Hybrid histidine kinase activation by cyclic di-GMP–mediated domain liberation

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Cytosolic hybrid histidine kinases (HHKs) constitute major signaling nodes that control various biological processes, but their input signals and how these are processed are largely unknown. In Caulobacter crescentus, the HHK ShkA is essential for accurate timing of the G1-S cell cycle transition and is regulated by the corresponding increase in the level of the second messenger c-di-GMP. Here, we use a combination of X-ray crystallography, NMR spectroscopy, functional analyses, and kinetic modeling to reveal the regulatory mechanism of ShkA. In the absence of c-di-GMP, ShkA predominantly adopts a compact domain arrangement that is catalytically inactive. C-di-GMP binds to the dedicated pseudoreceiver domain Rec1, thereby activating the canonical Rec2 domain from its central position where it obstructs the large-scale motions required for catalysis. Thus, c-di-GMP cannot only stabilize domain interactions, but also engage in domain dissociation to allosterically invoke a downstream effect. Enzyme kinetics data are consistent with conformational selection of the ensemble of active domain constellations by the ligand and show that autophosphorylation is a reversible process.

Significance

Expression of bacterial genes in response to various cues is predominantly regulated by 2- or multicomponent systems with autophosphorylation of a histidine kinase (HK), the first component, being controlled by an N-terminal sensor domain. This is followed by phosphotransfer to the receiver domain (Rec) of a cognate transcription factor. In about 20% of all cases, HK core and Rec are fused to form a hybrid HK (HHK). Here, we show the first full-length structure of an HHK and reveal how it gets activated by the second-messenger c-di-GMP that binds to a dedicated pseudo-Rec domain. The mechanism is fundamentally distinct from the canonical mechanism of HK regulation, but may be operational in many HHKs with a predicted pseudo-Rec domain.

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The authors declare no competing interest.

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Data deposition: Coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB codes 6QRJ and 6QRL). Sequence-specific resonance assignments have been submitted to the Biological Magnetic Resonance Data Bank (accession no. 27882).

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Results

Full-Length ShkA Crystal Structure Reveals an Autoinhibited State of ShkA. ShkA is a HHK composed of a DHp-CA core domain, which catalyzes autophosphorylation of H23, and a Rec2 receiver domain carrying the phospho-acceptor D430 (Fig. 1A). A second messenger. Activation of ShkA initiates a multistep phosphorylation cascade (Fig. 1L), consisting of the phosphotransferase protein ShpA and the transcription factor TacA. Activation of TacA by phosphorylation initiates a G1/S-specific transcriptional program ultimately leading to cell morphogenesis and S-phase entry (12, 17). Direct targets of TacA include spmX and staR, encoding a critical morphogen and a transcriptional regulator responsible for stalk elongation, respectively. In contrast to CckA, which upon binding of c-di-GMP switches into phosphatase mode, the ligand is required for ShkA kinase activity, suggesting a distinct mechanism of allosteric control.

Here, we reveal the molecular mechanism of ShkA regulation, which is based on c-di-GMP-mediated mobilization of a locked, autoinhibited domain arrangement. We have determined crystal structures of full-length ShkA and of its isolated Rec1 domain in complex with c-di-GMP and have analyzed the dynamics of the full-length enzyme by NMR spectroscopy, employing isoleucine methyl group isotope labeling. Comprehensive enzymatic analyses revealed the underlying thermodynamics of the regulatory mechanism. Finally, we propose a general model for the structural transitions during the catalytic cycle of HHKs.

