Structural asymmetry does not indicate hemiphosphorylation in the bacterial histidine kinase CpxA

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Histidine protein kinases (HKs) are prevalent prokaryotic sensor kinases that are central to phosphotransfer in two-component signal transduction systems, regulating phosphorylation of response regulator proteins that determine the output responses. HKs typically exist as dimers and can potentially autophosphorylate at each conserved histidine residue in the individual protomers, leading to phosphorylation. However, analyses of HK phosphorylation in biochemical assays in vitro suggest negative cooperativity, whereby phosphorylation in one protomer of the dimer inhibits phosphorylation in the second protomer, leading to ~50% phosphorylation of the available sites in dimers. This negative cooperativity is often correlated with an asymmetric domain arrangement, a common structural characteristic of autophosphorylation states in many HK structures. In this study, we engineered covalent dimers of the cytoplasmic domains of Escherichia coli CpxA, enabling us to quantify individual species: unphosphorylated, monophosphorylated, and diphosphorylated dimers. Together with mathematical modeling, we unambiguously demonstrate no cooperativity in autophosphorylation of CpxA despite its asymmetric structures, indicating that these asymmetric domain arrangements are not linked to negative cooperativity and hemiphosphorylation. Furthermore, the modeling indicated that many parameters, most notably minor amounts of ADP generated during autophosphorylation reactions or present in ATP preparations, can produce ~50% total phosphorylation that may be mistakenly attributed to negative cooperativity. This study also establishes that the engineered covalent heterodimer provides a robust experimental system for investigating cooperativity in HK autophosphorylation and offers a useful tool for testing how symmetric or asymmetric structural features influence HK functions.

Two-component systems, the prevalent signaling pathways in bacteria, are one of the best-studied models of signal transduction schemes (1, 2). A conserved phosphotransfer reaction between the sensor histidine kinase (HK) and the response regulator (RR) is used to couple a large variety of inputs and outputs. HKs, usually functioning as homodimers, sense stimuli through their variable sensing domains and modulate phosphorylation levels of their cognate RRs. HKs display multiple enzyme activities, including autokinase, phosphotransferase that is catalyzed in conjunction with the RR protein, and often phosphatase activity toward the phosphorylated cognate RR. The conserved catalytic core of an HK consists of a dimerization and histidine phosphotransfer (DHp) domain that contains the conserved histidine residue for receiving and transferring the phosphoryl group and a catalytic ATP-binding (CA) domain that contains residues critical for kinase activity. Structures of HKs and HK-RR complexes representing different enzymatic states have been captured, providing mechanistic details of HK activities. Signal-dependent regulatory mechanisms have been investigated for HKs in response to ligand binding (3–6) or light sensing (7, 8). The structures and mechanisms have been extensively reviewed (2, 9–12). Distinct structural features are often linked to individual catalytic functions and biochemical behaviors of HKs.

Except for several atypical HKs with unusual oligomeric or monomeric states (13–15), one predominant emerging theme in HK signaling is the symmetry/asymmetry transitions in HK dimer structures. The symmetric or asymmetric HK structures involve similar or different arrangements of structural elements between individual protomers within an HK dimer. Structures with both symmetric and asymmetric conformations have been observed in the periplasmic sensing domains (3–5), the signal-transducing transmembrane helices and HAMP domains (6), and the catalytic core domains (16–20). Transitions between the two conformations have often been associated with signal-dependent switching of HK activities (16–18). Bhat et al. (10) analyzed more than 20 HK structures and revealed a general trend of symmetry/asymmetry transitions in different HKs. Symmetry or asymmetry in the catalytic core domains refers to the packing of DHp helices and the relative positioning of the two CA domains. Although the phosphatase state of an HK is often symmetric, with two CA domains held at positions unfavorable for phosphorylation, the autokinase state harbors an asymmetric conformation with one CA domain in close prox-

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The abbreviations used are: HK, histidine kinase; CA, catalytic ATP-binding; DHp, dimerization and histidine phosphotransfer; PEP, phosphoenolpyruvate; RR, response regulator; SpyC, Spycatcher; SpyT, Spytag.
imity of one phosphorylatable histidine, whereas the other CA domain is located far from the other histidine. The CA domain that provides the reactive ATP and the histidine receiving the phosphoryl group can be from the same or different protomers, resulting in a cis- or a trans-phosphorylation mechanism in different HKs. Nevertheless, both mechanisms make use of similar asymmetric conformations that only allow phosphorylation of one histidine at a time (16, 17, 19, 21, 22).

Such asymmetric structures are often intuitively associated with asymmetric phosphorylation, in which phosphorylation of one protomer hinders phosphorylation of the second protomer, leading to hemiphosphorylation in the extreme case, with a single phosphorylation event per dimer. For example, observation of asymmetric conformations in CpxA led to implication of hemiphosphorylation despite the experimental observation of a 70% total phosphorylation level, where the phosphorylation exceeding 50% was attributed to subunit exchange of protomers within HK dimers (17). As structural asymmetry is repeatedly demonstrated for the kinase state, is phosphorylation asymmetry also a general trend in HKs?

Asymmetric phosphorylation, or negative cooperativity, has been biochemically explored in several HKs (19, 23, 24). Early studies on NRII indicated that the equilibrium constant for phosphorylation of the first protomer is ~78-fold larger than that of the second protomer (23). Negative cooperativity has been attributed to an exceptionally rapid reverse reaction, with transfer of the phosphoryl group from the diphosphorylated HK to ADP generating the hemiphosphorylated HK; thus, ADP has a large inhibitory effect on diphosphorylation of HK dimers. Hemiphosphorylated HKs have also been observed on native gels in studies of HK853 (19). A complete diphosphorylation of HK853 dimers was not achieved unless ADP present in the reaction mixture was continuously recycled back to ATP. An even stronger negative cooperativity has been demonstrated for DesK (24). The observed phosphorylation stoichiometry of DesK was ~0.5, and full phosphorylation was not achieved even in the presence of a coupled enzyme system that continuously converted ADP to ATP, indicating an extremely high negative cooperativity. Such extreme negative cooperativity may be uncommon for HKs because full phosphorylation at high ATP levels or in the presence of ATP regeneration has been documented for many HKs (19, 23, 25, 26). On the other hand, analyses of the hybrid HK SkkA indicate no cooperativity in autophosphorylation (25).

Biochemical analyses of negative cooperativity of phosphorylation often rely on deriving equilibrium constants by measuring HK phosphorylation levels across a wide range of ATP concentrations. However, likely due to the spontaneous hydrolysis of ATP, even the “high-purity” commercial ATP reagent contains a trace amount of contaminating ADP (26–28). The initial amount of ADP, relative to that generated during the phosphorylation reaction, is nontrivial when high concentrations of ATP are used. Under such conditions, the inhibitory effect of the contaminating ADP might not be negligible, as presumed in prior analyses, and could impact interpretation of negative cooperativity. Here, we take account of the initial ADP concentration in the kinetic equilibrium modeling and show that the neglect of ADP contamination can lead to significant overestimation of negative cooperativity. We develop a strategy to examine the phosphorylation cooperativity by measuring the un-, mono-, and diphosphorylated HK dimer species using a covalently linked HK dimer. Applying this strategy to E. coli CpxA, we demonstrate that autophosphorylation of CpxA is not negatively cooperative, or asymmetric, despite the structural asymmetry.

Results

Impact of ADP contamination on interpretation of negative cooperativity

Commercial ATP is typically contaminated by its hydrolysis product ADP, and even ultrapure ATP (e.g. Sigma) contains ~0.4% ADP (27, 28). The exact value of ADP contamination may vary depending on the provider, lot, storage conditions, and storage time. We measured the ADP level in our ATP reagent using the NADH-coupled enzyme assay (26). Approximately 1% ADP was present in the ATP stock solutions (Fig. S1). In phosphorylation equilibrium assays with ATP concentrations often exceeding 1 mM, 1% ADP contamination would provide more than 10 μM ADP and could significantly impact HK phosphorylation.

