Supporting Information for

Activity-Based Probe for Histidine Kinase Signaling

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1. List of Figures

2. General Methods and Information

Reagents were obtained from J.T. Baker, Mallinkrodt, Sigma, MP Biomedicals, IBI, EMD Biosciences, Bio-Rad and Fisher, except where otherwise noted.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Tris-glycine gels were typically 10% polyacrylamide, but 18% gels were used for analysis of PhoQ or RR468. Running parameters were 180 V, 400 mA, and 60 W for 1 h.

In-gel fluorescence detection. After SDS-PAGE, gels were washed 3 times with water. They were scanned on a Typhoon Variable Mode Imager 9210 (Amersham Biosciences) using 526-nm (short-pass filter) detection for BODIPY (λ_{ex}: 504 nm, λ_{em}: 514 nm). Integrated density measurements were determined using ImageJ.

Buffers. The reaction buffer used in several of the experiments was 50 mM Tris-HCl, 0.2 M KCl, 5 mM $MgCl_2$, final pH 7.8. Lysis buffer for HK853, RR468, and VicK was the same as the NiNTA buffer (5 mM imidazole). The lysis buffer for PhoQ was PBS for use with the glutathione affinity column. The 2X SDS-PAGE loading buffer contained 125 mM Tris, pH 6.8, 20% glycerol, 4% SDS (w/v), 5% BME (v/v), and 0.2% bromophenol blue (w/v).

BODIPY-Thiophosphorylation reactions. Reaction mixtures (50 µL) were prepared with 600 ng purified HK853, and labeling was achieved by adding 1 µL B-ATPγS (2 µM final concentration) in reaction buffer. Some experiments analyzing other proteins or concentrations are specifically addressed below. Individual mixtures were incubated at RT for 1 h in the dark to prevent fluorophore photobleaching. After incubation, reactions were quenched with 2X SDS-PAGE loading buffer before running on SDS-PAGE gels (samples were not heated). After analyzing gels by fluorescence, they were coomassie stained. Integrated densities of the gel bands were measured using ImageJ.

[γ-^{33}P]-ATP autophosphorylation or phosphotransfer assays. Specific protein and [γ-^{33}P]-ATP concentrations are addressed below. At indicated time intervals, 15 μL was removed from the reactions and quenched with 2X SDS-PAGE sample loading buffer by vigorously flicking the tube. Samples were not heated to preserve the phosphohistidine bond. For each quenched sample, 20 μL was loaded and run on 10% or 18% SDS-PAGE gels. Afterward, gels were soaked in 40% methanol/10% acetic acid/8% glycerol for 20 min and dried at 60° C for 1 h, 70° C for 1 h, and without heat for 1 h using a gel dryer (Bio-Rad). Gel(s) were exposed to a phosphor screen for 16-20 h and scanned using a Typhoon Variable Mode Imager 9210 under the "phosphorescence" setting.

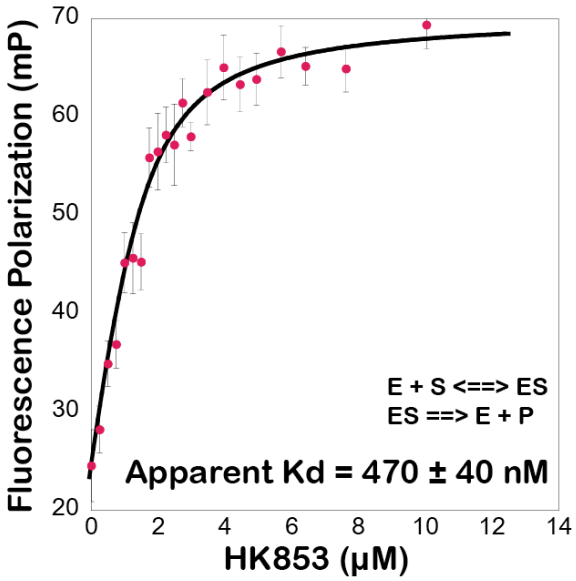
3. Experimental Methods and Results

Fluorescence polarization of HK853 and B-ATPγS

Fluorescence polarization was measured with a PC1 spectrofluorometer (ISS). During experimentation, the lamp was adjusted to 17 Å. A water bath was used to maintain constant temperature at 25° C. Slits at beam entrance were set to 0.5 mm, and slits on exit were set to 1.0 mm. The excitation monochromator was set to 504 nm, and emitted light was collected at 514 nm. For each read, 10-20 iterations were performed, and the G-factor was calculated once.

BODIPY-FL-ATPγS (B-ATPγS) was purchased from Invitrogen. To the quartz cuvette, 2 μM B-ATPγS was added in reaction buffer. After an initial reading of B-ATPγS, 1 μL aliquots of 0.5 mM or 4.2 mM HK853 were added. Sample was stirred 3 min before taking readings. Data was graphed and fit using DynaFit to solve for Keq dissociation using the following mechanism: ligand + protein <==> complex. Subsequently, the original data and fit coordinates were graphed in KaleidaGraph.

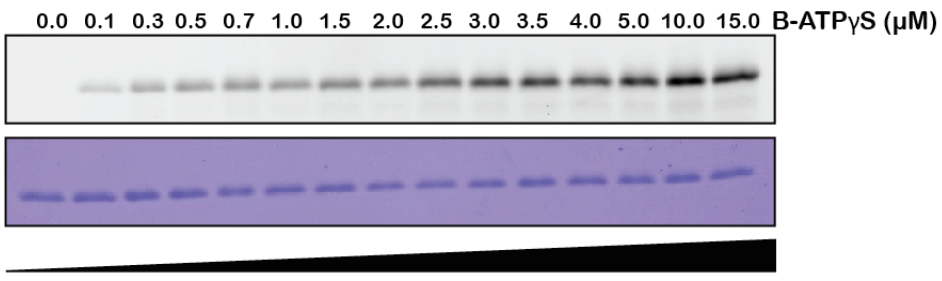
Figure S1. Fluorescence polarization (in milli) of BODIPY-FL-ATPγS upon binding to HK853.