crescentus with the cell cycle HK CckA being controlled by c-di-GMP (15). Increasing levels of c-di-GMP during the G1 phase cause CckA to switch from default kinase to phosphatase mode upon S-phase entry, thereby licensing cells for replication initiation. Based on structural and functional analyses, c-di-GMP-mediated cross-linking of the DHp and CA domain was proposed as the molecular mechanism of the switch (16). Most recently, it was found that ShkA, the other major kinase involved in the regulation of the C. crescentus cell cycle progression and morphogenesis (17), is also controlled by c-di-GMP (12). ShkA is a soluble HK that is activated during the C. crescentus cell cycle by increasing levels of
by NMR. A 2D $^{15}$N,$^1$H-TROSY spectra of uniformly deuterated ShkA and ShkA/AMPPNP show only around 40 strong signals in the random-coil region of the spectrum (Fig. 2A and SI Appendix, Fig. S3A), indicating that they arise from residues located in locally flexible regions of the protein. The large majority of the 489 nonproline residues of ShkA are, however, not detected in this spectrum, due to the large molecular size of the rigid dimer and the associated slow molecular tumbling.

Binding of AMPPNP to ShkA does not lead to major changes in the spectrum. In contrast, binding of c-di-GMP to either apo ShkA or ShkA/AMPPNP leads to dramatic spectral changes with around 120 well-resolved additional resonances being clearly detected, indicating a substantial change in the dynamics of ShkA for a large part of the protein (Fig. 2A and SI Appendix, Fig. S3A). This massive increase in signal intensity is exemplified for selected signals with their 1D $^1$H-cross sections (Fig. 2A and SI Appendix, Fig. S3D). A spectral overlay of ShkA/c-di-GMP with the isolated Rec2 domain shows that the new resonances perfectly overlap (SI Appendix, Fig. S3A), including Rec2 residues that are in contact with either the CA or Dhp domain in the ShkA crystal structure (A421, G422, R446, A447, K448, A471, A472, G473). This indicates that upon binding of c-di-GMP to full-length ShkA, the Rec2 domain loses its contacts with the other domains and detaches completely from the otherwise rigid main architecture of the protein (SI Appendix, Fig. S3A). Since no significant chemical shift differences are detected between the 2 spectra, the detached Rec2 domain in ShkA has essentially the same 3D structure as the isolated Rec2 domain.

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**Fig. 2.** C-di-GMP binding to ShkA leads to release of the Rec2 domain. (A) Two-dimensional $^{15}$N,$^1$H-TROSY spectra of ShkA/AMPPNP (blue spectrum) and ShkA/AMPPNP/c-di-GMP (red spectrum). The insets show 1D $^1$H-cross sections for representative amide protons. (B) Overlay of 2D $^{13}$C,$^1$H-HMQC spectra of the isolated $^{13}$C/1H-IleRec1 domain in apo state (blue) and c-di-GMP-bound state (red), and the isolated $^{13}$C/1H-IleRec2 domain (purple). (C) Spectral overlay of 2D $^{13}$C,$^1$H-HMQC spectra of $^{13}$C/1H-IleShkA in apo state (blue) and c-di-GMP-bound state (red). The 2 insets show 1D $^1$H-cross sections for representative methyl protons. Sequence-specific resonance assignments of the $\delta_1$ methyl groups are either indicated or assigned to the Rec2 or CA domain. (D) Crystal structure of ShkA/AMPPNP. Surface representation of the monomer composed of 4 domains: Dhp (dark gray), CA with bound AMPPNP (gray), Rec1 (light gray), and Rec2 (purple). The $\delta_1$ methyl groups of isoleucine residues in the Rec1 and Rec2 domains are shown as spheres with $\delta_1$ methyl groups of isoleucine residues in the Rec1 and Rec2 domains that are part of the Rec1-Rec2 linker. (E) Chemical shift perturbation of isoleucine $\delta_1$ methyl groups upon addition of AMPPNP to ShkA (Upper) and upon addition of c-di-GMP to ShkA/AMPPNP (Lower). (F) Transverse relaxation times of isoleucine $\delta_1$ methyl carbons of ShkA/AMPPNP (blue bars) and ShkA/ AMPPNP/c-di-GMP (red bars). The Lower panel shows the relative change of $T_2$($^{13}$C) upon addition of c-di-GMP ($\Delta T_2$). (E and F) Asterisks indicate methyl groups that are either not assigned or line-broadened beyond detection in at least 1 of the 2 states.
In order to obtain site-specific information of the effect of c-di-GMP on the structure and dynamics of ShkA, we increased the experimental sensitivity by employing methyl-NMR spectroscopy of specifically 61-[13C/1H]-isoleucine–labeled full-length ShkA and isolated Rec1 and Rec2 domains (Fig. 2 B–D and SI Appendix, Fig. S3B). Sequence-specific resonance assignments for Rec1 were obtained by identifying unambiguous nuclear Overhauser effects in agreement with the crystal structure. Additional assignments for isoleucine methyl groups 61-1340, 61-1390, and 61-1405 were obtained using selective mutagenesis of full-length ShkA (SI Appendix, Fig. S3C). The mutation I259V located within the Rec1 domain caused major spectral changes, indicating the importance of residue I259 in stabilizing the Rec1–CA domain interface (SI Appendix, Fig. S1B).