To assess the inhibitory effect of the contaminating ADP, we modeled the HK phosphorylation equilibrium with the simple bi-bi reaction model (Fig. 1A) that has been previously used to derive equilibrium constants and cooperativity (23). The
monophosphorylated species contains two indistinguishable forms, with phosphorylation occurring at each individual protomer. Thus, the macroscopic equilibrium constant for phosphorylation of the first His, \( K_{\text{mut}} \), equals twice the microscopic constant \( K \), whereas the constant for phosphorylation of the second His, \( K'_{\text{mut}} \), is half \( K \) if the cooperativity constant \( c \) is equal to 1 (see details in supporting information and Fig. S2A).

The steady-state phosphorylation percentages of all phosphorylatable His sites, which are often readily quantifiable by various experimental methods, such as assays with radiolabeled ATP or Phos-tag gels, were simulated at different ATP concentrations. Unsurprisingly, increasing the contaminating ADP percentage, \( ADP_0/ATP_0 \), has a negative impact on total phosphorylation levels (Fig. 1B). Despite a noncooperative system simulated with the \( c \) value at 1, at high ATP concentrations, ADP contamination at 2% yields a total phosphorylation level within the range of 40–60%, a range often estimated as ~50% phosphorylation and intuitively associated with hemiphosphorylation. Clearly, a total phosphorylation level close to 50% does not indicate hemiphosphorylation or negative cooperativity, and a range of 40–60% phosphorylation can be achieved with different combinations of equilibrium constants and ATP and ADP concentrations (Fig. 1B and Fig. S2B, shaded area).

Because both ADP contamination and negative cooperativity can negatively impact the total phosphorylation level, we examined whether the inhibitory effect of ADP contamination could be misinterpreted as negative cooperativity. Our simulations indicate that ATP-dependent phosphorylation profiles can be extremely similar for distinctly different scenarios, one with negative cooperativity and the other with no cooperativity but with a higher ADP contamination percentage (Fig. 1B, magenta and black dashed-dotted curves) or with a lower value of the equilibrium constant (Fig. S2C, magenta and black lines). Generally, stronger negative cooperativity causes the ATP-dependent phosphorylation to be more graded than that with no cooperativity, but such distinction can be obscured by data variations. Within the range of experimental error, different combinations of cooperativity, ADP contamination, and equilibrium constants can result in curves that are highly similar to each other (Fig. S2D) and thus prone to misinterpretation. As shown in Fig. 1C, data were simulated (black line) with no cooperativity, ADP contamination at 1% and an equilibrium constant of 0.1, a value within the same magnitude of measured constants for some HKs (23, 25). Total phosphorylation percentages simulated with small S.D. values under the above conditions (circles) could be fitted to a different set of parameters (magenta line) if the initial ADP contamination is neglected and assumed to be zero, leading to a conclusion of negative cooperativity. The fitted cooperativity constant \( c \) has a value at 0.14, corresponding to a nearly 30-fold difference between the two macroscopic constants \( K_{\text{mut}} \) and \( K'_{\text{mut}} \). Thus, neglect of ADP contamination can lead to significant overestimation of negative cooperativity for HK autophosphorylation.

Although the total phosphorylation levels shown in Fig. 1C are similar, populations of individual phosphorylated species differ remarkably between the two scenarios, with or without negative cooperativity (Fig. 1D). More importantly, a composite parameter \( h \), defined as 4 × \( U \times P_2/(P \times P) \) calculated from concentrations of individual phosphorylated species (Fig. 1A), is equal to the cooperativity constant \( c \) under the bi-bi reaction scheme (see Equation 7 under “Modeling”). The value of \( h \) is constant across different ATP concentrations independent of ADP contamination or the equilibrium constant \( K \). Thus, the cooperativity of HK phosphorylation can be derived from the concentrations of differently phosphorylated HK dimer species if the individual forms can be robustly quantified.

**Formation of covalently linked CpxA dimers**

Creation of covalent dimers provides a robust strategy for separating multiple phosphorylated forms of HKs based on their mobility difference on Phos-tag SDS-polyacrylamide gels. Furthermore, the covalent linkage eliminates potential subunit exchange of protomers among dimers. Instead of using an engineered disulfide linkage that could potentially introduce conformational strain or might interfere with naturally occurring cysteine residues in HKs, we employed Spycatcher–Spytag chemistry, a technology that utilizes two separate protein fragments that spontaneously form a covalent bond between a lysine and an aspartate residue (29). This strategy was used to create covalent dimers of cytoplasmic fragments of *Escherichia coli* CpxA, an HK in which structural asymmetry has been observed (17, 22). Spycatcher (SpyC-) or Spytag (SpyT-) was genetically fused to the N terminus of CpxAC, the cytoplasmic fragment (residues 188–457) of CpxA that was shown to have asymmetric domain arrangements (17), creating SpyT-CpxAC and SpyC-CpxA, C (Fig. 2A). These domains were fused with CpxA using flexible linkers at residue 188, a position spatially distant from the catalytic center, so it is unlikely that they will constrain the conformational changes of the DHp and CA domains or interfere with the enzymatic functions.

**Figure 2. Formation of covalent heterodimers of CpxA kinase domains.** A, schematic diagram of fusion proteins engineered with Spytag or Spycatcher domains joined by flexible linkers to CpxA cytoplasmic domains (SpyT-CpxAC and SpyC-CpxA) and their interactions. B, time course of covalent heterodimer formation analyzed using SDS-PAGE. SpyT-CpxAC and SpyC-CpxA were mixed at a final concentration of 1 μM each, and the linkage reaction was quenched by the addition of concentrated SDS sample loading buffer. For time point zero, individual CpxAC proteins were denatured using SDS sample loading buffer before mixing. C, size-exclusion chromatography of CpxAC proteins. SpyT-CpxAC, (dashed line), SpyC-CpxA, (dotted line), and a mixture of the two proteins (solid line) were analyzed using a Sephadex 200 size-exclusion column. Elution volumes of protein used for calibration are shown as circles. Protein standards are as follows: lysozyme (14 kDa), alcohol dehydrogenase (36 kDa), BSA (66 kDa), alcohol dehydrogenase dimer (73 kDa), and BSA dimer (132 kDa).
Formation of covalent dimers occurred rapidly after mixing SpyT-CpxAC and SpyC-CpxAC proteins (Fig. 2B). The majority of monomers were converted to covalent dimers after 5 min of incubation at room temperature, and dimers remained stable over time. However, this SDS-PAGE analysis does not exclude the existence of tetramers, specifically dimers of dimers that have been shown to exist in solutions at high protein concentrations (17) and could potentially be formed by covalent linkage of pairs of SpyT-CpxAC and SpyC-CpxAC noncovalent dimers. To address this possibility, the oligomerization states of the proteins in solution were examined using size-exclusion chromatography. Retention times of SpyT-CpxAC and SpyC-CpxAC proteins corresponded to apparent molecular masses of 68 and 88 kDa, in close correlation with the calculated molecular masses of the noncovalent homodimers, 67 and 86 kDa, respectively. The covalent dimer formed by mixing SpyT-CpxAC and SpyC-CpxAC eluted at a retention time corresponding to an apparent molecular mass of 78 kDa, similar to the calculated molecular mass of 79 kDa for the heterodimer. No peaks were observed at early retention times, as might be expected for higher-order oligomers.

To further interrogate autophosphorylation in the covalent dimer, the mode of autophosphorylation was examined with respect to cis or trans mechanisms. Crystal structures indicate that CpxA autophosphorylates in trans, with the CA domain of one protomer making contact with the conserved His residue within the Dhp domain of the second protomer (17). The covalent dimer allows biochemical differentiation of the trans and cis autophosphorylation mechanisms. Mutations were introduced to abrogate autophosphorylation either by disrupting the phosphorylation site in the Dhp domain (His-248 to Ala, H248A, H*) or by disrupting the ATP-binding site in the CA domain (Asn-356 to Lys, N356K, N*). If CpxA autophosphorylates by a trans mechanism, heterodimers designed with one protomer containing the H248A substitution and the second protomer containing the N356K substitution would be able to phosphorylate a single protomer within the dimer, whereas heterodimers designed with one protomer containing both H248A and N356K substitutions and the second protomer being WT would be unable to autophosphorylate (Fig. 4A). As expected, noncovalent homodimers carrying either H248A or N356K substitutions, or both, displayed no autophosphorylation when incubated with ATP for 30 min (Fig. 4C).