HK853 saturation with B-ATPγS

Reactions of 25 µL contained 600 ng HK853 and varying concentrations of B-ATPγS ranging from 0-15 µM. They were mixed well and incubated for 1 h at RT. Samples were quenched and analyzed as above.

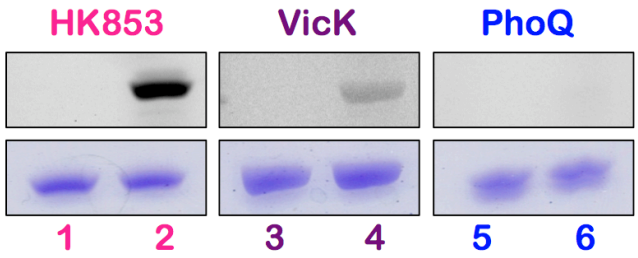
Figure S2. Concentration-dependent labeling of HK853 with B-ATPγS. Labeling saturated at ~10 µM B-ATPγS. The coomassie-stained gel illustrates even loading of HK853 such that fluorescent band intensity differences are a result of protein activity and not protein concentration (~90 ng/lane).



B-ATPγS probe labeling of HK853 and other HK proteins through thiophosphorylation

Reaction mixtures (50 µL) contained 4 µg HK853, 8 µg VicK, or 18 µg PhoQ and 2 µM B-ATPγS. They were mixed well and incubated for 1 h at RT. Samples were quenched and analyzed as above.

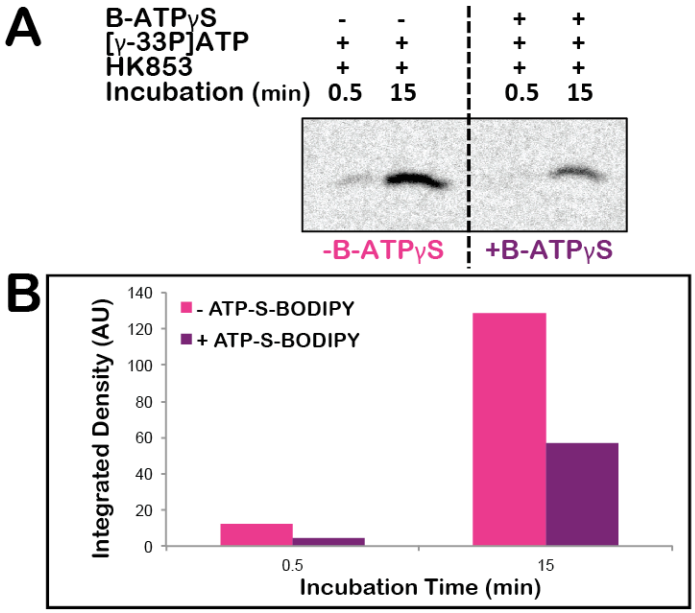
Figure S3. ABPP with three HK protein constructs illustrates B-ATPγS labeling by activity. Variation in labeling represents differences in reactivity or affinity for the probe of the HKs. PhoQ is an inactive construct because it lacks the dimerization domain housing the catalytic His. It was used as a negative control. (598 ng HK853, 1.2 µg VicK, and 2.7 µg PhoQ was loaded per lane.)



[γ-^{33}P]-ATP autophosphorylation: competition with B-ATPγS

Radioactive [γ-^{33}P]-ATP (3000 Ci/mmol, 10 mCi/mL, 1 mCi) was purchased from PerkinElmer. At RT, 100-µL reaction mixtures were prepared with final concentrations of 0.35 µM HK853 and 50 µM [γ-^{33}P]-ATP (0.32 Ci/mmol) in reaction buffer. To one reaction, 1 mM B-ATPγS was added and incubated with HK853 for 30 min prior to the addition of [γ-^{33}P]-ATP. Once mixed with [γ-^{33}P]-ATP, reactions were quenched at 30 s and 15 min and analyzed as above.

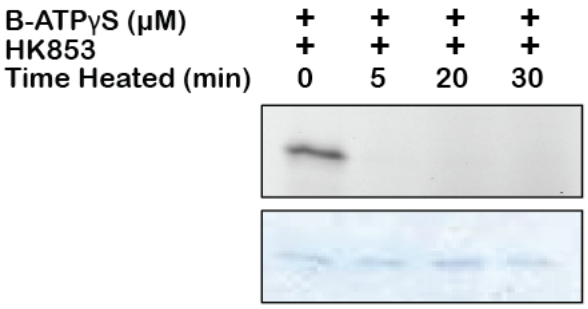
Figure S4. Examination of ability of B-ATPγS to compete with [γ-^{33}P]-ATP. (A) B-ATPγS competes with [γ-^{33}P]-ATP when reacted with HK853. (B) The integrated density representing γ-^{33}P-labeled HK853 is decreased more than 2-fold in the presence of B-ATPγS.



Heat-labile labeling of HK853 with B-ATPγS

Reactions contained 50 µL of 600 ng HK853 in reaction buffer. B-ATPγS (1 µL) was added to a final concentration of 2 µM. They were mixed well and incubated for 1 h at RT. Samples were quenched and heated at 95° C for 5, 20, or 30 min. After cooling to RT, samples were loaded onto gels and analyzed as above.

Figure S5. Examination of heat stability of labeled protein species. Heating of BODIPY-S-labeled HK853 results in loss of the thiophosphate bond. The fluorescence gel shows the bond is unstable even after 5 min at 95° C.