AMPPNP binding to the CA domain of full-length ShkA leads to only minimal chemical shift perturbation of the 61-Ile methyl groups of the entire protein, implying that the structures of ligand-free and AMPPNP-bound ShkA are very similar (Fig. 2E and SI Appendix, Fig. S3B). In contrast, binding of c-di-GMP to either apo ShkA or to ShkA/AMPPNP causes substantial spectral changes. All 61-Ile methyl group signals of the Rec2 domain strongly increase in intensity (SI Appendix, Fig. S3C), in full agreement with the spectral behavior of the backbone amides. Similar chemical shift changes were observed for c-di-GMP binding either to the isolated ShkA-Rec1 domain or to full-length ShkA, showing that c-di-GMP binds to the β5-α5 surface of prototypical Rec domains (Fig. 2 C and E and SI Appendix, Fig. S3B).

Overall, these data indicate that c-di-GMP binding to ShkA leads to liberation of the Rec2 domain from the protein core. To validate this hypothesis, we conducted NMR spin relaxation experiments in the presence and absence of c-di-GMP (Fig. 2F and SI Appendix, Fig. S3F). For the isolated receiver domains, average 13C transverse relaxation times for ILV methyl groups of Rec2 and Rec1 are quite similar (174 ms and 160 ms, respectively), in agreement with the similar molecular mass of both domains (16.3 kDa and 12.5 kDa, respectively). For full-length ShkA/AMPPNP in the absence of c-di-GMP, the transverse relaxation times of all 61-Ile resonances is smaller (25 to 75 ms), as expected from the respective protein sizes (Fig. 2F). Addition of c-di-GMP strongly increases the 13C T2 times of Rec2 such that 13C T2 times are effectively doubled, but does not significantly affect those of 61-Ile of Rec1 (Fig. 2F and SI Appendix, Fig. S3F). These experiments thus quantitatively confirm that c-di-GMP binding to ShkA leads to the specific detachment of the Rec2 domain from the protein core into a dynamic, multiconformational state, which enables the enzyme to undergo the large motions required for the phosphor transfer reactions.

C-di-GMP Competes with Rec1-Rec2 Linker for Binding to Rec1 Domain. To reveal the molecular mechanism of c-di-GMP-induced Rec2 domain mobilization, we set out to determine the structure of the ShkA/c-di-GMP complex. Well-diffracting crystals of the complex were obtained using selective mutagenesis of full-length ShkA (SI Appendix, Fig. S4). The asymmetric unit contains 2 monomers, each with a bound monomeric c-di-GMP ligand, which are virtually identical and superimpose closely with the corresponding domain in full-length ShkA (SI Appendix, Fig. S4B). In addition, an intercalated c-di-GMP dimer is observed that mediates a 2-fold symmetry contact between the 2 Rec1 monomers (SI Appendix, Fig. S4C) that is most likely a crystal artifact. Indeed, the iso-thermal titration calorimetry (ITC) data of full-length ShkA are consistent with a 1:1 stoichiometry (SI Appendix, Fig. S5 A–E).