Having verified the expected behavior of the substituted proteins, we examined autophosphorylation in covalent heterodimers. The covalent dimer with WT CpxAC domains, WT:WT (SpyT-CpxAC:CpxAC), migrates as a single species in the absence of phosphorylation. After incubation with ATP, two additional bands are observed (Fig. 4B). The lower band of the triple bands corresponds to the unphosphorylated dimer, and presumably, the middle band is a monophosphorylated species, and the upper band is a diphosphorylated species. A covalent dimer that contains one protomer with a mutation in the Dhp domain and the second protomer with a mutation in the CA domain, H*:N* (SpyT-CpxAC:CpxAC, N356K), displays only a single additional band of lower mobility than the unphosphorylated dimer, corresponding to a monophosphorylated species (Fig. 4B). A covalent dimer that con-

**Autophosphorylation of the covalently linked CpxAC dimers**

Autophosphorylation kinetics of the noncovalently linked proteins were analyzed for SpyC-CpxAC, SpyT-CpxAC, and CpxAC (Fig. 3). Quantitation of the bands from Coomassie Blue–stained Phos-tag gels produced highly similar autophosphorylation profiles (Fig. 3D), suggesting that the fused Spycatcher and Spytag fragments do not interfere with the autophosphorylation kinase activity of CpxAC. Unlike the noncovalently linked proteins that migrate as two bands corresponding to phosphorylated and unphosphorylated monomer proteins, the covalent CpxAC dimer with two phosphorylatable His residues, one in each of the two WT subunits (Fig. 4A, left), shows triple bands on the Phos-tag gel (Fig. 4B, top), suggesting separation of the di-, mono-, and unphosphorylated dimer species.

**Figure 3. Autophosphorylation kinetics of CpxAC proteins.** Phos-tag PAGE analysis of SpyT-CpxAC (A), SpyC-CpxAC (B), and CpxAC (C). D, quantification of the phosphorylation time course. Levels of phosphorylated and unphosphorylated proteins at each time point were quantified from gels stained with Coomassie Blue and were used to calculate the percentage of phosphorylated CpxA protein (SpyT-CpxAC, blue circles; SpyC-CpxAC, red squares; CpxAC, black triangles). Symbols show the mean of three independent experiments, and error bars indicate the S.D.

**EDITORS’ PICK:** Phosphorylation of HK CpxA is not cooperative


Figure 4. Assessment of a trans autophosphorylation mechanism using CpxA<sub>C</sub> mutant proteins. A, schematic diagram of a strategy to characterize the mode of autophosphorylation. Phosphorylation reactions in trans are depicted as solid arrows pointing from the CA domain of one protomer to the DHp domain of a second protomer. Phosphorylation reactions disrupted by an N356K mutation in the CA domain (N*) or an H248A mutation in the DHp domain (H*) are indicated by dotted lines, with mutated residues indicated by red crosses. B, kinase activities of covalently linked CpxA<sub>C</sub> dimers. Protein dimers at a final concentration of 1 μM were incubated at 25 °C with 0.5 mM ATP and then analyzed by Phos-tag PAGE. Bands corresponding to unphosphorylated, monophosphorylated, and diphosphorylated species are labeled as U, P, and P<sub>2</sub>, respectively. C, kinase activities of CpxA<sub>C</sub> mutant proteins. Proteins at a final concentration of 1 μM were incubated for 30 min at 25 °C with or without 0.5 mM ATP and then analyzed by Phos-tag PAGE. The faint band above N* likely is an impurity in the protein preparation and did not interfere with formation or phosphorylation of the covalent dimer.

Having established a method for quantifying individual phosphorylated dimer forms, the cooperativity of autophosphorylation for the covalent SpyT-CpxA<sub>C</sub>:SpyC-CpxA<sub>C</sub> dimer was examined in the presence of 1500 μM ATP and various concentrations of ADP (Fig. 6). With increasing ADP concentrations, levels of the diphosphorylated species (P<sub>2</sub>) decreased, whereas monophosphorylated species (P) increased initially and then gradually decreased, and the unphosphorylated species predominated (Fig. 6, A and B). Values of the composite parameter h, calculated with concentrations of individual phosphorylated species, 4 × U × P<sub>2</sub>/(P × P<sub>2</sub>), are close to 1 across a wide range of ADP concentrations and approach 2 at the lowest two ADP levels (Fig. 6C). Because the simple bi-bi reaction model predicts equal values of h and the cooperativity constant c, autophosphorylation of the covalent CpxA<sub>C</sub> dimer appears to be noncooperative, with a cooperativity constant close to 1, whereas h values smaller than 1, corresponding to negative cooperativity, were never observed.

Deviations of h values at low ADP concentrations from those at higher ADP levels are inconsistent with the model prediction that h values would be independent of ATP or ADP. Deviations may be an artifact of large quantification variations present at low ADP levels (Fig. 6C) or reflect inadequacy of the simple bi-bi model. When the simple model with the cooperativity constant c equal to 1 is used to simulate percentages of phosphorylated species (black lines in Fig. 6B), simulated data do not agree well with experimental data, suggesting that the simple model is not accurate in predicting autophosphorylation of CpxA<sub>C</sub>.

An apparent deficiency of the simple bi-bi model is the lack of consideration of the nucleotide-binding equilibria in HK autophosphorylation. Because the observed K<sub>m</sub> of ATP for autophosphorylation is ~10–200 μM for many HKs (10, 30, 31), ATP is expected to saturate the HK-binding sites in the above experimental assay with 1500 μM ATP. To address the discrepancy between experimental data and the bi-bi model, we developed a complex model that accounts for binding of ATP to the unphosphorylated HK and dissociation of ADP from the phosphorylated HK with their respective association constants K<sub>U</sub> and K<sub>P</sub> (Fig. 6D). Further, ATP can compete with ADP for binding to the phosphorylated HK (HK–P), inhibiting the reverse reaction; vice versa, ADP can inhibit the forward phos-
EDITORS’ PICK: Phosphorylation of HK CpxA is not cooperative

Phosphorylation reaction through competitive binding to the unphosphorylated HK (blue boxes in Fig. 6D). Binding coefficients $c_i$ and $c_{ij}$ are used to illustrate the two competitive binding events. In addition to the phosphorylation cooperativity constant $c$, a nucleotide-binding cooperativity constant $c_j$ is included for developing a cooperative model for nucleotide binding and autophosphorylation of HK dimers (Fig. S3A).

The model predicts that the value of $h$ approaches $c$ for a large parameter space if the ATP concentration greatly exceeds the binding constant $K_i$ (see supporting information for details). If $c_j$ is much smaller than 1 and the nucleotide-binding cooperativity constant $c_j$ is not equal to 1, the value of $h$ deviates from $c$ at low ADP concentrations but converges to $c$ at high ADP concentrations (Fig. S3B), consistent with the observed $h$ values shown in Fig. 6C. The $K_m$ of ATP for CpxA-C autophosphorylation is $\sim 147 \mu M$ (Fig. S4) and was used to approximate the dissociation constant of ATP for CpxA-C for fitting the $h$ values with the complex model. Estimation of multiple parameters based on a limited set of data points is challenging, leading to large S.E. of parameter values (Fig. 6C) except for the autophosphorylation cooperativity constant $c$. The value of $c$ is 0.98 with the 95% confidence range between 0.93 and 1.03, again suggesting that autophosphorylation of CpxA-C is not cooperative. Populations of phosphorylated spe-

![Figure 5](image1.png)

**Figure 5.** Kinetics of autophosphorylation of covalently linked CpxA$_C$ dimers. A, kinase assay of covalent CpxA$_C$ dimers. CpxA dimers at a concentration of 1 $\mu M$ were incubated at 25 °C with 0.5 mM ATP. Aliquots were quenched with 4 × SDS sample-loading buffer and analyzed by Phos-tag PAGE. B, kinase assay of covalent CpxA$_C$ dimers in the presence of an ATP regeneration system. Reactions were performed as in A with the inclusion of 20 units/ml pyruvate kinase and 3 mM PEP in the reaction mixture. C and D, kinetics of formation of phosphorylated species. In the absence C or presence D of ATP regeneration levels of phosphorylated species from reactions performed as in A and B were quantified from gel images (circles, unphosphorylated species; squares, monophosphorylated species; triangles, diphosphorylated species). Symbols show the mean of five independent experiments, and error bars indicate the S.D.