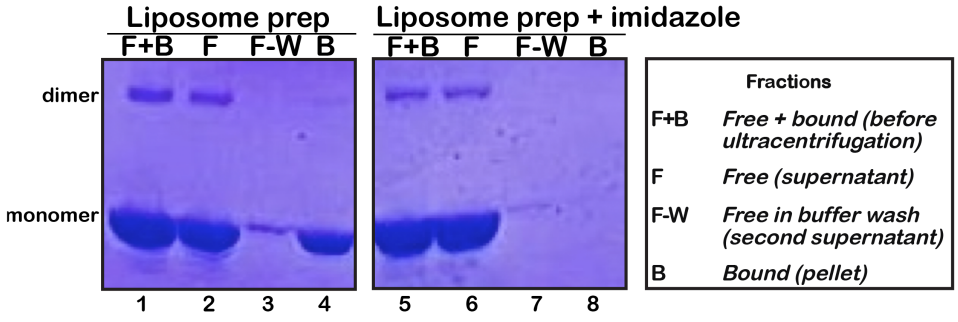


Liposome and proteoliposome preparation [1,2,3]

Lipids used in liposome preparation were 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1 carboxypentyl) iminodiacetic acid)succinyl] (nickel salt; DGS-NTA(Ni)) from Avanti Polar Lipids. The lipids were purchased suspended in chloroform. To a glass vial, 2 mg total lipid was added in a 1:1 ratio DOPC:DGS-NTA(Ni). To generate a lipid film on the glass, chloroform was removed by a stream of nitrogen, and residual solvent was removed under high vacuum overnight. The lipid film was hydrated with 1 mL reaction buffer to make 2 mg/mL lipid (~2 mM). Lipid multilayers were dispersed by vortexing, then agitating, the glass vial for 30 min. Suspensions were frozen on dry ice and thawed using a water bath ten times. A Mini-Extruder (Avanti Polar Lipids) and 0.1 μm polycarbonate filters were used to generate large unilamellar vesicles (LUVs) by extruding the suspension through the filter a minimum of 12 times at 30° C to yield LUVs with a typical mean diameter of 120-140 nm. Liposomes were stored at 4° C until use the same day.

A mixture of ~600 μM lipid and 35 μM His-tagged HK853 was prepared at a volume between 200 and 800 μL. As a control, 1 M imidazole in reaction buffer was also added to this lipid-protein mixture. The suspension was incubated at 4° C for 3 h. Ultracentrifugation at 125,000 x g for 20 min was used to separate free HK853 and HK853 bound to the liposomes. A wash step with equal volume of reaction buffer was used to remove all protein not associated with the liposomes. Final fractions were "free + bound" (before ultracentrifugation), "free," "free-wash," and "bound" (post-ultracentrifugation), and these fractions were analyzed by SDS-PAGE.

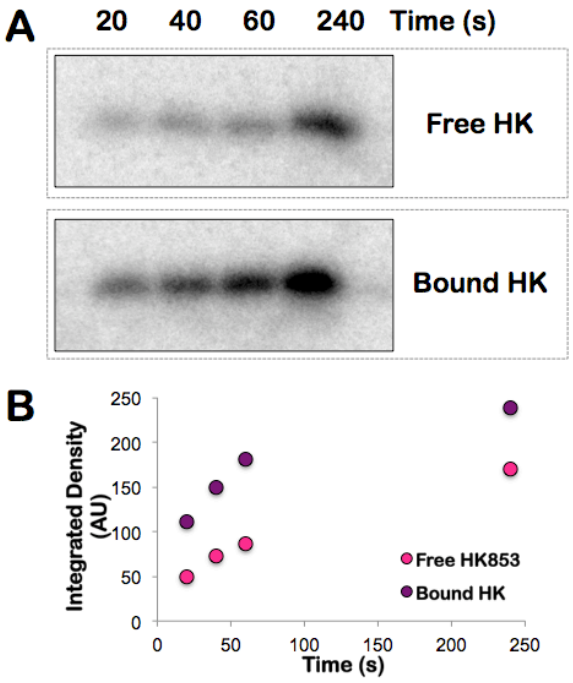
Figure S6. Proteoliposome preparation. Coomassie staining demonstrates HK853 was associated with the liposomes. HK853 was found in all fractions, representing HK853 bound and unbound to the liposomes. The wash step removed a small amount of protein that remained after the removal of supernatant after one round of ultracentrifugation (lanes 3 and 7). Importantly, when 1 M imidazole was added to the reaction buffer during proteoliposome preparation, no HK853 was bound to liposomes (lane 8), indicating that HK853 is associated with the liposomes through a nickel-His-tag interaction.



[γ-^{33}P]-ATP autophosphorylation of HK853 proteoliposomes

To analyze the effect of the liposomes on HK853 activity, free and bound HK853 were prepared as above. Free or bound HK853 (0.875 μM) was autophosphorylated with 100 μM [γ-^{33}P]-ATP (0.27 Ci/mmol) in reaction buffer (100 μL total volume). Reactions were quenched at 20 s, 40 s, 60 s, and 4 min, and the remainder of the procedure was the same as above.

Figure S7. [γ-^{33}P]-ATP autophosphorylation of HK853 proteoliposomes. (A) HK853 bound to liposomes was more active than HK853 free in solution, which is shown in time-based assays from 20 s to 4 min. (B) Integrated density values of the HK853 autophosphorylated in Part A illustrate that bound HK853 has higher activity.