Monomeric c-di-GMP is bound to the α6-β5-α5 face of Rec1 (Fig. 3 A and B), that is, to the surface that is involved in the dimerization of canonical Rec domains. The Watson–Crick edges of both guanine bases form H-bonds with the backbone of the exposed edge of the β5 edge and the β5-α5 loop. In addition, Y338 and R344 are forming stacking and caten–π interactions with the guanyl bases, respectively. S347 is H-bonded to a guanyl edge of both guanines, forming a π-stacking and cation–π interaction with the guanines respectively. S347 is H-bonded to a guanyl face of both guanines.

In full-length ShkA, the c-di-GMP binding site is occupied by the Rec1-Rec2 linker (Fig. 3C and D). Specifically, residues shown to be engaged in c-di-GMP binding (Y338, R324, and R344) are interacting with a conserved DDR motif of the linker region. Thus, we reasoned that if the DDR segment were to compete with c-di-GMP binding, its deletion should increase c-di-GMP affinity. Indeed, affinity measurements by ITC and by a fluorescence competition assay (FCA) showed that the DHp-CA-Rec1 construct (residues 1 to 366) had a lower dissociation constant compared to the other variants, which can be attributed to the absence of the competing DDR segment. Full data are given in SI Appendix, Fig. S5 (E) Sequence logo encompassing the c-di-GMP binding site of the ShkARec1 domain.
dynamic equilibrium between a closed, autoinhibited state \( (E_c) \) and an ensemble of open, inhibition-relieved states \( (E_o) \) that are characterized by a liberated Rec2 domain. In the absence of the ligand, this equilibrium would be populated largely on the \( E_c \) side, in line with the NMR results and the constitutive inactivity of the enzyme \( (12) \). C-di-GMP–mediated ShkA activation would then proceed by conformational selection \( (20) \) of the \( E_o \) states with their unobstructed Rec1 binding site, shifting the equilibrium in a dose-dependent fashion to these active states.

The regulatory model was tested in vivo by analyzing the transcriptional activity of the ShkA-ShpA-TacA pathway with a β-Gal assay \( (9) \). In this assay, plasmid-borne variants of \( shkA \) were expressed in a \( C. \) crescentus \( shkA \) deletion background that harbors a plasmid-borne translational fusion between the TacA target gene \( spmX \) and \( lacZ \) \( (spmX′-lacZ) \). This allowed following the activity of the ShkA-ShpA-TacA pathway directly by reading out the activity of β-Gal, encoded by \( lacZ \). The assay was performed in the wild-type and in a c-di-GMP minus strain \( (\text{rcdG}^0 \text{ strain} \ 21) \). While in absence of c-di-GMP \( (\text{rcdG}^0 \text{ strain}) \) wild-type ShkA showed no activity, introducing D369N or placing an insertion between the DDR motif and Rec2 (but not between Rec1 and DDR) rendered ShkA active \( (\text{Fig. } 4C) \). Thus, the results fully corroborate the regulatory model, as illustrated in the scheme \( (\text{Fig. } 4D) \). Additional support for the model is provided by analytical ultracentrifugation sedimentation velocity (SV-AUC) experiments \( (\text{SI Appendix, Fig. } 5G) \) that show a decrease in the sedimentation coefficient upon ligand addition, indicative of a more open structure. In the following, the LCCE model \( (\text{Fig. } 4A, \text{Inset}) \) is scrutinized and its thermodynamic parameters are determined by quantitative functional investigations.