![Figure 6](image2.png)

**Figure 6.** Determination of cooperativity of autophosphorylation in covalently linked CpxA$_C$ dimers. A, steady-state phosphorylation of CpxA$_C$ dimers. CpxA$_C$-covalent dimers at a concentration of 1 $\mu M$ were incubated with the indicated concentrations of ATP and ADP for 40 min at 25 °C and then analyzed by Phos-tag PAGE. B, effects of ADP on phosphorylation of CpxA$_C$ dimers. Percentages of unphosphorylated (triangles), monophosphorylated (circles), and diphosphorylated (squares) dimers were quantified from gel images of experiments performed as in A. Black lines are simulated with the simple bi-bi model using $c = 1$ and $K = 0.05$. Blue lines are simulated using the complex nucleotide-binding model with the following: $K_{pt} = 0.35$; $K_{pt}$, and $K_{ct}$ 0.0068 $\mu M^{-1}$, derived from Fig. 5C; $c_{ij} = 1$; $c_i = 4$, $c_j = 0.04$, which are derived from C. C, determination of cooperativity of phosphorylation. The value of parameter $h$ was fitted with the complex model (blue line), yielding the following parameter values: $c = 0.98 \pm 0.03$, $c_i = 4.1 \pm 4.6$, $c_j = 0.04 \pm 0.01$. D, effects of competitive binding of ATP to HK–P on total phosphorylation levels. A complex model (top and Fig. S3) has been developed to investigate how competitive nucleotide binding impacts autophosphorylation (bottom). The nonproductive dead-end complexes are highlighted in blue boxes. Circles indicate total phosphorylation percentages calculated from B. Curves are data simulated using the simple (black) or the complex model (blue). When $c_i = 1$, the two models yield identical phosphorylation (dotted lines) for noncooperative systems ($c = 1$). Solid blue and dashed black lines are simulated with parameter values indicated in B. Symbols in all panels show the mean of five independent experiments, and error bars indicate the S.D.
**EDITORS’ PICK:** Phosphorylation of HK CpxA is not cooperative

**Figure 7.** Steady-state phosphorylation levels of HKs under cellular concentrations of ATP and ADP. A, total phosphorylation levels; B, percentages of diphosphorylated species (solid lines) and the corresponding RR phosphorylation levels (dashed-dotted lines) are simulated with the complex model using the indicated parameter values of $c_P$ and $c_0$. Other parameter values are as follows: $c_P = 1; c_0 = 0.4$ (when $c_P = 0.04$); $K_m = 0.0068 \mu M^{-1}; K_c = 0.0068 \mu M^{-1}$ or $0.068 \mu M^{-1}$ (dashed lines). The total HK dimer concentration is $1 \mu M$, and the RR concentration is $10 \mu M$. The equilibrium constant for the reversible phosphotransfer to RR is set at 10. Magenta lines, data simulated with the negativity cooperativity constant $c_P = 0.05$, derived by the macroscopic constants measured for NRII (23). Solid circles indicate HKs with known equilibrium constants $K^c_{CpxA}$. CpxA, 0.05; NRII, 0.35 (23); ShkA, 0.13 (23). Gray symbols indicate HKs with $K_c/K_v$ valued at 0.1 based on $K_m$ and equilibrium constants derived from $k_{cat}$ for both forward and reverse reactions: diamond, NarQ, $\sim 0.02$ (30); pentagon, AgrA ($\pm$ stimulus), 0.4 (34); circle, CheA, 1 (26).

Negative cooperativity of autophosphorylation, with nucleotide competition might impact HK activities under cellular conditions, we modeled the autophosphorylation and phosphotransfer to RRs at cellular concentrations of ATP and ADP (Fig. 7). An ATP concentration of 1.5 mM and an ADP concentration of 0.2 mM were used based on the reported ATP level and ATP/ADP ratio in *E. coli* cells (32, 33). If there is no cooperativity ($c_P = 1, c_0 = 1$) and nucleotides have equal affinity for HK and HK-P ($c_0 = 1, c_0 = 1$), the total phosphorylation level of the HK and the concentration of phosphorylated RR (RR-P) correlate with the autophosphorylation equilibrium constant $K_{pt}$ (black lines in Fig. 7A and B)). Under this scenario, with nucleotide competition considered, the complex model appears identical to the simple bi-bi model, and the overall equilibrium constant equals $K_{pt} \times K_c/K_v$ as shown in Equation 10. A higher affinity of ADP for HK-P than that of ATP for HK reduces the effective equilibrium constant and shifts the autophosphorylation reaction to the unphosphorylated reactant (dashed line in Fig. 7A). Several HKs, such as CheA (26), NarQ (30), and AgrA (34), have been shown to have a $K_m$ for ATP that is about one-tenth of the $K_m$ for ATP, suggesting a reduced overall autophosphorylation activity, although phosphorylation cooperativities of these HKs are unknown.

Two additional scenarios were modeled: one with no cooperativity but with less inhibition of the reverse reaction, as suggested by CpxA ($c_P = 0.04$, blue lines) and the other with $c_0$ valued at 1 but with negative cooperativity (magenta lines), with $c$ valued at 0.05, which corresponds to an ~80-fold difference in macroscopic equilibrium constants observed for NRII (23). Both greatly reduce the total phosphorylation levels of HK (Fig. 7A). In contrast, their effects on RR-P levels are modest, with only ~10% decrease at the measured equilibrium constant values of NRII and CpxA (positioned at the vertical dotted line in Fig. 7B), suggesting that negative cooperativity or reduced reverse reaction inhibition may not impact RR phosphorylation as much as it impacts HK phosphorylation at cellular levels of ATP and ADP. This is due to RR-P nearing saturation because of a large equilibrium constant for RR phosphorylation, reflecting the fact that phosphotransfer is believed to greatly favor the forward reaction (18). For NRII, it has been shown that disrupting the negative cooperativity effect by removal of ADP has little impact on RR phosphorylation (23), consistent with the model prediction that a greatly favored phosphotransfer minimizes the effect of negative cooperativity on RR-P. The impact of cooperativity and nucleotide competition on RR phosphorylation could be significant for HKs with smaller values of equilibrium constants for autophosphorylation and phosphotransfer activities. Under the two scenarios described above, the diphosphorylated species remains low across different $K_{pt}$ values, consistent with the monophosphorylated species dominating the phosphorylated HK dimers. However, for an HK without the two phosphorylation-inhibitory effects in the above scenarios, such as ShkA (25), the diphosphorylated species is expected to constitute a nontrivial population of the phosphorylated HK proteins.

**Discussion**

Negative cooperativity of autophosphorylation, with phosphorylation of one protomer inhibiting the phosphorylation of the other protomer of an HK dimer, has sometimes been presumed when an asymmetric structure corresponding to the kinase state has been observed (17). Our biochemical analyses of the covalent CpxA$_2$ dimer indicate that autophosphorylation of CpxA is not negatively cooperative despite observations of hemiphosphorylated and asymmetric structures captured by crystallography (17, 22). In addition to CpxA, a cytosolic hybrid HK, ShkA, has also been shown to display no cooperativity in autophosphorylation (25). The biochemical reaction of autophosphorylation appears not always to be negatively cooperative, even though the kinase state of HKs shares common asym-
metric structures with only one CA domain positioned in a phosphorylation-competent conformation.

Noncooperativity is not necessarily inconsistent with asymmetric catalytic structures. The basis for structural asymmetry is believed to result from asymmetric bending of the DHp helix (10, 17, 21, 35), which leads to asymmetric DHp surfaces that promote different interactions with the two ATP-binding CA domains via the interacting helix in the CA domain, known as the Gripper helix. Such structural asymmetry precludes simultaneous phosphorylation of the two His sites at a single time but does not prevent a proposed sequential phosphorylation mechanism with the two CA domains alternating their interactions with the His sites (21). It is not clear how phosphorylation of one protomer would impact the conformation of the other due to the lack of structures of hemiphosphorylated HKs. One structural snapshot of a hemiphosphorylated (i.e. monophosphorylated) HK is available for CpxA<sub>C</sub> (22). In this structure, the unphosphorylated His site forms the typical catalytic complex with the CA domain, ready for phosphorylation without any apparent structural hindrance from phosphorylation of the other protomer. Noncooperativity and structural asymmetry appear not to be mutually exclusive, and asymmetric catalytic structures are not equivalent to half-of-sites reactivity.

