Optimized assay for the quantification of histidine kinase autophosphorylation

Takahiro B. Ueno¹, Roger A. Johnson², Elizabeth M. Boon^{1*}

¹Department of Chemistry, Stony Brook University, Stony Brook, NY 11794-3400.

²Department of Physiology and Biophysics, Stony Brook University, Stony Brook, NY 11794-8661.

*Corresponding Author

Department of Chemistry, Stony Brook University, Stony Brook, NY 11794-3400. Telephone: (631) 632-7945. Fax: (631) 632- 7960. E-mail: elizabeth.boon@stonybrook.edu.

ABSTRACT

Although two-component signaling systems, comprising a sensory histidine kinase and a response regulator, are a primary means by which bacteria detect and respond to environmental stimuli, they are poorly characterized. Here we report optimized conditions for detecting histidine phosphorylation using a facile medium-throughput filter paper-binding assay. Employing this assay we report the kinetic parameters of previously uncharacterized histidine kinases from *Vibrio haveyi*, *Vibrio parahaemolyticus*, *Shewanella oneidensis*, and *Legionella pneumophila*. In characterizing these kinases, we effectively double the number of kinetically characterized histidine kinases that have been reported in the literature.

KEYWORDS

Histidine kinase, TCS, two-component signaling, His-Asp phosphorelay, phospho-His, autophosphorylation, filter binding assay, autoradiography.

INTRODUCTION

Two-component signaling (TCS) systems are a predominant mechanism for stimulus response in bacteria [1,2,3,4]. TCS consists of a sensor histidine kinase (HK) and a response regulator (RR) [5]. The variable sensor domain of the HK is responsible for detecting a stimulus, which results in a change in the phosphorylation state of the HK. The signal is relayed downstream by means of phosphoryl transfer from the HK histidine to an aspartic acid in the receiver domain of the RR. The activated RR then elicits a response to the initial stimulus [6,7]. Many TCS systems deviate from this canonical system, however. In three-component systems, an accessory protein that detects a signal and regulates HK autophosphorylation *in trans* replaces the HK sensor domain

[8,9]. In a hybrid TCS, the HK has a receiver domain, thus a His-to-Asp phosphotransfer takes place within the hybrid HK, and then a histidine-containing phosphotransfer protein (Hpt) is required to relay the signaling phosphate from the HK to the appropriate RR in two additional phosphotransfer steps [10,11,12]. TCS is widely prevalent in bacteria; bacteria encode HKs capable of detecting a wide range of environmental stimuli that regulate biological functions essential for survival [13,14,15].

Most *in vitro* TCS studies analyze basal HK autophosphorylation rates, phosphotransfer, and changes in these rates in response to environmental stimuli using [γ - ^{32}P]-ATP as the HK substrate and analysis of phosphorylation by polyacrylamide gel electrophoresis (PAGE) followed by autoradiography [16,17]. Although these PAGE-based assays are labor intensive, time-consuming, and have limited sample capacities, they remain the primary means for analysis of phosphorylated His (pHis) [18,19,20]. In part this is due to the inherent lability of pHis [21,22,23,24], which hinders the application of techniques widely used for analysis of phosphorylated serine, threonine, and tyrosine residues [25]. Although several alternative analysis methods have been proposed to overcome the instability of pHis [26,27], each is nonetheless a PAGE-based assay. Here we report a carefully optimized filter paper-binding assay to determine the kinetic parameters of several previously uncharacterized HKs, nearly doubling the total number of kinetically characterized HKs [18,19,20,28,29,30].

MATERIALS AND METHODS

Unless otherwise noted, all the reagents were purchased at their highest available qualities and used as received.