ShkA Autophosphorylation Is c-Di-GMP–Dependent, Reversible, and Noncooperative. To determine the reaction kinetics of the full-length ShkA and mutants thereof, we acquired progress curves of enzyme net phosphorylation \( (E^+) \) by autoradiography and of ATP to ADP turnover by on-line ion exchange chromatography (oIEC) \( (\text{SI Appendix, Methods}) \) quantification. First, we determined the kinetics of autophosphorylation separately using phosphotransfer-deficient variants that had the phospho-acceptor D430 mutated (ShkAD/A) or the Rec2 domain deleted (construct \( \text{Dhp-CA-Rec1} \) or, in short, \( \text{ShkA}_{\text{Rec2}} \)). As found previously \( (12) \) and consistent with the proposed regulatory model, ShkAD/A is constitutively active \( (\text{Fig. } 5A \text{ and } B) \), since it lacks the obstructing Rec2 domain. ShkA\(_{\text{Rec2}}\) attains very quickly (in less than 15 s) a stable phosphorylation state with or without the ligand; that is, it proceeds with an autophosphorylation rate constant \( k_1 = 1/(15 \text{ s}) = 0.07 \text{ s}^{-1} \). For ShkA\(_{\text{rec2}}\), the same fast kinetics is observed, but only in presence of c-di-GMP \( (\text{Fig. } 5C \text{ and } D \text{ and } \text{SI Appendix, Fig. } 5T/A) \), which demonstrates that under this condition the mutant is fully active. In the absence of the ligand, the turnover number is strongly reduced \( (k_{\text{cat}}/c_{\text{di-GMP}} = 0.012 \text{ s}^{-1}) \) but, within error limits, phosphorylation is profound to almost the same degree as for the activated enzyme. The observations are consistent with the regulatory LCCE model \( (\text{Fig. } 4) \) under the assumption that the samples had reached equilibrium after the about 15 min before start of the enzymatic reaction. No attempts were made to determine the kinetics of the conformational equilibrium or of c-di-GMP binding, but the ITC spikes induced upon ligand binding didn’t exhibit unusual broadening \( (\text{width} < 300 \text{ s}) \) \( (\text{SI Appendix, Fig. } 5O/K/D) \). Since the initial reaction velocity is at least 20 times larger in the presence of c-di-GMP \( (\text{Fig. } 5D) \), a lower boundary of 20 can be estimated for the conformational equilibrium constant \( K = [E_0]/[E_c] \).

To obtain more quantitative information about the autophosphorylation reaction, we measured ATP to ADP turnover by oIEC. \( \text{Fig. } 5B \text{ and } D \) show the ADP progress curves \( (\text{Fig. } 5B \text{ and } D, \text{filled symbols}) \) of the phosphotransfer-deficient ShkA mutants that turn out to be congruent with the appropriately scaled phosphorylation curves \( (\text{Fig. } 5B \text{ and } D, \text{open symbols}) \). This indicates stable histidine phosphorylation at the employed condition as verified by long-term measurements \( (\text{SI Appendix, Fig. } 5S/D) \). Intriguingly, for both mutants, the progress curves \( (\text{Fig. } 5B \text{ and } D) \) indicate that the phosphotransfer-deficient mutants do not proceed to full quantitative modification \( (\text{i.e., did not reach the level corresponding to the enzyme concentration, } 10 \mu M) \). We reasoned that this may be due to reversibility of the autophosphorylation reaction, and that only a high ATP substrate concentration would shift the equilibrium completely to the product state. Indeed, upon ATP titration, the equilibrium ADP concentration was increased and reached the expected value close to the enzyme concentration indicating complete phosphorylation \( (\text{Fig. } 5E) \). A simple reversible bi-bi reaction model \( (\text{SI Appendix, Methods}) \) reproduced the data well and yielded an equilibrium constant \( K_1 = k_1/k_{-1} \) of 0.13 for activated ShkA\(_{\text{D/A}}\) Enzyme titration \( (\text{SI Appendix, Fig. } 5B/B) \) confirmed the result.
C-di-GMP Shifts Conformational ShkA Equilibrium to the Active State.