Biochemical investigations of autophosphorylation cooperativity are often carried out by measuring the total phosphorylation levels of HKs, and ~50% phosphorylation is often intuitively associated with half-of-sites reactivity. Our modeling indicates that ~50% phosphorylation can result from different combinations of cooperativity, equilibrium constants, and ADP present as contaminants in ATP or generated during reactions. Only when the reverse reaction of autophosphorylation is strongly inhibited by removing ADP with an ATP regeneration system can a 50% total phosphorylation level be used to conclude half-of-sites reactivity. Such a scenario has been reported previously for DesK (24), indicating a strong negative cooperativity. ATP-dependent phosphorylation profiles, often used to derive the two equilibrium constants for autophosphorylation, are also affected by ADP contamination. Higher ADP contamination or larger data variation produces less confidence in predicting negative cooperativity. A sufficiently strong negative cooperativity, as shown for NRII (23), is less prone to misinterpretation caused by the neglect of ADP contamination. Predetermination of the ADP level in ATP reagents can facilitate accurate measurement of the cooperativity of phosphorylation.

Quantification of individual phosphorylated species of CpxA<sub>C</sub> at different ATP and ADP concentrations revealed the necessity of including the competitive ATP/ADP-binding equilibrium for correct modeling of HK autophosphorylation. The overall equilibrium constant, $K_{prot} \times K_\alpha / K_\beta$, depends on the ratio of binding affinities of ATP for HK over ADP for HK~P. This provides additional control over the autophosphorylation equilibrium. No apparent trend in these relative affinities seems to exist for different HKs. Binding of ADP can be stronger than (26, 30, 34, 36), equal to (23, 30, 37), or weaker than (38, 39) binding of ATP to different HKs. Binding of ADP to HK~P has a negative impact on the overall phosphorylation by promoting the reverse reaction (23, 26, 38). Competitive binding of ATP to HK~P results in formation of a dead-end complex, HK~P-ATP, and reduces the amount of HK~P-ADP, a complex that is required for the reverse reaction. Thus, a weaker affinity of ATP for HK~P will lead to less competition with ADP, a more favored reverse reaction, and less phosphorylation. This mechanism is distinct from negative cooperativity because it involves a dead-end complex of HK~P-ATP and does not require a dimer. Formation of the dead-end complex has been suggested for CheA with ATP having a lower affinity for CheA~P than for unphosphorylated CheA (40). Much less is known for typical HKs in the common HisKA or HisKA3 subfamilies. A reduced affinity of ATP for HK~P relative to unphosphorylated HK implies that phosphorylation occurring in the DHp helices impacts nucleotide binding in the CA domains. Because the Gripper helix involved in DHp-CA interaction is directly connected to the ATP lid that is important for nucleotide binding, it is not surprising that phosphorylation-induced changes could propagate to the nucleotide-binding site.

Although both negative cooperativity and reduced inhibition of the reverse reaction can have large negative impacts on HK phosphorylation levels, the two mechanisms are predicted to have modest impact on RR phosphorylation at cellular concentrations of ATP and ADP. A large phosphotransfer equilibrium constant used in our model results in the reaction being heavily favored toward the product side and yields nearly saturating RR phosphorylation, overshadowing the inhibitory effect on HK phosphorylation. Experimentally examined phosphotransfer for typical HKs appears to have an even larger constant than that used in the modeling, with little back-transfer from RR~P to HK (18, 41–44). Negative cooperativity would have little effect on RR phosphorylation levels in such systems. For many HKs involved in phosphorelays with a more reversible phosphotransfer (18), negative cooperativity might play a more significant role in determining overall RR phosphorylation levels.

Creation of covalent HK dimers with Spytag–Spycatcher technology has proved to be a successful strategy for examining the biochemical activities of dimeric HK proteins. It provides a robust alternative method to native gels (19) for differentiating the un-, mono-, and diphosphorylated species for analyzing the cooperativity of phosphorylation. Furthermore, because of the covalent complex, the potential complicating effect caused by subunit exchange between HK dimers is eliminated (45, 46). Covalent heterodimers of HK mutants enable a straightforward method to differentiate the <i>cis</i> or <i>trans</i> mechanism of autophosphorylation. Here, we demonstrate that autophosphorylation of CpxA<sub>C</sub> is through a <i>trans</i> mechanism, consistent with the domain arrangement revealed by crystal structures (17, 22). Investigations of <i>cis</i> or <i>trans</i> mechanisms often rely on kinase assays of HKs with active-site mutations. In such studies, phosphorylation observed upon mixing H<sup>H</sup>H<sup>H</sup> and N<sup>N</sup>N<sup>N</sup> mutant proteins is definitive evidence of a <i>trans</i> mechanism, but lack of phosphorylation is not definitive evidence of a <i>cis</i> mechanism, unless it can be demonstrated that subunit exchange produces structurally sound heterodimers. Covalent linkage ensures heterodimer formation, strengthening conclusions of a <i>cis</i> mechanism from a null phosphorylation result with H<sup>H</sup>:N<sup>N</sup> covalent dimers and unequivocal identification of a <i>cis</i> mechanism if phosphorylation is observed in WT:H<sup>H</sup>N<sup>N</sup> covalent dimers. Fur-
ther, because structural asymmetry is a general theme in HK signaling, the Spytag–Spycatcher covalent heterodimer will be a useful tool for testing how individual asymmetric features influence the structure and function of HKs.

**Experimental procedures**

**Construction of plasmids**

All cloning was done using the *E. coli* strain DH5α. Two DNA fragments encoding either Spycatcher or Spytag 002 were synthesized based on sequences of pET28-trc-Streptag-trc-Streptag-YFP, and pDEST14-SpyCatcher002 from Addgene (ID no. 35050 and 35044 respectively) (29) and were integrated into pET21 between NdeI and EcoRI sites, resulting in pSB18 (pET21-Spycatcher) and pSB20 (pET21-Spytag), respectively. Using the two plasmids as templates, vectors pSB41 (pET-trc-Streptag-Spytag-YFP), pRG463 (pET-trc-Histag-Spycatcher-YFP), and pSB46 (pET-trc-Streptag-Spytag-YFP) were assembled using Golden Gate cloning protocols (47). These vectors contain a trc promoter to allow control of expression levels, a His6 or Streptag for affinity purification, a flexible linker GSGESGGSGA (nucleotide sequence, ggtagttggaagttgtggtcgcgggtcgtt) between Spy fragments and the downstream gene as recommended (29), and a gene encoding YFP for fluorescence-based clonal screening. A gene encoding the cytoplasmic domains of *E. coli* CpxA (CpxAC, amino acids 188–457) was then integrated using Golden Gate cloning protocols to replace yellow fluorescent protein between the Bsal sites, resulting in vectors pSB42 (pET-trc-Streptag-Spytag-CpxA(C)), pSB58 (pET-trc-Histag-Spycatcher-CpxAC), and pSB47 (pET-trc-Streptag-CpxAC), respectively. The Streptag coding sequence from pSB47 was replaced by the Histag coding sequence to obtain pSB80 (pET-trc-Histag-CpxAC). All final plasmids were sequenced.

Single-site substitutions H248A and N356K were introduced using a QuikChange kit (Agilent), following the manufacturer’s protocol, ending with transformation into *E. coli* DH5α. The mutations in the resulting vectors, pSB76 (Spycatcher-CpxAC H248A-N356K), pSB77 (Spytag-CpxAC H248A), and pSB79 (Spytag-CpxAC H248A-N356K), were confirmed by sequencing.