Protein expression and purification

All the bacterial strains, plasmids, and primers used in this work are shown in Table 1. *Shewanella oneidensis* MR-1 was a gift from Dr. Jeffrey Gralnick of the University of Minnesota. VIBHAR_01913 (*Vibrio harveyi*, BB120, ATCC BAA-1116) WT and its mutants were expressed then purified with methods published previously [31]. PCR was used to amplify VP1876 with Phusion High-Fidelity DNA Polymerase (New England Biolabs) from *Vibrio parahaemolyticus* genomic DNA (*Vibrio parahaemolyticus*, EB101, ATCC 17802). Upstream and downstream primers contained NdeI and XhoI restrictions sites, respectively. SO_2145 was amplified by PCR from *Shewanella oneidensis* genomic DNA (*Shewanella oneidensis*, MR-1) with Phusion High-Fidelity DNA Polymerase. Upstream and downstream primers contained restriction sites EcoRI and XhoI, respectively. PCR was used to amplify lpg0278 with Phusion High-Fidelity DNA Polymerase from genomic DNA (*Legionella pneumophila*, subsp. *Pneumophila* str. Philadelphia 1). Upstream and downstream primers contained restriction sites NdeI and XhoI, respectively. Amplified VIBHAR_01913, VP1876 and SO2145 were cloned into expression vector pET-23aHis-TEV to yield N-terminus 6× His tagged plasmids. Amplified lpg0278 was cloned into pET-20b(+) to yield C-terminus 6× His-tagged plasmid. Cloned PCR products were transformed into *E. coli* DH5α and positive transformants were verified by sequencing (Stony Brook DNA sequencing facility). Site directed mutagenesis was carried out to make the VIBHAR_01913 D459A and VP1876 D499A mutant constructs following the QuikChange protocol from Stratagene.

All proteins were expressed and purified as follows. Inserts containing plasmids were transformed into *E. coli* BL21(DE3)pLysS. Cells were grown in 2XYT media (16 g/L Tryptone, 10 g/L yeast extract and 5 g/L NaCl), supplemented with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol at 37 °C with agitation at 250 rpm (New Brunswick Scientific, Innova 4230) until A_{600nm} reached 0.6-0.9. Protein expression was induced with 100 µM IPTG for 15 hours at 16 °C, then cells were harvested. Proteins containing a 6× His tag and were purified by Ni-NTA agarose. This single column yielded proteins that were at least 95% pure as estimated by Commassie-stained SDS-PAGE (sodium dodecyl polyacrylamide sulfate gels). Protein concentrations were determined by Bradford assay with bovine serum albumin as a standard [32].

Phosho-His stability test

[γ -³²P]-ATP (6000 Ci/mmol, 10 mCi/mL) was purchased from PerkinElmer Health Sciences Inc. All reactions were performed at room temperature. Histidine kinases (10 or 25 µM) were equilibrated in the reaction buffer (40 mM Tris-Cl, 150 mM KCl, 10% glycerol, and 4 mM MgCl₂ at pH 8.0) at room temperature for five minutes before initiation of the reaction by the addition of the ATP (2 or 4 mM) with trace amount of [γ -³²P]-ATP (10 µCi). Reactions were stopped with either 2.5× SDS loading dye (0.13% bromophenol blue, 0.25 M dithiothreitol, 25% Glycerol, 5% sodium dodecyl sulfate, 0.13 M Tris-HCl at pH 6.8), for PAGE assays, or ice cold 5.77 mM phosphoric acid, for filter paper-binding assays. Reactions quenched with phosphoric acid were kept on ice and then neutralized with 2.5× SDS loading dye at various time points to assess the effect of acid exposure.

Phospho-His visualization

Proteins were separated by electrophoresis on sodium dodecyl polyacrylamide sulfate gels (SDS-PAGE). Ten microliters of quenched reactions, without boiling, were loaded onto a 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gel. Proteins were separated by electrophoresis at 140 V for 80 min. The resulting chromatogram was fixed (30% methanol and 5% glycerol) for 30 min, then stained (10% acetic acid, 45% methanol and 0.25 g/100 mL Coomassie Brilliant Blue), followed by destaining (10% acetic acid and 20% methanol), and then dried (Promega Gel Drying kit V7120/7131). Dried gels were exposed to a storage phosphor-imaging screen (Molecular Dynamics) for 24 to 48 hours and images were scanned with the Typhoon scanner (Typhoon 9400, Amersham Biosciences). Band intensities from scanned images were quantified with image processing software ImageJ v1.46. Resulting band intensities were normalized to the band of a sample not exposed to phosphoric acid.