Full-length ShkA is effectively an ATPase, converting ATP to ADP via phospho-enzyme intermediates. Progress curves of ATP turnover acquired under various conditions by oIEC (Fig. 6A and SI Appendix, Fig. S9C) were fitted with a competitive product inhibition Michaelis–Menten model (SI Appendix, Fig. S9A) to derive the enzymatic parameters. The $K_m$ and $K_i$ values are both about 60 μM, a value that agrees well with dissociation constants of AMPPNP and ADP obtained by FCA, while the ITC measurements agreed less, probably due to the intrinsic limitations of this method for low-affinity binders (SI Appendix, Fig. S10 A–D). Interestingly, the FCA measurements clearly showed that c-di-GMP has no significant effect on mononucleotide affinity (SI Appendix, Fig. S10 E–H). In the presence of c-di-GMP, ShkA is rather active ($k_{cat} = 0.20 \text{ s}^{-1}$) (Fig. 6A), considering that the reaction involves 3 individual steps (autophosphorylation, phosphotransfer, dephosphorylation) and requires several domain rearrangements (Discussion). Importantly, low but significant activity ($k_{cat} = 0.0034 \text{ s}^{-1}$) (Fig. 6A) is observed also in absence of the activator, which corroborates the LCCE model and allows an estimation of the conformational equilibrium constant to $K = 58$. Assuming the same $K$, the microscopic autophosphorylation rate of activated ShkA$_{ΔΔ}$ can be calculated to $k_1 = k_{1,\text{macro}} \times K = 0.7 \text{ s}^{-1}$. Regarding wild-type ShkA phosphorylation, we confirmed that it gets boosted by c-di-GMP, but the phosphorylation level is clearly lower than that of the phosphotransfer-deficient ShkA$_{ΔΔ}$ mutant (SI Appendix, Fig. S7B), which can be attributed to the dephosphorylation activity of the former.

To quantitatively describe the activating effect of c-di-GMP, progress curves were acquired at various c-di-GMP concentrations (SI Appendix, Fig. S9B) and fitted with the Michaelis–Menten model. The resulting $k_{cat,\text{macro}}$ values yielded a sigmoidal activation profile with $K_{act} = 0.25 \mu \text{M}$, as shown in Fig. 6B. This agrees reasonably well with the (apparent) dissociation constant of c-di-GMP as determined by ITC (1.5 μM in the presence of AMPPNP) (SI Appendix, Fig. S5B). The microscopic dissociation constant in the absence of the competing DDR motif would then be considerably smaller, namely $K_d = K_{act}(1 + K) = 4 \text{ nM}$ (SI Appendix, Methods). This value is smaller than that observed.
for the DHp-CA-Rec1 construct by FCA and ITC (80 nM and 250 nM, respectively) (Fig. 6A) and dephosphorylation. The data fully support the regulatory LCCE model and thus confirm that the c-di-GMP activation profile is governed not only by the affinity of the ligand to its binding site, but also by the free-energy difference between the conformational states.

Discussion

The structural, dynamic, and functional results presented in this study consistently show that activation of ShkA proceeds via the c-di-GMP–mediated liberation of a locked, autoinhibited state (Fig. 4). Thereby, c-di-GMP competes with the tethering of a domain linker (Fig. 3A and D) to unleash the C-terminal domain allowing the enzyme to step through the catalytic cycle that involves large domain motions (Fig. 7). C-di-GMP interference with protein–protein interactions as a regulatory mechanism was also proposed for a YajQ–like transcription factor/coactivator complex (22), but structural information is missing.