**Protein purification**

Recombinant proteins Spytag-CpxAC, Spycatcher-CpxAC, CpxAC, Spycatcher-CpxAC, CpxAC, Spytag-CpxAC, CpxAC, and Spytag-CpxAC, CpxAC were produced from *E. coli* BL21(DE3) containing the plasmids pSB42, pSB58, pSB80, pSB76, pSB77, and pSB79, respectively. Cells from frozen permanents were grown in Luria broth supplemented with 1% glucose and 100 μg/ml ampicillin at 37 °C with shaking. Overnight cultures were diluted 1:100 into Luria broth containing 100 μg/ml ampicillin and incubated at 37 °C with shaking. At A600 = 0.6, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM to induce expression of CpxAC and Spytag-CpxAC protein and variants, and incubation was continued for 3 h at 37 °C. Expression of Spycatcher-CpxAC protein and variants was induced with 20 μM isopropyl β-D-thiogalactopyranoside, and incubation was continued for 3 h at 30 °C.

Cells were harvested by centrifugation, resuspended at a density of 0.3 g cells/ml in binding buffer, and lysed by sonication. Binding buffer for purification of Strep-tagged proteins was composed of 100 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA. Binding buffer for purification of His-tagged proteins was composed of 24 mM KH2PO4/Na2HPO4 buffer, pH 6.8, 0.5 M NaCl, 24 mM imidazole supplemented with 0.5 mM phenylmethylsulfonyl fluoride. This and all subsequent purification procedures were performed at 4 °C. Lysates were centrifuged at 35,000 rpm for 1 h. The supernatant was collected, passed through a 0.2-μm filter, loaded onto Strep-Tactin resin (IBA) for Strep-tagged proteins or HiTrap FF resin (GE Healthcare) for His-tagged proteins, and purified using the manufacturer’s recommendations. His-tagged proteins were eluted using Histag binding buffer supplemented with 0.5 M imidazole and Strep-tagged proteins using StrepTag binding buffer supplemented with 2.5 M biotin. Fractions containing the eluted proteins were pooled and concentrated using Amicon Ultra 15-ml centrifugal filters with a cut-off of 10,000 nominal molecular weight limit (MilliporeSigma). Fractions were passed through a 0.2-μm filter and loaded onto a Superdex 75 26/60 column (GE Healthcare) equilibrated with 50 mM Tris, pH 7.4, 0.1 M NaCl, and 2 mM β-mercaptoethanol. Fractions containing CpxAC proteins were pooled and stored in aliquots at −80 °C.

**Formation of covalent dimers**

Covalently linked dimers were produced by mixing equimolar quantities of Spytag-CpxAC and of Spycatcher-CpxAC at a final concentration of 3 μM each. To determine the kinetics of formation of covalent dimers, aliquots of the reaction mixture were removed at the indicated times, and the linkage reaction was stopped by the addition of concentrated SDS loading buffer. To prepare covalent dimers for kinase assays, Spytag-CpxAC and Spycatcher-CpxAC were incubated at room temperature for 30 min, a point at which kinetics experiments indicated that dimer formation is complete. Trace amounts of nonspecific proteins potentially produced as proteolytic products of tagged proteins or noncovalent CpxA proteins remaining after covalent dimer formation due to a small excess of one of the protomers may be present in the samples, but they did not interfere with phosphorylation analyses because they migrate at positions distinct from the covalent dimers during Phos-tag SDS-PAGE.

**Size-exclusion chromatography**

Proteins (0.25 ml Spytag-CpxAC at 6 μM, 0.3 ml of Spycatcher-CpxAC at 8 μM, and a mixture of 0.21 ml of Spytag-CpxAC and 0.29 ml of Spycatcher-CpxAC incubated for 30 min at room temperature to form 5 μM covalent dimers of Spytag-Spycatcher-CpxAC) were chromatographed on a Superdex S200 10/30 GL column pre-equilibrated with 100 ml Tris-HCl, pH 7.5, 150 mM NaCl. The column was calibrated using the following standards: 0.3 ml of blue dextran at 5 mg/ml, 0.2 ml of BSA at 6.8 mg/ml, 0.3 ml of lysozyme at 5.9 mg/ml, and 0.3 ml of alcohol dehydrogenase at 1 mg/ml.
Preparation of regenerated ATP

Reactions for regeneration of ATP contained 10 mM ATP, 3 mM PEP, and 20 units/ml pyruvate kinase in 50 mM Tris-HCl, pH 7.4, 4 mM MgSO4, 7 mM KCl. After incubation for 1 h at room temperature, the pyruvate kinase was removed using a Microcon 10K centrifugal filter (MilliporeSigma). The concentration of the recovered regenerated ATP was determined using the measured absorbance at 260 nm and an extinction coefficient of 15.4 mM⁻¹ cm⁻¹. PEP remained in the regenerated ATP and was experimentally confirmed not to influence the kinase assay. For regeneration of ATP in kinase assays, 20 units/ml pyruvate kinase was added to the reaction mixture (PEP being present in the regenerated ATP).

Measurement of ADP contamination in ATP

An assay was conducted in which NADH oxidation is coupled to ADP consumption via pyruvate kinase and lactate dehydrogenase to measure ADP concentrations in solutions of ATP. Reactions contained 700 μM NADH, 3 mM PEP, 30 units/ml lactate dehydrogenase, and 20 units/ml pyruvate kinase in 50 mM Tris-HCl, pH 7.4, 4 mM MgSO4, 7 mM KCl, and 25 mM ATP (the sample being assayed for contamination). Reactions were initiated by the addition of pyruvate kinase and incubated for 15 min at room temperature. The decrease in NADH was monitored by absorbance at 340 nm using a Beckman DU 800 spectrophotometer. The difference in A340 at t = 0 and t = 15 min, a time substantially beyond completion of the reaction, was used to calculate the concentration of oxidized NADH, equivalent to the concentration of ADP in the sample, using an extinction coefficient of 6.22 mM⁻¹ cm⁻¹.

Autophosphorylation assays and kinetics

Autophosphorylation reactions contained 50 mM Tris-HCl, pH 8, 25 mM KCl, 10 mM MgCl2, 0.1 mM NaCl, and covalent dimers at a final concentration of 1 μM. Reactions were initiated by the addition of ATP and/or ADP. Autophosphorylation reactions were always performed using regenerated ATP (described above). Experiments were performed at room temperature; incubation times and ATP/ADP concentrations for individual experiments are specified in the figure legends. Aliquots were removed, and the reaction was quenched by the addition of concentrated SDS loading buffer at a 2:1 sample/loading buffer ratio. SDS Phos-tag gels were prepared as described previously with 8% acrylamide, 40 μM Phos-tag acrylamide (Wako Chemicals), and 160 μM MnCl2 (48). Electrophoresis was performed at room temperature at a constant voltage of 110 V, and proteins were visualized using Coomassie Blue. Intensities of bands were measured using ImageJ software (National Institutes of Health), and adjacent bands were deconvoluted using the peak analysis algorithm of Origin software.

Modeling

Considering the simplest bi-bi reaction scheme of phosphorylation (Fig. 1A), the reaction proceeds from unphosphorylated HK dimers (U) to monophosphorylated species (P) and eventually to diphosphorylated dimers (P₂). This scheme is similar to others described previously (23, 25). Because the hemiphosphorylated species contains two indistinguishable microscopic forms with phosphorylation occurring at each monomer, the macroscopic equilibrium constant Kₗₘ₉ equals twice the microscopic constant K (see details in supporting information). For the same reason, the macroscopic constant for the second phosphorylation reaction (Kₗ₉) equals half K if no cooperativity is present. A cooperativity parameter c is used to describe how phosphorylation of one site impacts phosphorylation of the second site.

The steady state can be described by the following five equations.

\[ \frac{P \times ADP}{U \times ATP} = K_{ma} = 2K \]  
\[ \frac{P_2 \times ADP}{P \times ATP} = K_{ma} = cK/2 \]  
\[ U + P + P_2 = HK_0 \]  
\[ ADP + ATP = ADP_0 + ATP_0 \]  
\[ ATP_0 - ATP = P + 2P_2 \]

HK₀, ATP₀, and ADP₀ are initial concentrations of HK dimers and nucleotides. Equations 1 and 2 describe the equilibrium, whereas Equations 3–5 represent mass conservation of HK proteins, nucleotides, and phosphoryl groups. Solutions for the equation system were determined using Matlab for a wide range of parameter values and initial concentrations. Total phosphorylation fractions of HK proteins were calculated as follows.

\[ TotalP = \frac{P + 2P_2}{2HK_0} = \frac{P}{2HK_0} + \frac{P_2}{HK_0} \]

Dividing Equation 2 by Equation 1 and multiplying by 4 gives the following.

\[ h = \frac{U \times P_2}{P \times P} \times 4 = c \]

A composite parameter h is defined with Equation 7. The value of the cooperativity parameter c is equal to h and can be calculated using concentrations of individual HK dimer species.

