

Activity-Based Probe for Histidine Kinase Signaling

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S Supporting Information

ABSTRACT: Bacterial two-component systems (TCSs) are signaling pathways composed of two proteins: a histidine kinase (HK) and a response regulator (RR). Upon stimulation, the HK autophosphorylates at a conserved histidine. The phosphoryl group is subsequently transferred to an aspartate on an RR, eliciting an adaptive response, often up- or downregulation of gene expression. TCS signaling controls many functions in bacteria, including development, virulence, and antibiotic resistance, making the proteins involved in these systems potential therapeutic targets. Efficient methods for the profiling of HKs are currently lacking. For direct readout of HK activity, we sought to design a probe that enables detection of the phosphotransfer event; however, analysis of the phosphohistidine species is made difficult by the instability of the P–N bond. We anticipated that use of a γ -thiophosphorylated ATP analogue, which would yield a thiophosphorylated histidine intermediate, could overcome this challenge. We determined that the fluorophore-conjugated probe, BODIPY-FL-ATP γ S, labels active HK proteins and is competitive for the ATP binding site. This activity-based probe provides a new strategy for analysis of TCSs and other HK-mediated processes and will facilitate both functional studies and inhibitor identification.

Two-component systems (TCSs) are stimulus-and-response signaling pathways composed of a histidine kinase (HK) and a response regulator (RR). TCSs exist primarily in bacteria but have also been identified in some lower eukaryotes.^{1,2} They contribute to many critical bacterial functions, including virulence, resistance mechanisms, and survival, making the TCS proteins potential therapeutic targets.^{3–5} For example, TCS signaling has been implicated in pneumonia, toxic shock syndrome, tuberculosis, and a plethora of other acute and chronic infections.⁶ Moreover, TCSs have been correlated with antibiotic resistance in multiple Gram-negative and Gram-positive bacterial systems.^{1,4,7} HKs are known to be regulated by a number of stimuli such as temperature, peptides, and metal ions, which are sensed by an extracellular input domain.^{1,6,8} HK activation promotes conformational changes in the catalytic and ATP-binding (CA) domain as well as the dimerization and His phosphotransfer (DHp) domain (Figure 1A). Once the protein is in the activated state, a conserved His in the DHp domain attacks the γ -phosphate of ATP, resulting in autophosphor-

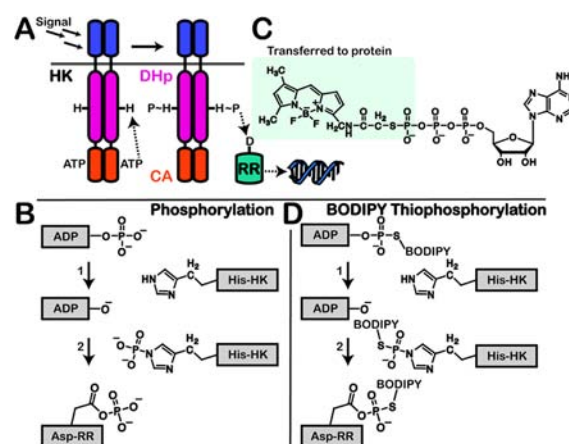


Figure 1. (A) HK is activated by an extracellular signal, which results in phosphorylation of a conserved histidine by ATP. The phosphoryl group is transferred to the aspartate on an RR. The activated RR then triggers a response, typically acting as a transcription factor. Domains: DHp, dimerization and histidine phosphotransfer; CA, catalytic and ATP-binding. (B) Mechanisms of HK autophosphorylation and phosphotransfer to RR. (C) Structure of B-ATP γ S, highlighting the thiophosphate–BODIPY moiety that is transferred during autophosphorylation. (D) Proposed mechanism of B-ATP γ S labeling of the active HK and cognate RR.

ylation (Figure 1B).^{3,9} The phosphoryl group is subsequently transferred to an aspartate (Asp) on a cognate RR, eliciting an adaptive response, often up- or downregulation of gene expression.^{3,9} Most bacteria express 20 or more TCSs,¹⁰ many of which may have redundant or overlapping functions. Thus, the development of facile methods to facilitate their study is an important goal.