Filter binding assay

Whatman P81 ion exchange chromatography paper (catalog # 21426-202) was purchased from VWR international. Histidine kinase activities were quantified with respect to time, enzyme concentration, and substrate concentration. In general, histidine kinases (10 or 25 μ M) were equilibrated in the reaction buffer (40 mM Tris-Cl, 150 mM KCl, 10% glycerol, and 4 mM MgCl_2 at pH 8.0) at room temperature for five minutes before initiation of the reaction by the addition of ATP (2 or 4 mM) with trace amount of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ (10 μ Ci). Reactions were stopped with ice-cold 5.77 mM phosphoric acid and then were spotted onto Whatman P81 chromatography paper. The chromatography paper was immediately dropped into a container containing ice-cold 5.77 mM phosphoric acid (10 mL/paper). The spotted chromatography papers were then washed by rocking on a benchtop rotator (25 rpm) for 10 minutes. The acid was

replaced anew and washed two to three more times until no radioactivity was detected from the wash solution by a Geiger counter. After the wash, chromatography papers were rinsed briefly with acetone then air dried for 1 hour. The ^{32}P content of the dried chromatography papers was quantified in a liquid scintillation spectrometer (PerkinElmer, Tri-Carb 2900TR), without the addition of water or scintillation fluid. The specific activity of ATP solutions were determined from the radioactivity of the $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ solutions relative to amount of unlabeled ATP in the reaction. The resulting values were used to quantify enzyme specific activities. Dependence of autophosphorylation on reaction time and enzyme concentration were determined by stopping the reaction at different time points (1 – 150 min) or by conducting reactions with varying enzyme concentrations (5 – 80 μM). These data were used to determine reaction times and enzyme concentrations that were within the linear range for use in monitoring the dependence of enzyme activity on substrate concentration.

Data fitting and determination of kinetic parameters

Data obtained from experiments with varying ATP concentrations, under conditions with which pHis formation was linear with respect to time and enzyme concentration, were fitted to the Michaelis-Menten model by use of KaleidaGraph software to determine V_{max} , K_{m} and $k_{\text{cat}}/K_{\text{m}}$ values.

RESULTS AND DISCUSSION

TCS is a primary mechanism used by bacteria for adaptive responses to changes in their environment. TCS pathways are critically important in pathogenesis, as well as many other aspects of bacterial responses to changes in their environments [33,34]. Enzyme kinetic analyses

have proven to be essential for understanding signal transduction in many different signaling pathways in many different organisms, both for fundamental characterization of these signaling pathways, as well as in drug discovery efforts [35,36,37,38]. Nonetheless, only a handful of bacterial HKs have been kinetically characterized. This is due in part to the lack of facile assay methods. Here we report the detailed kinetic parameters of several previously uncharacterized HKs as well as optimized conditions for detecting pHis using a medium-throughput filter paper-binding assay.

Phospho-His stability

Kinetic characterizations of HKs have primarily been accomplished by use of PAGE followed by autoradiography or alkali-based filter binding assays, but both of these assays have critical shortcomings. The PAGE/autoradiography method is labor intensive, time consuming, and has a small sample capacity. The alkali-based filter binding assay frequently suffers from considerable background [39]. In search of a rapid and efficient HK kinase activity quantification method, we revisited an acid-based filter binding assay [40,41] and optimized the experimental conditions to overcome the inherent acid lability of pHis. We employed phosphoric acid at moderate concentration (final concentration, 5.77 mM) both to stop the phosphorylation reaction and to wash away unreacted ATP. We used this phosphoric acid concentration, because we found this to be enough acid to stop the reaction with minimal pHis cleavage. Furthermore, samples were kept near freezing during quenching and washing to avoid pHis degradation.