The reaction scheme used to derive Equation 7 is oversimplified, without considering the binding of ATP or ADP to HK proteins. A more complex model, detailed in the supporting information and illustrated in Fig. 6D and Fig. S3A, accounts for competitive nucleotide binding of ATP and ADP and phosphorylation equilibria. The difference between the two schemes can be illustrated using the following example of the phosphorylation equilibrium of the first site. For the simple scheme, Equation 1 can be rewritten as follows.

\[ \frac{P}{U} = 2K \times \frac{ATP}{ADP} \]
For the complex scheme, considering the nucleotide binding constants \( K_t \) (ATP to unphosphorylated HK) and \( K_d \) (ADP to HK–P),
\[
P = \frac{2K_{pt} \times \alpha}{\beta} \times \frac{F_1}{F_0} = 2K_{pt} \times \frac{K_t}{K_d} \times \frac{\text{ATP}}{\text{ADP}} \times \frac{F_1}{F_0}
\]
(Eq. 9)
in which \( \alpha = K_t \times [\text{ATP}] \) and \( \beta = K_d \times [\text{ADP}] \), and \( F_1 \) and \( F_0 \) are composite factors related to the cooperative and competitive binding of nucleotides (see details in supporting information), whereas \( K_{pt} \) is the equilibrium constant of phosphorylation. If there is no cooperativity in nucleotide binding, and nucleotides have equal affinity to HK and HK–P, then the value of \( F_1/F_0 \) equals 1, and Equation 8 can be rewritten as follows:
\[
P = 2K_{pt} \times \alpha \times \beta = 2K_{pt} \times K_t \times \frac{\text{ATP}}{\text{ADP}} \times \frac{F_1}{F_0}
\]
(Eq. 10)

This equation is essentially identical to Equation 8 derived from the simple bi-bi reaction scheme except that the nucleotide association constants are now considered, and the overall equilibrium constant \( K \) equals \( K_{pt} \times K_t/K_d \). If nucleotides have different affinities for HK and HK–P, or if there is cooperativity in nucleotide binding, the value of \( F_1/F_0 \) will be different from 1; thus, Equation 8 and the simple bi-bi reaction scheme are no longer applicable.

The value of \( h \), calculated from concentrations of individual phosphorylated species, can still be used to derive the phosphorylation cooperativity with the following equation:
\[
h = \frac{U \times P_2}{P \times P} \times 4 = c \times \frac{F_2 \times F_0}{F_1^2}
\]
(Eq. 11)
in which \( F_2, F_1, \) and \( F_0 \) are composite factors related to the cooperative and competitive binding of nucleotides to the di-, mono-, and unphosphorylated HK dimer species (see details in supporting information). When there is no cooperativity in nucleotide binding, the value of \( F_2 \times F_0/F_1^2 \) equals 1 and \( h \) equals the phosphorylation cooperativity constant \( c \). When ATP and ADP concentrations are both very high, saturating the binding sites, the value of \( h \) converges to \( c \). When one is high and the other is low, such as occurs in the common in vitro phosphorylation experiment in which ATP is high and ADP is low, the value of \( h \) may deviate from \( c \). If nucleotide binding is positively cooperative, the value of \( h \) is larger than \( c \); vice versa, \( h \) is smaller than \( c \) for a system with negative cooperativity in nucleotide binding. Thus, measuring the values of \( h \) at different concentrations of ATP and ADP can be used to determine the phosphorylation cooperativity constant \( c \).

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**References**

1. Gao, R., and Stock, A. M. (2009) Biological insights from structures of two-component proteins. *Annu. Rev. Microbiol.* 63, 133–154

2. Buschiazzo, A., and Trajtenberg, F. (2019) Two-Component sensing and regulation: How do histidine kinases talk with response regulators at the molecular level? *Annu. Rev. Microbiol.* 73, 507–528

3. Neiditch, M. B., Federle, M. J., Pompeani, A. I., Kelly, R. C., Swen, D. L., Jeffrey, P. D., Bassler, B. L., and Hughson, F. M. (2016) Autoinducer-2-induced asymmetry regulates LuxPQ quorum-sensing signal transduction. *Cell 126*, 1095–1108

4. Cheung, J., and Hendrickson, W. A. (2009) Structural analysis of ligand stimulation of the histidine kinase NarX. *Structure 17*, 190–201

5. Moore, J. O., and Hendrickson, W. A. (2012) An asymmetry-to-symmetry switch in signal transmission by the histidine kinase receptor for TMAO. *Structure 20*, 729–741

6. Gushchin, I., Melnikov, I., Polovinkin, V., Ishchenko, A., Yuzhakova, A., Buslaev, P., Bourenkov, G., Grudinin, S., Round, E., Balandin, T., Borshchevskiy, V., Willbold, D., Leonard, G., Büldt, G., Popov, A., and Gordeliy, V. (2017) Mechanism of transmembrane signaling by sensor histidine kinases. *Science 356*, eaah6345

7. Berntsson, O., Diensthuber, R. P., Panman, M. R., Björling, A., Gavstsson, E., Hoernke, M., Hughes, A. J., Henry, L., Nielson, S., Takala, H., Ihala, A., Newby, G., Kerruth, S., Heberle, J., Liebi, M., et al. (2017) Sequential conformational transitions and α-helical supercoiling regulate a sensor histidine kinase. *Nat. Commun. 8*, 284

8. Diensthuber, R. P., Bommer, M., Gleichmann, T., and Mößig, A. (2013) Full-length structure of a sensor histidine kinase pinpoints coaxial coiled coils as signal transducers and modulators. *Structure 21*, 1127–1136

9. Casino, P., Rubio, V., and Marina, A. (2010) The mechanism of signal transduction by two-component systems. *Curr. Opin. Struct. Biol.* 20, 763–771

10. Bhate, M. P., Molnar, K. S., Goulain, M., and DeGrado, W. F. (2015) Signal transduction in histidine kinases: insights from new structures. *Structure 23*, 981–994

11. Zschiedrich, C. P., Keidel, V., and Szurmant, H. (2016) Molecular mechanisms of two-component signal transduction. *J. Mol. Biol. 428*, 3752–3775

12. Mößig, A. (2019) Signal transduction in photoreceptor histidine kinases. *Protein Sci.* 28, 1923–1946

13. Dikjij, I., Edupuganti, U. R., Abzalimov, R. R., Borbat, P. P., Srivastava, M., Freed, J. H., and Gardner, K. H. (2019) Insights into histidine kinase activation mechanisms from the monomeric blue light sensor EL346. *Proc. Natl. Acad. Sci. U.S.A. 116*, 4963–4972

14. Wojnowska, M., Yan, J., Sivalingam, G. N., Cryar, A., Gor, J., Thalassinos, K., and Djordjevic, S. (2013) Autophosphorylation activity of a soluble hexameric histidine kinase correlates with the shift in protein conformational equilibrium. *Chem. Biol. 20*, 1411–1420

15. Willett, J. W., and Crosson, S. (2017) Atypical modes of bacterial histidine kinase signaling. *Mol. Microbiol. 103*, 197–202

16. Albanesi, D., Martin, M., Trajtenberg, F., Mansilla, M. C., Haouz, A., Alzari, P. M., de Mendoza, D., and Buschiazzo, A. (2009) Structural plasticity and catalysis regulation of a thermosensor histidine kinase. *Proc. Natl. Acad. Sci. U.S.A. 106*, 16185–16190

17. Mechaly, A. E., Sassoon, N., Beton, J. M., and Alzari, P. M. (2014) Segmental helical motions and dynamical asymmetry modulate histidine kinase autophosphorylation. *PLoS Biol.* 12, e1001776

18. Trajtenberg, F., Imelio, J. A., Marques, M. R., Larrieux, N., Marti, M. A., Ohal, G., Mechaly, A. E., and Buschiazzo, A. (2016) Regulation of signaling directionality revealed by 3D snapshots of a kinase-regulator complex in action. *Elife 5*, e21422