Total gene synthesis of HK853, RR468, and PhoQ[4]

HK853 and RR468 sequences were retrieved from PDB code 3DGE (*Thermotoga maritima*) and the PhoQ sequence was retrieved from PDB code 3CGY (*Salmonella*

typhimurium).[5,6] All sequences were submitted to DNAWorks (Helix Systems) to generate primers for total gene synthesis using codons optimized for expression in *E. coli.* For template synthesis, a mixture was prepared containing 2.0 μM of each oligonucleotide. 2.0 μL of each primer stock was mixed with 1 X Pfu buffer, 1.0 U Taq, 1.0 U Pfu, and 0.2 mM dNTPs to a final volume of 30 μL. The PCR reaction included 30 cycles of 95° C for 30 s, 46° C for 2.5 min, and 72° C for 1.5 min. To amplify the synthesized template, 2.5 μL PCR product was mixed with 0.25 mM dNTPs, 1 X Pfu buffer, and 0.5 μM the outermost sense and antisense primers. The PCR reaction was 95° C for 60 s; 30 cycles of 95° C for 30 s, 60° C for 2 min, and 72° C for 1.5 min; and 72° C for 360 s. Amplified PCR products were gel purified, and extracted DNA was digested with *Eco*RI and *Nco*I in *Eco*RI buffer. After purification with the QIAquick PCR Purification Kit, DNA coding for either HK853 or RR468 was ligated into the pHis-parallel vector using Fermentas Rapid DNA Ligation Kit.[7] DNA encoding PhoQ was ligated into the pGST-parallel vector.[7] Ligated vectors were transformed into DH5α *E. coli* cells and plated on LB agar containing 100 μg/mL ampicillin (amp), and a single colony was used to inoculate 3.0 mL LB broth containing amp. Positive clones selected based on restriction mapping were confirmed via sequencing (Indiana Molecular Biology Institute).

HK853 (TM0853). Typically a membrane-bound protein, HK853 was designed as a membrane-truncated construct only containing the cytosolic DHp and CA domains, residues 232-489. An *Nco*I restriction site was added to the sense primer at the 5′ end that included the ATG start codon. A stop codon and *Eco*RI site were added to the 3′ end. Marina et al describe the helical domain as residues 232-317, coiled-coil domain as residues 232-253, linker domain as residues 318-322, and C-terminal domain, containing the ATP binding site, as residues 323-489.[8]

RR468 (TM0468). The RR468 construct was generated for production of full-length protein. To accommodate subcloning into the *Nco*I site of pHis-parallel, a Ser was changed to Ala in the initial sense primer. HK853 and RR468 are cognate pairs.

PhoQ. The PhoQ protein construct was designed to contain only the catalytic, ATP-binding domain, residues 332-487.[6] To adjust for the *Nco*I site, a Met was added at the 5′ end followed by a Ser substitution to Ala. PhoQ is from the PhoP-PhoQ TCS.

VicK (WalK$_{Spn}$ΔN35 (N)-Sumo)

A frozen glycerol stock of BL21(DE3)-pLysS Rosetta *E. coli* cells harboring the plasmid coding for the VicK protein construct was received as a gift from the laboratory of Malcolm Winkler (IU) and can be found in Gutu, et al.[9]

Protein overexpression

DNA for HK853, RR468, or PhoQ was transformed into BL21(DE3)-pLysS Rosetta *E. coli* cells. Transformed *E. coli* cells were plated on LB agar containing 100 μg/mL amp and 34 μg/mL chloramphenicol (Cm). A single colony was transferred to 50 mL sterile LB media supplemented with 100 μg/mL amp and 34 μg/mL Cm and incubated at 37° C while shaking at 220 rpm. Once the inoculum culture reached OD^{620} of 0.4-0.6, 1 L cultures containing antibiotics were inoculated and grown by shaking at 220 rpm at 37° C. After reaching an OD ~0.6, cultures were induced with 0.22 mM IPTG (Calbiochem) and incubated at 20° C while shaking for 16 h. Cultures were centrifuged at 8000 x g at 4° C for 20 min, and the pellets were collected, weighed, and quickly frozen on dry ice prior to storage at -80° C. Samples collected before and after induction were analyzed using SDS-PAGE to confirm protein expression.

LB broth with 30 μg/mL kanamycin (kan) and 34 μg/mL Cm was inoculated from a frozen stock of BL21(DE3)-pLysS Rosetta *E. coli* cells carrying the plasmid for VicK. Growth conditions were the same as above. VicK protein overexpression was induced with 0.1 mM IPTG and incubated at 16° C for 22 h at 220 rpm.

Cell lysis

Pellets were resuspended in lysis buffer containing 20 units Deoxyribonuclease I (Sigma Cat. DN25) per L culture and one Complete Mini EDTA-free protease inhibitor tablet (Roche) per 2 g pellet. Thirty mL of buffer was prepared for every 2 g pellet. Pellets were disrupted using a Dounce homogenizer. Resuspended cells were then lysed by applying high pressure with a cell cracker apparatus (M110 Microfluidizer; greater than 750 psi). Once lysed, the cell suspension was centrifuged at 14,000 x g for 45 min at 4° C, and the supernatant was decanted and filtered (0.22 μm).

Protein purification

With the exception of the glutathione sepharose column, purification was performed in stages using fast protein liquid chromatography (FPLC; GE, AKTApurifier). All buffers, lysates, and proteins were filtered (0.22 μm). Details of chromatography are included in subsequent sections. HK853 and RR468 were purified using nickel affinity and size exclusion. PhoQ was first purified from lysate using a glutathione sepharose gravity column. After TEV cleavage, the GST tag was removed from the sample by again using the glutathione sepharose column. Nickel affinity chromatography was used to isolate PhoQ from the His-tagged TEV protease, and size

exclusion was the final purification step. Lastly, VicK was purified using nickel affinity, ion exchange, and size exclusion chromatography.

Nickel affinity. Samples were loaded onto a nickel-nitriloacetic acid column (Ni-NTA; Qiagen) with buffer (25 mM Tris-HCl, pH 8, 1 M NaCl, 10 % glycerol, and 2 mM DTT). A reducing agent and high salt concentration were maintained to prevent protein aggregation. An elution gradient of 5 mM imidazole to 1 M imidazole was used, and the protein was detected via absorbance at 260 nm and 280 nm.