Generation of straightforward methods for the profiling of HK activity has been a long-standing challenge because of the instability of the phosphohistidine (pHis) species.¹¹ In comparison with the study of phosphorylation at serine, threonine, and tyrosine, which has enjoyed great advancement in the past decade,^{12,13} detection of phosphorylated histidine residues has lagged far behind. Some success has been achieved by the application of Phos-tag, a phosphate-binding molecule embedded in a polyacrylamide gel matrix, to facilitate the separation and quantitation of phosphorylated HKs from nonphosphorylated ones.^{14,15} However, this technique has not seen wide application. The use of LC–MS-based

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techniques for detecting pHis-containing proteins/peptides is also severely hindered by the acid lability and short half-life of the P–N bond.^{16,17} Careful optimization can enable detection of specific phosphorylated HKs, but a global strategy does not exist. pHis instability is also likely responsible for the failure to generate an antibody for detection of phosphorylated histidine-containing proteins to date. Promisingly, some recent progress has been made using stable synthetic pHis analogues.¹⁸ Because of the described difficulties, most TCS studies still depend upon the use of radioactivity-based assays or analysis of the isostable phosphorylated RR species.

For direct readout of HK activity, we sought to design a probe that would enable detection of the phosphotransfer event; however, it was clear that a strategy for generating a more stable intermediate species would be required. We anticipated that the use of a γ -thiophosphorylated ATP analogue (ATP γ S), which would yield a thio-pHis, could overcome this challenge because of the increased stability that the sulfur confers on the P–N bond.^{16,17} Several laboratories have demonstrated that ATP γ S can act as a substrate for HKs.^{19,20} Recently, Shokat and Marletta used this alternative substrate to facilitate immunodetection of HK activity²⁰ in a manner analogous to that previously developed for Ser/Thr kinases.²¹ Their studies demonstrated that ATP γ S is a widely accepted substrate analogue, as it was turned over by five of six tested enzymes. Taking advantage of the ATP γ S scaffold, we sought to devise a direct-readout strategy that could detect HK activity in one step and be visualized immediately.

With this goal in mind, we examined a fluorescently labeled ATP analogue, BODIPY-FL-ATP γ S (B-ATP γ S), for its utility as an activity-based probe (ABP) for HKs (Figure 1C).²² ABPs commonly are substratelike scaffolds that covalently modify their enzyme target(s) to facilitate detection of the activity state.²³ Although B-ATP γ S is generally considered to be nonhydrolyzable, we hypothesized that it might be accepted as a substrate of HKs and could serve as an ABP, since autothiophosphorylation with this probe would yield a fluorescently labeled histidine conjugate (Figure 1D). We examined this possibility with HK853, an HK protein from *Thermotoga maritima*.^{24,25}

Initial experiments demonstrated that HK853 has good affinity for this probe [$K_{d,app} = 470 \pm 40$ nM, as measured using a fluorescence-polarization-based assay; Figure S1 in the Supporting Information (SI)], in stark contrast to reported ATP affinity values (~ 14 – 500 μ M).^{26,27} Next, we assessed the ability of the enzyme to autophosphorylate with the γ -thiophosphate–BODIPY group. HK853 was incubated with B-ATP γ S (2 μ M) at 25 °C for 1 h prior to running sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for in-gel fluorescence detection. Addition of the probe resulted in labeling of HK853 (Figure 2A). Examination of the concentration dependence of the B-ATP γ S reaction revealed that half-maximal labeling occurred at ~ 2 μ M (Figure 2B and Figure S2). Labeling was also observed with ViCK from *Streptococcus pneumoniae* (Figure S3).²⁸ In addition, when B-ATP γ S was incubated with PhoQ from *Salmonella typhimurium*,²⁹ an HK construct containing the ATP-binding domain but lacking the DHp domain, rendering it inactive, no labeling was observed (Figure S3), further demonstrating the activity dependence of probe labeling.

To provide further evidence that protein labeling occurs in an activity-dependent fashion, we utilized various ATP analogues (ATP, AMP-PNP, ATP γ S) as competitive sub-

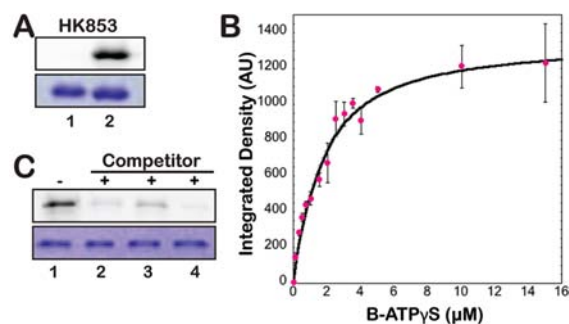


Figure 2. (A) B-ATP γ S used as an ABP to show in vitro thiophosphorylation of active HK853 (*T. maritima*). Top: in-gel fluorescence detection of the BODIPY fluorophore showed signal only with addition of probe (2 μ M). Bottom: Coomassie staining revealed even protein loading. Lanes: 1, no probe; 2, B-ATP γ S added. (B) Concentration dependence of HK853 labeling by B-ATP γ S. Labeling was saturated at ~ 10 μ M and measured by in-gel fluorescence scanning (integrated band intensities given in arbitrary units). (C) B-ATP γ S labeling of HK853 was inhibited when ATP analogues (200 μ M, 10 min) were added prior to B-ATP γ S (2 μ M). Lanes: 1, no inhibitor; 2, ATP; 3, AMP-PNP; 4, ATP γ S.