We used this assay to characterize kinetic behavior of four histidine kinases [*V. harveyi* (gene ID: 5555316), *V. parahaemolyticus* (gene ID: 1189383), *S. oneidensis* (gene ID: 1169886), and *L.*

pneumophila (gene ID: 19831845)]. *S. oneidensis* and *L. pneumophila* HKs are orthodox HKs that lack receiver domains. HKs from *V. harveyi* and *V. parahaemolyticus* are hybrid histidine kinases with receiver domains. We used receiver domain-aspartate mutant constructs of the hybrid *Vh* and *Vp* kinases (D459A and D499A, respectively), to ensure measurement of the autophosphorylation kinetics without interference from phosphotransfer and/or autophosphatase activities. As such, for each kinase in this study, we report kinetics determined by measuring the pHis autophosphorylated product. To establish the stability of pHis under our experimental conditions, each HK was incubated with ATP/[γ - 32 P]-ATP for 60 min, and then phosphorylated HKs were incubated in ice-cold phosphoric acid. Samples were subjected to SDS-PAGE and the degradation of pHis over time was measured by autoradiography of the gels. All four HK tested in these experiments showed no measurable degradation of pHis upon exposure to ice-cold phosphoric acid (5.77 mM, final concentration) for up to 60 min (Figure 1). This was very encouraging, as it is widely accepted that pHis is acid-labile; [23,25] however, our results indicate that pHis degradation is negligible at a moderate acid concentration in solution near the freezing point of water.

To further characterize the extent of pHis stability in our assay, the phosphorylation state of the HK from *S. oneidensis* was monitored over a 2 h incubation period in ice-cold phosphoric acid. Consistent with the earlier experiment, we observed no degradation of pHis up to 60 min, followed by gradual degradation over the next 60 min. Overall, we observed a 7% reduction in phosphorylation of *So*HK after 90 min of incubation in acid and a 21% reduction after 120 min of acid-incubation. In subsequent experiments, all of the enzymes reported here were exposed to acid for no more than 60 min over the course of reaction quenching, filter paper binding, and

successive washing steps. Therefore, we conclude that under our reported conditions, the integrity of pHis protein phosphorylation is maintained.

Effect of time and enzyme concentration

To allow an accurate assessment of the kinetic parameters of each HK enzyme studied here, the linear range of HK enzyme activity, as a function of time and enzyme concentration, was determined. HKs were incubated with ATP with trace $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, and at various time points the phosphorylated HKs were subjected to ice-cold phosphoric acid to stop the reaction. As illustrated in Figure 2, plots of phosphorylation as a function of time, for all four HKs tested here, showed the expected enzyme saturation curves, with reactions reaching saturation between 15 min (*L. pneumophila*) and 100 min (*V. harveyi*). The linear range of the autophosphorylation reaction with respect to time was determined from these plots [41]. To determine the linear range of the HK autophosphorylation activity as a function of enzyme concentration, pHis formation at varying enzyme concentrations was measured with fixed incubation time (40 min for *V. harveyi*, 20 min for *V. parahaemolyticus*, 5 min for *S. oneidensis* and 1 min for *L. pneumophila*) and ATP concentration (2 mM) (Figure 3). As expected, product formation increased linearly as a function of enzyme concentrations for all HKs tested at the given reaction times and substrate concentrations

Substrate kinetics

Finally, in order to determine the k_{cat} and K_{m} for each HK, pHis formation as a function of ATP concentration was measured for each HK. Incubation time and enzyme concentration for each HK was chosen based on the results of the time- and enzyme-dependent reactions described

above (Figures 2 and 3). As illustrated in Figure 4, all four HKs showed the expected linear double reciprocal plot with increasing ATP concentration. Data were fitted to the Michaelis-Menten model to determine the kinetic parameters. The kinetic parameters for each HK studied here, as well as those of previously characterized HKs, are summarized in Table 2.

Kinetic constants for the kinases from *V. parahaemolyticus*, *S. oneidensis*, and *L. pneumophila* are reasonably similar to those of many previously characterized *E. coli* HKs (Table 2). In our hands *V. harveyi* HK exhibited a relatively low catalytic efficiency ($0.02 \text{ M}^{-1}\text{sec}^{-1}$) and a large K_m (2.26 mM). The reasons for the high K_m and low catalytic efficiency remain unclear. However, since *Vh*HK is a dual-functioning enzyme, with both kinase and phosphatase activities [ENREF 8](#)[8], it is conceivable that the primary function of this enzyme may be as a phosphatase. It is also possible that the autophosphorylation activity of *Vh*HK may be enhanced upon detection of a ligand through its partner sensory protein.