Glutathione affinity purification. A gravity column was packed with glutathione sepharose resin (GE Healthcare). Once equilibrated, lysate was loaded on the column using binding buffer (Phosphate-buffered saline; 140 mM NaCl, 2.7 mM KCl, 10 mM $NaHPO_4$, 1.8 mM KH_2PO_4, 10 mM DTT, 10% glycerol, pH 7.3). Flow-through and wash was collected. Adding 1 mL of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, 2 mM DTT, 10 % glycerol, pH 8) at a time, eluent was collected in fraction tubes. Protein concentration of fractions was monitored by UV absorbance (280 nm) to determine when elution was complete. TEV protease was used to cleave the GST tag by preparing a mixture of 1:15 of TEV:PhoQ (mass ratio; TEV protease was less than 20 % of the total volume.) The reaction was incubated at 4° C overnight. After cleaving PhoQ from the GST tag, the GST tag and PhoQ protein were separated from one another using glutathione affinity chromatography again.

Source Q. Protein was loaded onto the anion exchange resin (GE Healthcare) in low ionic strength buffer (20 mM Tris-HCl, pH 8, 2 mM DTT). A salt gradient that increased from 0 to 1 M NaCl was applied to elute the protein with detection at 260 nm and 280 nm.

Size exclusion. The final purification step for each protein was size exclusion using a HiLoad 16/60 Superdex 75 or 200 pg column (GE Healthcare). Protein was eluted into a storage buffer (10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 0.5 M NaCl, 12 % glycerol, and 2 mM DTT).

Protein concentration determination

The concentration of protein (after purification or free/bound protein from proteoliposome preparation) was determined by two means. Concentration was first measured on a nanodrop spectrophotometer at 280 nm. For accuracy, a DC colorimetric assay (Bio-Rad) was also used and performed according to the Bio-Rad DC Assay manual.

[γ-^{33}P]-ATP autophosphorylation or phosphotransfer assays

Time-based assays. At room temperature, reaction mixtures were prepared with final concentrations of 0.35 μM HK853/ VicK and 100 μM [γ-^{33}P]-ATP (0.28 Ci/mmol) in reaction buffer. At specified time intervals (15 s, 30 s, 50 s, 2.5 min, and 10 min), 15 μL was removed from the reactions and quenched as above.

[γ-^{33}P]-ATP phosphotransfer reactions. HK853 (2.7 μM) was autophosphorylated for 30 min in 100-μL reactions containing 200 μM [γ-^{33}P]-ATP (0.41 Ci/mmol) in reaction buffer. At 30 min, 4X or 7X RR468 was added. At 10 s, 3 min, and 20 min, 15 μL was removed from the reactions and quenched as above. Two controls were prepared, one without HK853 and one without RR468.

B-ATPγS thiophosphorylation of HK

Competition assays. ATP analogs were used as competitors of B-ATPγS labeling. Competition assays were performed using 600 ng HK853 and 200 μM ATP, AMP-PNP, ATPγS. Concentrations of ATP analogs were confirmed by measuring the absorbance at 259 nm (ε 15,400 $M^{-1}cm^{-1}$). After 10 min of HK-competitor incubation, 2 μM B-ATPγS was added. The reaction was incubated 1 h in the dark, and samples were quenched and analyzed as above.

HK853 thiophosphorylation in IPTG-Induced Samples. *E. coli* grown and induced to overexpress HK853 was collected before and after addition of IPTG. Pelleted cells were lysed and separated by centrifugation. The soluble proteome was adjusted to 3 mg/mL, and 50 μL was mixed with 1 μL B-ATPγS (2 μM, final concentration). Reactions were incubated, quenched, and analyzed as above.

HK853-RR468 thiophosphotransfer. After 600 ng HK853 was incubated with B-ATPγS for 1 h, 7-fold RR468 was added. The HK-RR-probe reaction was incubated 1-5 h, quenching with 2X loading buffer over hour intervals to observe increasing thiophosphoryl transfer. Samples were analyzed as above.

HK853 thiophosphorylation in proteoliposomes. To analyze activity of free HK853 and HK853 bound to liposomes, the proteins were labeled with B-ATPγS using the same concentrations and conditions as listed above for thiophosphorylation and competition. In addition, a sample of HK853 from a stock solution was run alongside the fractioned HK853 that had undergone the full proteoliposome preparation.

Figure S8. ATP analogs used in competition assays with B-ATPγS. (A) Structure of competitors. (B) Integrated density values of HK853 labeling (Figure 2) indicate competition of ATP analogs with B-ATPγS.

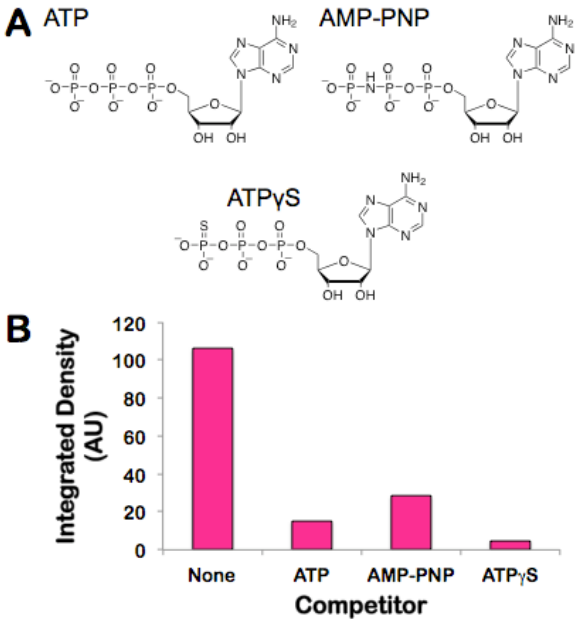


Figure S9. Quantitation of BODIPY-thiophosphate transfer between HK853 and RR468. Integrated density values of HK853-RR468 phosphotransfer (Figure 3) demonstrate the changes in labeling during the 5 h time period.