strates/inhibitors. Upon incubation with each of these compounds (200 μ M, 10 min) followed by addition of the probe, a dramatic decrease in labeling was detected, indicating that the ABP binds in the ATP binding pocket (Figure 2C). We also examined the ability of B-ATP γ S to compete with [γ -³³P]ATP and observed the expected decrease in the amount of radioactive protein upon addition of the probe (Figure S4). Finally, although the sulfur atom in our probe imparts added stability to the formed thiophosphoramidate, these species are still heat-sensitive.^{16,17} To confirm that labeling occurs by formation of such a bond, we subjected probe-labeled protein to heat treatment (95 °C, 5–30 min), which resulted in immediate disappearance of the labeled band (Figure S5).

We have strong evidence for the thiophosphotransfer of BODIPY- γ -thiophosphate to the conserved histidine of HK853. However, during TCS signaling, this is not the end of the pathway: the phosphoryl group undergoes another transfer event to RR468, the cognate RR (Figure 1B). Thus, we hypothesized that the fluorescently tagged thiophosphate group could be transferred to the aspartic acid on the RR (Figure 1D), but it was unclear whether the bulky BODIPY group would prevent recognition between the HK and the RR. To test this, we first incubated HK853 with B-ATP γ S for 1 h and then added the cognate RR468. The reaction was quenched in hour intervals and run on an SDS-PAGE gel. Gratifyingly, the band corresponding to RR468 grew increasingly fluorescent over time, indicating successful transfer of the BODIPY- γ -thiophosphate from the HK to the RR (Figure 3). As a control, B-ATP γ S was incubated with RR468 alone, and no labeling of the RR was observed, indicating that HK-dependent transfer of the BODIPY- γ -thiophosphate to RR468 had occurred.

Transfer of the phosphate group between an HK and an RR is usually an exceptionally fast process (seconds to minutes). The exchange that we observed was dramatically slower (hours). This slow RR thiophosphorylation may be due to alteration of the transfer kinetics by the oxygen-to-sulfur substitution. Furthermore, the size of the BODIPY molecule may hinder HK/RR recognition. In either case, the delayed transfer of the thiophosphate to the RR makes B-ATP γ S an

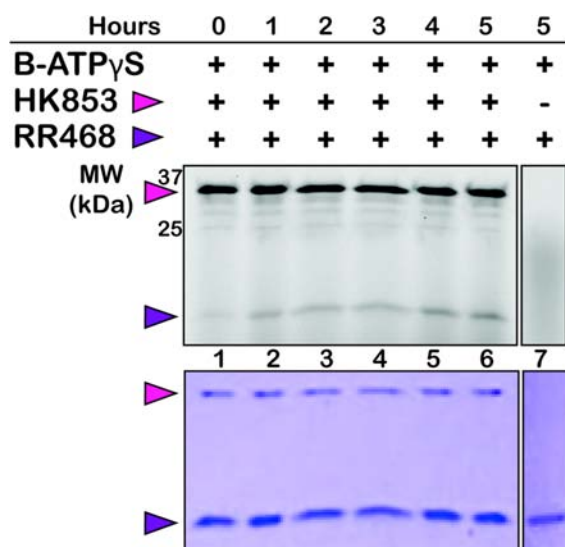


Figure 3. BODIPY- γ -thiophosphate is transferred to RR468 (16 kDa) over time only when HK853 (32 kDa) is present. HK853 was first incubated with the probe for 1 h at 25 °C. The RR468 was then added at 7-fold the concentration of HK853. Top: In-gel fluorescence detection of the BODIPY fluorophore. Bottom: Coomassie staining of the same gel, revealing even protein loading. The control lane (7) reveals that RR labeling is HK-dependent.

excellent tool for visualizing the often fleeting phosphorylated HK species while at the same time still enabling the examination of their cognate RR partners.

The activity of any enzyme is ideally studied *in vivo*. Currently, *in vivo* HK regulation (i.e., phosphorylation) has been examined only indirectly, likely because of inherently low protein concentrations, minimal basal activity levels, and the instability of the phosphorylated species. Most common in the literature are β -galactosidase reporter assays in which the *lacZ* gene is engineered into the regulon target of phosphorylated RRs. β -Galactosidase activity denotes RR-dependent transcription in cells, thus indicating stimulated HK autophosphorylation.³⁰ While well-accepted, this technique is an indirect measure of HK activity.

