6 g) was obtained as a white solid.

Our best procedure prepared diammonium thiophosphoramidate as above (78% yield). A portion of this ammonium salt (1.0 g) was dissolved in 3 mL of 50% KOH and heated at 60° for 30 min. The clear solution[29] was cooled to room

(29) A green solution was sometimes observed at this stage. Although the cause of this discoloration is not known, it led to decreased purity of the final product. Factors that seem to contribute to the green color include insufficient drying of the ammonium thiophosphoramidate, excessive heating of the KOH solution, and insufficient KOH solution.

temperature and added to 100 mL of EtOH. The heterogeneous mixture was gently agitated while acetic acid was added dropwise. Acidification was terminated upon the deposition of a white precipitate from the now homogeneous solution. The precipitate (potassium thiophosphoramidate) was filtered and washed with EtOH and ether (71% yield). The dry compound was stored sealed under argon.

Diammonium Thiophosphoramidate. ^{31}P NMR (D_2O): δ 41.51, >97%; unknown impurity $-\delta$ 54.43, <3%. UV (H_2O): λ_{max} 224 nm (log ϵ 1.50). Mp: 160 °C (dec). Anal. Calcd for $H_{10}N_3O_2PS$: H, 6.85; N, 28.56; S, 21.79. Found: H, 6.84; N, 28.33; S, 21.56. IR (KBr): 3150, 1610, 1111, 1002, 809, 609 cm^{-1}.

Potassium Thiophosphoramidate. ^{31}P NMR (D_2O): δ 38.8. UV (H_2O): λ_{max} 212 nm (log ϵ 1.34). Mp: >350 °C. Anal. Calcd for H_3KNO_2PS: H, 2.00; N, 9.27; S, 21.21. Found: H, 2.22; N, 9.09; S, 20.52. IR (KBr): 3392, 1642, 1414, 1113, 1055, 901, 614 cm^{-1}.

3-Phosphohistidine. In a 5 mm NMR tube were placed histidine (15 mg), potassium phosphoramidate (100 mg), and D_2O (1 mL). The reaction was monitored at 25 °C. ^{13}C NMR (D_2O): δ 174.38, 139.03 (d, J = 4.63), 134.36 (d, J = 8.10), 118.35 (d, J = 4.96), 54.70, 28.81. ^{1}H NMR (D_2O): δ 8.20 (bs, 1H), 7.00 (bs, 1H), 3.80 (dd, J = 5.1, 4.8, 1H), 3.40 (dd, J = 15.9, 4.0, 1H), 3.20 (dd, J = 15.9, 7.5, 1H). ^{31}P NMR (D_2O): δ −3.7.

3-Thiophosphohistidine. To histidine (0.6 g) in 25 mL of H_2O was added 1 g of freshly prepared potassium thiophosphoramidite. After 3 d at room temperature, the reaction mixture was diluted with 100 mL of H_2O and loaded onto a 3 cm × 13 cm Dowex-1 column preequilibrated with 0.1 M Tris·HCl, pH 8.25. The column was eluted with a linear gradient of 0 → 0.5 M LiCl in 0.1 M Tris·HCl. Fractions giving strong ninhydrin tests were combined. This solution was chilled and added dropwise with stirring to chilled EtOH. After 10 min, the precipitated lithium salt (0.45 g, 45%) was collected by filtration and dried under vacuum. ^{13}C NMR (D_2O): δ 173.93, 138.35 (d, J = 4.95), 133.70 (d, J = 7.16), 118.40 (d, J = 5.19), 54.60, 28.46. ^{31}P NMR (D_2O): δ 35.2.FAB MS (negative ion, glycerol/amine matrix): calcd for $C_6H_9O_4N_3PS$ 250.0051, found 250.0052; calcd for $C_6H_9O_4N_3PSLi$ 257.0211, found 257.0211. IR (KBr): 3403, 1632, 1518, 1057, 604, 494 cm^{-1}.

Kinetics of 3-Thiophosphohistidine Formation. In a 5 mm NMR tube were placed histidine (15 mg), potassium thiophosphoramidate (100 mg), and D_2O (1 mL). The reaction was monitored at 25 °C.

HPLC. Reverse phase HPLC (C-18 Econosil, 5 μm, Alltech) was used to separate histidine and 3-phosphohistidine. The following solvent system and gradient gave baseline resolution. A, 10 mM NH_4HCO_3; B, acetonitrile. %B: 0, 1 min; 0 to 10 over 5 min; 10 to 50 over 5 min; 50 to 0 over 5 min.

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Supporting Information Available: Progress curves for histidine-catalyzed phosphoramidate hydrolysis and thiophosphoramidate hydrolysis in the presence and absence of histidine. ^{13}C and ^{31}P NMR spectra for thiophosphohistidine. This material is available free of charge via the Internet at http://pubs.acs.org.

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