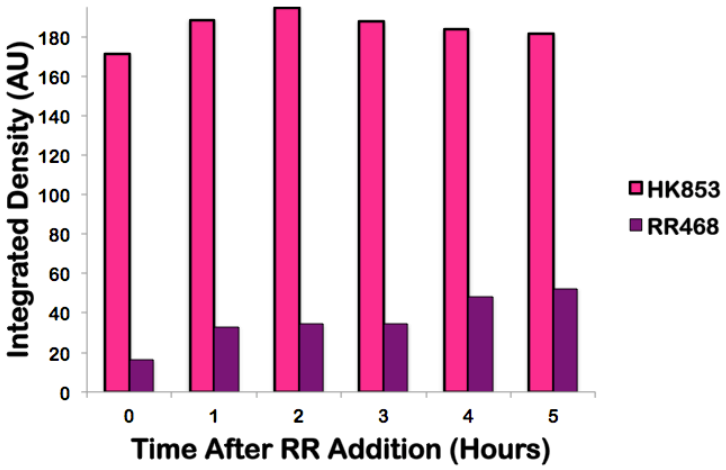


Figure S10. Quantitation of labeling of protein in proteoliposomes. Integrated density values of HK853 (Figure 4) from a purified protein stock (S), free in solution (F), or bound to liposomes (B) are shown in the graph. Numbers 0, 0.2, and 2 represent the concentration of competitor (ATP in mM). Integrated density values were determined for both fluorescence-detected and coomassie-stained bands. The fluorescence intensities were divided by the corresponding coomassie intensities to give ratios of activity:abundance. Ratios were normalized such that the value for HK853 (S) without competitor was set to 1.0000.

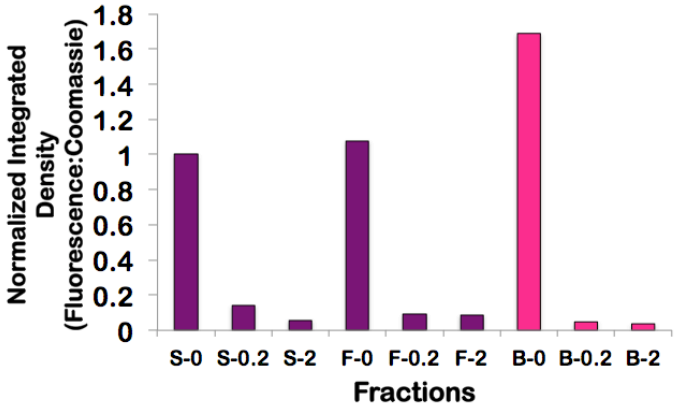


Table S1. Primers for total gene synthesis of HK853.

NO.	PRIMER SEQUENCE	SITE INFO
KEW001	GGGCCATGGAAAATGTCACTGAATCTAAGGAGCTCGAACGTCTC	NcoI
KEW003	TCATACGATCGATGCGTTTGAGACGTTCGAGCTCCTTAGA	
KEW004	AAACGCATCGATCGTATGAAAACCGAATTTATCGCGAACA	
KEW005	GGGTACGGAGTTCGTGAGAGATGTTCGCGATAAATTCGGT	
KEW006	CTCACGAACTCCGTACCCCGCTGACCGCGATCAAAGCGTA	
KEW007	GCTGTTGTAGATGGTTTCCGCATACGCTTTGATCGCGGTC	
KEW008	GCGGAAACCATCTACAACAGCCTCGGCGAGCTGGATCTCT	
KEW009	TTCCAGGAACTCCTTCAGCGTAGAGAGATCCAGCTCGCCG	
KEW010	GCTGAAGGAGTTCCTGGAAGTTATCATCGACCAGAGCAAC	
KEW011	TCAGCAGGTTCTCCAGGTGGTTGCTCTGGTCGATGATAAC	
KEW012	ACCTGGAGAACCTGCTGAACGAACTGCTGGACTTCTCTCG	
KEW013	GGAGGGATTTGCGTTCCAGACGAGAGAAGTCCAGCAGTTC	
KEW014	CTGGAACGCAAATCCCTCCAGATCAACCGTGAAAAAGTCG	
KEW015	TCAACCAGGTCGCACAGGTCGACTTTTTCACGGTTGATCT	
KEW016	CCTGTGCGACCTGGTTGAATCTGCGGTTAACGCTATCAAG	
KEW017	TGTGAGAGCTCGCAAATTCCTTGATAGCGTTAACCGCAGA	
KEW018	AATTTGCGAGCTCTCACAATGTTAACGTTCTGTTTGAGTC	
KEW019	ACCGGGCACGGGACGTTGGACTCAAACAGAACGTTAACAT	
KEW020	GTCCCGTGCCCGGTGGAGGCGTACATTGACCCAACGCGTA	
KEW021	GAGGTTGAGGAGCACCTGGCGGATACGCGTTGGGTCAATG	
KEW022	CAGGTGCTCCTCAACCTCCTGAACAATGGTGTAAAGTACTCC	
KEW023	GTCTGGTGCGTCCTTCTTGGAGTACTTTACACCATTGTTCAG	
KEW024	AAGAAGGACGCACCAGACAAGTACGTGAAAGTCATTCTCGA	
KEW025	CACACCACCGTCTTTCTCATCGAGAATGACTTTCACGTACTT	
KEW026	TGAGAAAGACGGTGGTGTGCTGATCATCGTGGAGGATAATG	
KEW027	GGTCCGGGATGCCGATACCATTATCCTCCACGATGATCAG	
KEW028	GTATCGGCATCCCGGACCACGCGAAGGACCGCATCTTTGA	
KEW029	GCTGTCAACACGGTAGAACTGTTCAAAGATGCGGTCCTTC	
KEW030	AGTTCTACCGTGTTGACAGCTCTCTGACCTACGAAGTTCC	
KEW031	CCAGGCCCAGACCGGTACCCGGAACTTCGTAGGTCAGAGA	
KEW032	CCGGTCTGGGCCTGGCGATCACCAAAGAAATCGTTGAGCT	
KEW033	CCAAATACGGCCACCGTGGAGCTCAACGATTTCTTTGGTG	
KEW034	CCACGGTGGCCGTATTTGGGTAGAGTCCGAGGTTGGTAAA	
KEW035	ACACGAAGAAACGAGAGCCTTTACCAACCTCGGACTCTAC	
KEW036	GGCTCTCGTTTCTTCGTGTGGATCCCGAAAGACCGTGCGG	
KEW037	GGGGAATTCCTAGTTGTCCTGACGGTTGTCTTCGCCCGCACGGTCTTTCGG	EcoRI + Stop

Table S2. Primers for total gene synthesis of RR468.