In ShkA, c-di-GMP binds to the edge strand (β5) of the Rec1 β-sheet and the following β5–a5 loop. The Watson–Crick edges of both guanines form H-bonds with the main chain (Fig. 3B), reminiscent of the backbone interactions in a β-sheet. The same kind of interaction occurs in the CckA/c-di-GMP complex, where 1 of the guanines binds to the β-sheet edge of the CA domain (16). In the accompanying study (12), Y338 was identified as a c-di-GMP binding residue in a targeted alanine scan. In addition to Y338A, several other mutants (R324A, I340A, S347A, and Q351A) showed severely impaired activity in vivo (12). Since all these residues contribute to c-di-GMP binding (Fig. 3B), this can be attributed to an impaired activation by the cellular c-di-GMP pool and suggests that the affinity of wild-type ShkA to c-di-GMP is finely tuned to its rise in concentration during cell cycle progression (21).

A mutation of R344, the remaining c-di-GMP binding residue, showed a wild-type–like phenotype. This may be explained by the dual role of R344 being involved in c-di-GMP binding as well as Rec1-Rec2 linker tethering (Fig. 3A and D), resulting in opposing effects on activation upon mutation. Although R324 also has such a dual role, its strong phenotype suggests that this residue contributes more to c-di-GMP binding than to linker tethering. These observations demonstrate that identification of ligand binding residues is not straightforward if they are also involved in opposing activities like protein–protein interactions. However, mutation of the DDR motif or inserting a loop between the motif and the Rec2 domain clearly resulted in constitutively active enzyme and, thus, confirmed the importance of Rec2 tethering for autoinhibition (Fig. 4C).

The NMR studies on full-length ShkA using specific isotope labeling of the isoleucine methyl groups provided atomic scale information of structural and dynamic changes upon ligand binding (Fig. 2). Binding of c-di-GMP, but not AMPPNP, changed the dynamics of Rec2 substantially and exclusively, leading to the conclusion that the mobility of the Rec2 domain is restricted in the absence of c-di-GMP but is drastically enhanced upon addition of the ligand such that its relaxation times become comparable to those of an individual protein of this size. The binding of c-di-GMP to Rec1 therefore releases Rec2 allosterically.

In order to relate the mechanistic “activation by domain liberation” model of ShkA with the function of this hybrid kinase, comprehensive enzymatic data were acquired. The specially developed oIEC method proved highly valuable as it enabled the efficient recording of quantitative enzyme progress curves. We found, using phosphotransfer-deficient mutants, that ShkA auto phosphorylation is clearly reversible. Under various titration regimes, the equilibrium concentrations were consistent with a simple reversible bi–bi reaction with the equilibrium largely on the side of the reactants (Fig. 5E and SI Appendix, Fig. S8B). Thus, the degree of phosphorylation depends on the mononucleotide concentrations, and a high ADP concentration promotes enzymedephosphorylation by back transfer of the phosphoryl group onto ADP. Reversibility of the HK autophosphorylation reaction was originally demonstrated for CheA (23) (see also ref. 24 and references therein) and KinA (25), and later for NRII (26). Although largely neglected in the literature, this is probably a general feature of ATP-driven histidine phosphorylation. It should be tested whether it can explain the ADP dependence of the kinase reaction as observed in vitro for HK853 (27) and other HKs (28). The rate constant of autophosphorylation is with $k_1 = 0.7 \pm 0.1$ s$^{-1}$ at least 30 times faster than measured for other HKs (16, 29). Possibly, some of these enzymes were partly impaired by truncation or not fully activated. ATP turnover of full-length ShkA proceeds somewhat slower, which demonstrates that phosphotransfer and P~Asp hydrolysis are not much slower. To answer whether the latter reaction is entirely due to the intrinsic lability of the phosphoaspartate or catalyzed by docking of the Rec2 domain to the DHp bundle (4, 19) requires further investigations. Although dephosphorylation of ShkA has not been investigated in vivo and is not part of the current model of cell cycle control (30), it may be controlled by yet unknown factors that, for example, affect the DHp bundle geometry as in DesK (31). Additionally, the
counteracting dephosphorylation activity may be relevant for concentration robust signaling (32).