The method described in this paper provides an optimized protocol for analyzing the autophosphorylation activity of histidine kinases. The most commonly used PAGE-based method for analysis of HK-autophosphorylation is labor intensive, limits the sample number, and is time consuming. Our method is facile; it is less labor-intensive and allows a researcher to process a large number of samples in a short period of time, without risking degradation of pHis. This method proved to be effective for determining the kinetic parameters of four previously uncharacterized HKs. We propose that this assay can be used to determine the rate of autophosphorylation of any HK and may be applied further to determine the effect of ligands on HK kinase activity.

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FIGURE CAPTIONS

Figure 1. Stability of pHis on HKs from *V. harveyi* (A), *V. parahaemolyticus* (B), *S. oneidensis* (C), and *L. pneumophila* (D) pHis. The reaction mixture contained 40 mM Tris-Cl, 150 mM KCl, 10% glycerol and 4 mM MgCl₂ at pH 8.0. Histidine kinase and ATP concentrations respectively, were 10 μ M and 2 mM for *V. harveyi*, 25 μ M and 2 mM for *V. parahaemolyticus*, 22 μ M and 2 mM for *S. oneidensis*, and 16 μ M and 2.5 mM for *L. pneumophila*. Autoradiography of PAGE gels showed phosphorylated histidine kinases after exposure to ice-cold 5.77 mM phosphoric acid for the amount of time indicated. Band intensities were quantified using ImageJ. No measurable degradation was observed within 60 min for any HK tested. Phosphorylated *S. oneidensis* (panel C) was exposed to these acidic conditions for an additional 60 minutes, during which gradual degradation was observed: 7% degradation at 90 min and 21% degradation at 120 min.

Figure 2. Time-dependent autophosphorylation of HKs from *V. harveyi* (A), *V. parahaemolyticus* (B), *S. oneidensis* (C), and *L. pneumophila* (D). Reactions were carried out at 23 °C $\pm$ 1 °C. ATP concentrations and enzyme concentrations, respectively, were 2.1 mM and 16 μ M for *V. harveyi*, 2.1 mM and 14 μ M for *V. parahaemolyticus*, 2.1 mM and 14 μ M for *S. oneidensis*, and 2.1 mM and 50 μ M for *L. pneumophila*. Product formation was monitored over time by use of the filter-binding assay described in under Materials and Methods. All four tested HKs showed saturation curves in a time dependent manner. Reactions reach saturation within 15 to 100 min.

Figure 3. Enzyme concentration-dependent autophosphorylation of HKs from *V. harveyi* (A), *V. parahaemolyticus* (B), *S. oneidensis* (C), and *L. pneumophila* (D). ATP concentrations and incubation times were 2 mM and 40 min for *V. harveyi*, 2 mM and 20 min for *V. parahaemolyticus*, 2 mM and 5 min for *S. oneidensis*, and 2 mM and 1 min for *L. pneumophila*. Reactions were carried out at $23\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$.

Figure 4. Substrate concentration-dependent autophosphorylation of HKs from *V. harveyi* (A), *V. parahaemolyticus* (B), *S. oneidensis* (C), and *L. pneumophila* (D). Double reciprocal plots are shown. Reactions were carried out at $23\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$. Enzyme concentrations and incubation times, respectively, were 35 μM and 40 min for *V. harveyi*, 14 μM and 20 min for *V. parahaemolyticus*, 18 μM and 5 min for *S. oneidensis*, and 52 μM and 1 min for *L. pneumophila*. Error bars represent the standard error of the mean from a minimum of three assays.

Table 1. Bacterial strains, plasmids, and primers used in this work.