NO.	PRIMER SEQUENCE	SITE INFO
KEW038	GGGCCATGGCTAAAAAAGTTCTGCTCGTTGA	NcoI + S2A mutation
KEW039	CGGAGAACCGCAGAGTCGTCAACGAGCAGAACTTTTTTAGA	
KEW040	CGACTCTGCGGTTCTCCGCAAGATCGTCTCCTTCAATCTCA	
KEW041	TCACTTCGTAGCCTTCTTTCTTGAGATTGAAGGAGACGATCTT	
KEW042	AGAAAGAAGGCTACGAAGTGATTGAGGCTGAGAATGGTCAG	
KEW043	AGACAGTTTTTCCAGCGCGATCTGACCATTCTCAGCCTCAA	
KEW044	CGCGCTGGAAAAACTGTCTGAATTTACCCCGGACCTGATCG	
KEW045	GACCGGCATCATAATATCCAGAACGATCAGGTCCGGGGTAA	
KEW046	TCTGGATATTATGATGCCGGTCATGGACGGTTTCACCGTTC	
KEW047	TCCTTTTCCTGCAGCTTTTTGAGAACGGTGAAACCGTCCAT	
KEW048	CAAAAAGCTGCAGGAAAAGGAAGAATGGAAGCGTATCCCGG	
KEW049	CCTTTCGCGGTCAGAACAATAACCGGGATACGCTTCCATTC	
KEW050	TTGTTCTGACCGCGAAAGGTGGTGAAGAAGACGAATCTCTG	
KEW051	GCGCACCCAGAGACAGCGCCAGAGATTCGTCTTCTTCACCA	
KEW052	CTGTCTCTGGGTGCGCGTAAGGTAATGCGCAAGCCTTTTTC	
KEW053	TCTTCGATAAACTGGGAAGGGGAAAAAGGCTTGCGCATTAC	
KEW054	CCCTTCCCAGTTTATCGAAGAGGTTAAACACCTGCTGAACG	
KEW055	GGGGAATTCCTATTCGTTCAGCAGGTGTTTAACC	EcoRI + Stop

Table S3. Primers for total gene synthesis of PhoQ.

NO.	PRIMER SEQUENCE	SITE INFO
KEW056	GGGCCATGGCTGTGCTGCTGTCTCGTGAACTGCATCCGGTTGCG	NcoI + MA
KEW057	GAGATCAGGTTGTCGAGCAGCGGCGCAACCGGATGCAGTT	
KEW058	CTGCTCGACAACCTGATCTCTGCGCTGAACAAAGTCTACC	
KEW059	AATGTTTACACCCTTGCGCTGGTAGACTTTGTTCAGCGCA	
KEW060	AGCGCAAGGGTGTAAACATTAGCATGGACATCTCCCCGGA	
KEW061	CTGCTCGCCAACAAAGCTAATTTCCGGGGAGATGTCCATG	
KEW062	AGCTTTGTTGGCGAGCAGAACGACTTCGTTGAGGTAATGG	
KEW063	GCGTTGTCCAGGACGTTGCCCATTACCTCAACGAAGTCGT	
KEW064	CAACGTCCTGGACAACGCGTGCAAATATTGCCTGGAGTTT	
KEW065	ACGCGCAGAAATCTCCACAAACTCCAGGCAATATTTGCAC	
KEW066	GTGGAGATTTCTGCGCGTCAGACCGACGACCACCTGCACA	
KEW067	CGGACCGTCGTCTTCCACGAAGATGTGCAGGTGGTCGTCG	
KEW068	TGGAAGACGACGGTCCGGGCATCCCGCACTCTAAGCGTTC	
KEW069	CTGACCACGGTCGAAAACCAGAGAACGCTTAGAGTGCGGG	
KEW070	GGTTTTCGACCGTGGTCAGCGTGCGGACACCCTGCGTCCA	
KEW071	AACGGCCAGACCCACACCTTGACCTGGACGCAGGGTGTCC	
KEW072	GTGTGGGTCTGGCCGTTGCGCGTGAAATCACCGAGCAGTA	
KEW073	ACGCGATGATTTGACCCGCGTACTGCTCGGTGATTTCACG	
KEW074	CGGGTCAAATCATCGCGTCTGACTCTCTGCTGGGTGGTGC	
KEW075	GACCGAAAACAACTTCCATACGCGCACCACCCAGCAGAGA	
KEW076	CGTATGGAAGTTGTTTTCGGTCGTCAGCACCCGACCCAAA	
KEW077	GGGGAATTCCTATTCTTCTTTTTGGGTCGGGTGCTGA	EcoRI + Stop

Figure S11. HK853, RR468, and PhoQ overexpression. The three lanes of each gel correspond to protein ladder, non-induced *E. coli* lysate, and induced *E. coli* lysate.