In vitro study of HKs incorporated into proteoliposomes represent the most complex systems in which HK phosphorylation has been directly measured. Because liposomes mimic the native membrane lipid environment, they have been exploited in the reconstitution of transmembrane (TM) proteins for both solubility and *in vitro* studies.³¹ Use of a liposomal scaffold provides the two-dimensional landscape and motional organization native to membrane proteins.³² Even for proteins lacking a TM domain, the association of His-tagged proteins to nickel-conjugated liposomes has effectively imparted the influence of the membrane on the coordinated protein(s). In particular, template-directed assembly of multiple chemotaxis proteins in liposomes utilizing NiNTA-bearing lipids has been utilized to study the CheA-mediated TCS of *Escherichia coli*.³²

Given the importance of the study of HKs in liposomal environments, we sought to test the efficacy of our probe with membrane-associated HK853. For ease of preparation and use, our construct does not contain the transmembrane domain. In place of this domain is the His tag used for protein purification, which could also be utilized to anchor this HK to the liposome (Figure S6). It is thought that the coiled-coil portion of HK853

plays an important role in transducing conformational changes from the membrane domain to the cytoplasmic domains that affect catalytic activity.²⁴ Because the His tag on HK853 is located at the N-terminus directly adjacent to the coiled-coil region, we hypothesized that the incorporation of HK853 into liposomes may confer a native structural impact and poise the coiled-coil domain for greater activity, which could be detected by labeling with B-ATP γ S.

The efficacy of B-ATP γ S for the activity readout of HK-proteoliposomes was analyzed by comparison of the labeling of free HK853 in solution with protein bound to liposomes. The bare liposomes were mixed with protein to facilitate protein association. Unbound protein (free) was then removed by ultracentrifugation, yielding purified proteoliposomes (bound) (Figure 4A). We compared the activity of HK853 prior to

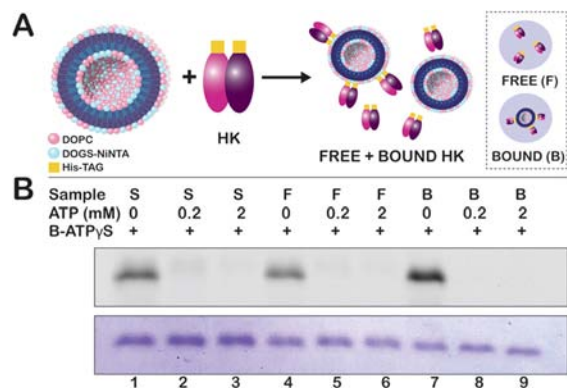


Figure 4. (A) His-tagged HK853 was conjugated to NiNTA-bearing liposomes and separated into free and bound HK853. (B) HK853 (600 ng) from purified stock solution (S), free HK853 after ultracentrifugation (F), and liposome-bound HK853 (B) were labeled with 2 μ M B-ATP γ S. Inhibition of fluorescent labeling was achieved by the addition of ATP prior to B-ATP γ S. Use of B-ATP γ S revealed that HK853 bound to liposomes is more active than that free in solution.

mixing (stock) with those of the liposomes from the “free” fraction and the protein associated with the liposomes (bound) (Figure 4B). Labeling of all three protein samples was observed and found to be ATP-competitive, indicating that the liposome does not interfere with probe labeling. In addition, although the protein concentrations of the free and bound samples were roughly equivalent (as judged by Coomassie staining), there was a noticeable difference in the intensities of the fluorescence signals, with the bound protein showing increased activity. This trend was also noted when liposome-bound HK was phosphorylated with [γ -³³P]ATP, indicating that liposome association likely influences the activity state of HK853 (Figure S7). These results confirmed B-ATP γ S to be akin to other phosphorylation readout strategies while at the same time boasting superior bond stability and a highly fluorescent signal that readily distinguishes differences in activity.

We anticipated that use of a γ -thiophosphorylated ATP analogue, which would yield a thiophosphorylated histidine species, could enable detection of the phosphotransfer event. We have identified BODIPY-FL-ATP- γ -S as an activity-based probe that shows specific labeling of active HK proteins and is competitive for the ATP binding site. Surprisingly, the fluorophore is also transferred to the RR. The application of B-ATP γ S exemplifies the most straightforward and rapid way of visualizing not only HK activity but also RR phosphotransfer

events and will facilitate both functional studies of HKs and inhibitor identification.

■ ASSOCIATED CONTENT

● Supporting Information

Methods and results for the following: protein overexpression and purification, [γ - ^{33}P]ATP assays, fluorescence polarization, ATP analogue information, B-ATP γ S assay conditions, integration of gel bands, and liposome preparation. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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