Strains, plasmids and primers	Genotype	Reference
Bacterial strains		
<i>V. harveyi</i>		
WT	<i>Vibrio harveyi</i> , BB120, ATCC BAA-1116	ATCC
<i>V. parahaemolyticus</i>		
WT	<i>Vibrio parahaemolyticus</i> , EB101, ATCC 17802	ATCC
<i>S. oneidensis</i>		
WT	<i>Shewanella oneidensis</i> , MR-1	
<i>L. pneumophila</i>		
WT	<i>Legionella pneumophila</i> , Philadelphia-1, ATCC 33152	ATCC
<i>E. coli</i>		
DH5 α	Cloning competent cell	Invitrogen
BL21(DE3)pLysS	Expression competent cell	Invitrogen
Plasmids		
pVh_HqsK(D459A)	pET-23aHis-Tev with VIBHAR_01913, N-terminus 6x His-tag, Amp ^r	[28]
pVp_HqsK(D499A)	pET-23aHis-Tev with VP1876, N-terminus 6x His-tag, Amp ^r	This work
pSo_HK(WT)	pET-23aHis-Tev with SO_2145, N-terminus 6x His-tag, Amp ^r	This work
pLp_HK(WT)	pET-20b(+) with lpg0278, C-terminus 6x His-tag, Amp ^r	This work
Primers		
pVh_HqsK(WT)-Fwd	GCG AAT TCG TGG CGT TAA AGA AAC TCG	
pVh_HqsK(WT)-Rvs	CAA TGA AGC TTT TCG CCG AGC CAT TTA CAC	
pVp_HqsK(WT)-Fwd	GGA TCC ATA TGA TGA CGG GTA ACT CC	
pVp_HqsK(WT)-Rvs	GCA TTC TCG AGC TAC TGC TCA AGC	
pSO_HK(WT)-Fwd	CCA TCC GAA TTC ATG ACT GAC AGC GAA AAT CCC	
pSO_HK(WT)-Rvs	CCA GCA GAG CTC CTA GGT TAT CGA GCT AGA AGT	
pLp_HK(WT)-Fwd	NNN CAT ATG ACA GAA ATG CAT CGG TTG TTG CAG	
pLp_HK(WT)-Rvs	NNN CTC GAG CCT CGT ACT CAA GGT TTT GGG ATT G	

Abbreviation: Amp^r: Ampicillin resistance

Table 2. Summary of histidine kinase autophosphorylation kinetic parameters for all characterized HK enzymes to date.

Protein	$V_{\max}$ (nmol/mg•min)	k_{cat} (/sec)	K_m (μ M)	k_{cat}/K_m (/Msec)	Ref.
<i>V. harveyi</i> HK	0.050 $\pm$ 0.006	(4.95 $\pm$ 0.63) * 10 ⁻⁵	2260 $\pm$ 340	0.02	This work
<i>V. parahaemolyticus</i> HK	0.056 $\pm$ 0.003	(6.01 $\pm$ 0.33) * 10 ⁻⁵	177 $\pm$ 12	0.34	This work
<i>S. oneidensis</i> HK	0.476 $\pm$ 0.032	(2.83 $\pm$ 0.19) * 10 ⁻⁴	88 $\pm$ 14	3.23	This work
<i>L. pneumophila</i> HK	1.320 $\pm$ 0.218	(1.18 $\pm$ 0.20) * 10 ⁻³	173 $\pm$ 19	6.80	This work
<i>E. coli</i> , EnvZ	-	8.10 * 10 ⁻⁵	218	0.37	[28]
<i>E. coli</i> , NarQ	-	(2.20 $\pm$ 1.10) * 10 ⁻⁴	23 $\pm$ 9	9.65	[29]
<i>B. subtilis</i> , KinA	-	(1.90 $\pm$ 0.50) * 10 ⁻³	74 $\pm$ 11	25.7	[20]
<i>E. coli</i> , CheA	-	(2.60 $\pm$ 0.40) * 10 ⁻²	770	33.8	[19]
<i>E. coli</i> , NRII (NtrB)	-	(2.37 $\pm$ 0.05) * 10 ⁻²	31 $\pm$ 1	763.4	[30]
<i>E. coli</i> , PhoQ	-	(1.70 $\pm$ 0.81) * 10 ⁻³	21 $\pm$ 13	81.2	[18]

Figure 1

A.

B.

C.

D.

Figure 2

A.

B.

C.

D.

Figure 3

A.

B.

C.

D.

Figure 4

A.

B.

C.

D.