19. Casino, P., Miguel-Romero, L., and Marina, A. (2014) Visualizing autophosphorylation in histidine kinases. *Nat. Commun.* 5, 3258

20. Willett, J. W., Herrou, J., Brielig, A., Rotskoff, G., and Crosson, S. (2015) Structural asymmetry in a conserved signaling system that regulates division, replication, and virulence of an intracellular pathogen. *Proc. Natl. Acad. Sci. U.S.A. 112*, E3709–E3718
structure of a histidine kinase with signal transducer and sensor domains. *PLoS Biol.* **11**, e1001493 CrossRef Medline

22. Mechaly, A. E., Soto Díaz, S., Sassoon, N., Buschiazzo, A., Betton, J. M., and Alzari, P. M. (2017) Structural coupling between autokinase and phosphotransferase reactions in a bacterial histidine kinase. *Structure* **25**, 939–949 e3 CrossRef Medline

23. Jiang, P., Peliska, J. A., and Ninfa, A. J. (2000) Asymmetry in the autophosphorylation of the two-component regulatory system transmitter protein nitrogen regulator II of *Escherichia coli*. *Biochemistry* **39**, 5057–5065 CrossRef Medline

24. Trajtenberg, F., Graña, M., Ruétalo, N., Botti, H., and Buschiazzo, A. (2010) Structural and enzymatic insights into the ATP binding and autophosphorylation mechanism of a sensor histidine kinase. *J. Biol. Chem.* **285**, 24892–24903 CrossRef Medline

25. Dubey, B. N., Agustoni, E., Böhmm, R., Kaczmarczyk, A., Mangia, F., von Ara, C., Jenal, U., Hiller, S., Plaza-Menacho, I., and Schirmer, T. (2020) Hybrid histidine kinase activation by cyclic di-GMP-mediated domain liberation. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 1000–1008 CrossRef Medline

26. Tawa, P., and Stewart, R. C. (1994) Kinetics of CheA autophosphorylation and dephosphorylation reaction. *Biochemistry* **33**, 7917–7924 CrossRef Medline

27. Young, C., and Karbstein, K. (2012) Analysis of cofactor effects on RNA hybrid histidine kinase activation by cyclic di-GMP-mediated domain liberation. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 1000–1008 CrossRef Medline

28. Hazra, S., Henderson, J. N., Liles, K., Hilton, M. T., and Wachter, R. M. (2012) Kinetics of CheA autophosphorylation and dephosphorylation reaction. *Biochemistry* **33**, 7917–7924 CrossRef Medline

29. Keeble, A. H., Banerjee, A., Ferla, M. P., Reddington, S. C., Anuar INAK, and Howarth, M. (2017) Evolving accelerated amiation by SpyTag/SpyCatcher to analyze membrane dynamics. *Angew. Chem. Int. Ed. Engl.* **56**, 16521–16525 CrossRef Medline

30. Noriega, C. E., Schmidt, R., Gray, M. J., Chen, L. L., and Stewart, V. (2008) Autophosphorylation and dephosphorylation by soluble forms of the nitrate-responsive sensors NarX and NarQ from *Escherichia coli* K-12. *J. Bacteriol.* **190**, 3869–3876 CrossRef Medline

31. Ueno, T. B., Johnson, R. A., and Boon, E. M. (2015) Optimized assay for the quantification of histidine kinase autophosphorylation. *Biochem. Biophys. Res. Commun.* **465**, 331–337 CrossRef Medline

32. Yaginuma, H., Kawai, S., Tabata, K. V., Tomiyama, K., Kakizuka, A., Komatsu, T., Noji, H., and Imamura, H. (2014) Diversity in ATP concentrations in a single bacterial cell population revealed by quantitative single-cell imaging. *Sci. Rep.* **4**, 6522 CrossRef Medline

33. Tran, Q. H., and Unden, G. (1998) Changes in the proton potential and the cellular energetics of *Escherichia coli* during growth by aerobic and anaerobic respiration or by fermentation. *Eur. J. Biochem.* **251**, 538–543 CrossRef Medline

34. Wang, B., Zhao, A., Novick, R. P., and Muir, T. W. (2014) Activation and inhibition of the receptor histidine kinase AgrC occurs through opposite helical transduction motions. *Mol. Cell* **53**, 929–940 CrossRef Medline

35. Cai, Y., Su, M., Ahmad, A., Hu, X., Sang, J., Kong, L., Chen, X., Wang, C., Shuai, J., and Han, A. (2017) Conformational dynamics of the essential sensor histidine kinase WalK. *Acta Crystallogr. D Struct. Biol.* **73**, 793–803 CrossRef Medline

36. Grimshaw, C. E., Huang, S., Hanstein, C. G., Strauch, M. A., Burbuly, D., Wang, L., Hoch, J. A., and Whiteley, J. M. (1998) Synergistic kinetic interactions between components of the phosphorylase controlling sporulation in *Bacillus subtilis*. *Biochemistry* **37**, 1365–1375 CrossRef Medline

37. Wilke, K. E., Francis, S., and Carlson, E. E. (2015) Inactivation of multiple bacterial histidine kinases by targeting the ATP-binding domain. *ACS Chem. Biol.* **10**, 328–335 CrossRef Medline

38. Yeo, W. S., Zwi, I., Huang, H. V., Shin, D., Kato, A., and Groisman, E. A. (2012) Intrinsic negative feedback governs activation surge in two-component regulatory systems. *Mol. Cell* **45**, 409–421 CrossRef Medline

39. Plesniak, L., Horiuchi, Y., Sem, D., Meinenger, D., Stiles, L., Shaffer, J., Jennings, P. A., and Adams, J. A. (2002) Probing the nucleotide binding domain of the osmoregulator EnvZ using fluorescent nucleotide derivatives. *Biochemistry* **41**, 13876–13882 CrossRef Medline

40. Surette, M. G., Levit, M., Liu, Y., Lukat, G., Ninfa, E. G., Ninfa, A., and Stock, J. B. (1996) Dimerization is required for the activity of the protein histidine kinase CheA that mediates signal transduction in bacterial chemotaxis. *J. Biol. Chem.* **271**, 939–945 CrossRef Medline

41. Albanesi, D., Mansilla, M. C., and de Mendoza, D. (2004) The membrane fluidity sensor Desk of *Bacillus subtilis* controls the signal decay of its cognate response regulator. *J. Bacteriol.* **186**, 2655–2663 CrossRef Medline

42. Fisher, S. L., Kim, S.-K., Wanner, B. L., and Walsh, C. T. (1996) Kinetic comparison of the specificity of the vancomycin resistance kinase VanS for two response regulators, VanR and PhoB. *Biochemistry* **35**, 4732–4740 CrossRef Medline

43. Potter, C. A., Ward, A., Laguri, C., Williamson, M. P., Henderson, P. J., and Phillips-Jones, M. K. (2002) Expression, purification and characterisation of full-length histidine protein kinase RegB from *Rhodobacter sphaeroides*. *J. Mol. Biol.* **320**, 201–213 CrossRef Medline

44. Janiak-Spens, F., Cook, P. F., and West, A. H. (2005) Kinetic analysis of YPD1-dependent phosphotransfer reactions in the yeast osmoregulatory phosphorelay system. *Biochemistry* **44**, 377–386 CrossRef Medline

45. Cai, S. J., and Inouye, M. (2003) Spontaneous subunit exchange and biochemical evidence for trans-autophosphorylation in a dimer of *Escherichia coli* histidine kinase (EnvZ). *J. Mol. Biol.* **329**, 495–503 CrossRef Medline

46. Park, S. Y., Quezada, C. M., Bilwes, A. M., and Crane, B. R. (2004) Subunit exchange by CheA histidine kinases from the mesophile *Escherichia coli* and the thermophile *Thermotoga maritima*. *Biochemistry* **43**, 2228–2240 CrossRef Medline

47. Engler, C., Kandzia, R., and Marillonnet, S. (2008) Universally applicable methods for monitoring response regulator aspartate phosphorylation both in vitro and in vivo using Phos-tag-based reagents. *Anal. Biochem.* **376**, 73–82 CrossRef Medline