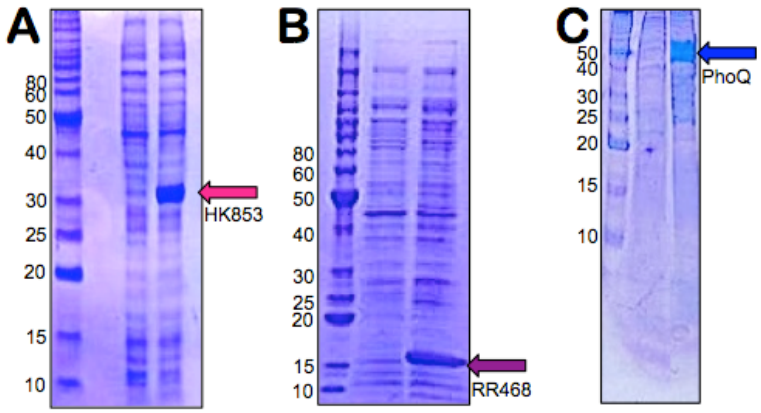


Table S4. Final sequences of the constructs generated from total gene synthesis. The catalytic His and phosphate-receiving Asp are in red. Gray residues comprise His-tags or the remainder of the GST-tag (PhoQ) after cleavage.

Protein	Sequence	Domains	MW (kDa)	pI	ε ($M^{-1}cm^{-1}$)
HK853	MSYYHHHHHHDYDIPTTENLYFQGAMENVTESKELERLKRIDRMKTEFIANISHEL RTPLTAIKAYAETIYNSLGELDLSTLKEFLEVIIDQSNHLENLLNELLDFSRLERKSLQI NREKVDLCDLVESAVNAIKEFASSHNVNVLFESNVPCPVEAYIDPTRIRQVLLNLLN NGVKYSKKDAPDKYVKVILDEKDGGVLIIVEDNGIGIPDHAKDRIFEQFYRVDSSLT YEVPGTGLGLAITKEIVELHGGRIWVESEVGKGSRFFVWIPKDRAGEDNRQDN	CA, DHp	32.5	5.20	27,390
RR468	MSYYHHHHHHDYDIPTTENLYFQGAMAKKVLLVDDSAVLRKIVSFNLKKEGYEVIE AENGQIALEKLSEFTPDLIVLDIMMPVMDGFTVLKKLQEKEEWKRIPVIVLTAKGGE EDESLALSLGARKVMRKPFSPSQFIEEVKHLLNE	RR	16.9	5.97	12,950
PhoQ	GAMAVLLSRELHPVAPLLDNLISALNKVYQRKGVNISMDISPEISFVGEQNDFVEV MGNVLDNACKYCLEFVEISARQTDDHLHIFVEDDGPGIPHSKRSLVFDRGQRADT LRPGQGVGLAVAREITEQYAGQIIASDSLLGGARMEVVFGRQHPTQKEE	CA	17.6	5.04	4470

Figure S12. Labeling of overexpressed HK in cell lysate. Overexpressed HK853 from *E. coli* lysate was labeled with 2 μM HK853. (NI=non-induced HK853; I=induced HK853)

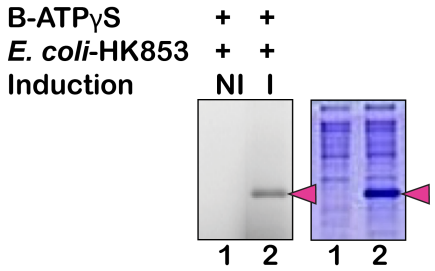


Figure S13. Time-based autophosphorylation assay of HK853. HK853 was incubated with 100 μM [γ-^{33}P]-ATP (0.28 Ci/mmol). Activity also assessed in Marina, et al (2005).[8]

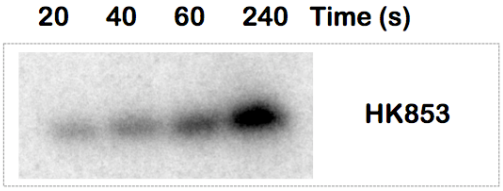


Figure S14. Time-based autophosphorylation assay of VicK. VicK was incubated with 100 μM [γ-^{33}P]-ATP (0.28 Ci/mmol). VicK kinetic details can be found in Gutu, et al (2010).[9]

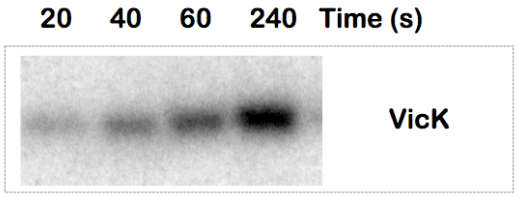
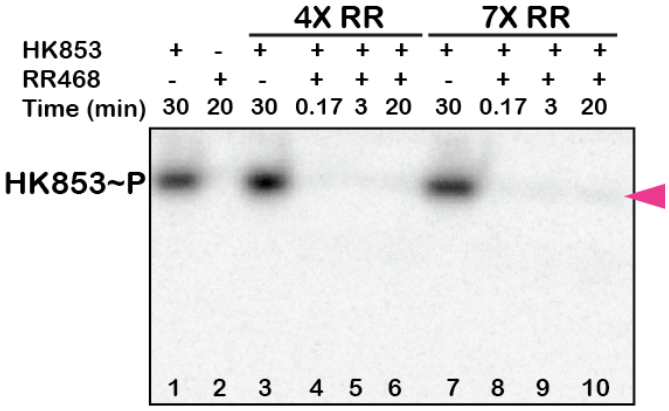


Figure S15. Assessment of phosphate transfer from HK853 to RR468 with radioactivity assay. Phosphotransfer occurs at 4X and 7X RR468. (7X RR was used in the experiments with B-ATPγS.) In all cases, RR468 phosphorylation was not visible, as discussed in Casino, *et al*. (2009).[5] Lanes 1 and 2 are controls. HK853 was phosphorylated at 30 min (pink arrow; lanes 1, 3, and 7), and phosphoryl transfer to RR468 occurred within 10 s.



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