Evidence has accumulated that HKs form asymmetric dimers (9) that can catalyze in concert both autophosphorylation and phosphotransfer and a structural model has been put forward for CpxA (33). The corresponding model for activated (i.e., c-di-GMP–complexed) ShkA is shown in Fig. 7A, Upper, where domains have been placed on the basis of homologous structures with competent autophosphorylation or phosphotransfer domain arrangement. Canonical autophosphorylation and phosphotransfer mechanisms can be anticipated, since the functional interfaces show no severe clashes or polarity mismatch. Both CA and Rec2 of subunit A (CA<sub>A</sub>, Rec2<sub>A</sub>) (Fig. 7A, bright colors) would be engaged in concerted catalysis resulting in a net transfer of a phosphoryl group from CA<sub>A</sub> bound ATP to the active aspartate of Rec2<sub>A</sub> (Fig. 7A, red arrows), while the corresponding domain of subunit B (Fig. 7A, tinted colors) would be in stand-by mode. Subsequently, both domains would dislodge (Fig. 7A, black arrows), with CA<sub>A</sub> moving to a near-by parking position and Rec2<sub>A</sub> becoming available for downstream phosphoryl transfer (Fig. 7A, Lower). Concurrently, the catalytic domains of subunit B would move to catalytically competent positions, resulting in the same structure as at the beginning, although 2-fold related and with the roles of the subunits interchanged (state 2). After concerted catalysis, equivalent domain rearrangements would bring back the enzyme to state 1.

In line with the left-handed connectivity of the 2 DHp helices, the model predicts that autophosphorylation occurs in trans (34). Due to the almost equal distance between either of the C-termini of the Rec1 domains and the N terminus of the competently docked Rec2 domain (about 60 Å), phosphotransfer may occur in trans or in cis as modeled. In any case, the Rec1-Rec2 linker of ShkA is long enough (27 residues) to allow simultaneous execution of autophosphorylation and phosphotransfer. Notably, also for canonical HHKs (without an intervening domain like Rec1), this appears possible, since their CA–Rec linkers appear long enough (23 ± 3 residues for the 23 HHKs in C. crescentus) to bridge the distance (again 60 Å) (dashed line in Fig. 7A).

![Diagram](https://example.com/diagram.png)

Fig. 7. Proposed domain arrangements during the catalytic cycle of ShkA. (A) During the catalytic cycle, the enzyme adopts 2 asymmetric dimer states that are structurally identical, but with domain roles interchanged. Subunits are distinguished by color; “A” : strong, “B” : weak, the active His and Asp and ligands are shown in full. State 1 (Upper): CA/ATP and Rec2 of chain A are poised for trans-autophosphorylation and cis-phosphotransfer, respectively. After concerted execution of the reactions (red arrows), CA/ADP and P<sup>~</sup>Rec2 dislodge and CA/ATP, Rec2 of chain B engage with the DHp histidines (black arrows) to yield state 2 (Lower). The competent DHp/CA and DHp/Rec2 domain arrangements have been modeled based on the structures of CpxA [4BIW (18)] and ChpT/CtrA(Rec) [4QPJ (37)], respectively. In canonical HHK, CA would be linked directly to Rec2 (dashed line). (B) Part of DHp α1 helix structures of the HKs of B after superposition of C-terminal parts. The structures with reported autophosphorylation competent domain arrangement (CpxA, 4BIW; WalK, 5C93; EnvZ, 4KP4) and those in phosphotransfer or dephosphorylation arrangement (HK853, 3DGE; ChpT, 4QPJ) and ShkA form structural clusters distinguished by the degree of helix kinking. (Right) The kinase competent structures have the N-terminal part of α1 bent toward the center of the DHp bundle (ShkA in gray).
Most likely the asymmetry is rooted in the inward kinking of the N-terminal part of DHp α helix upon interaction with the ATP complexed CA domain (Fig. 7C), which would be sterically incompatible with an analogous motion of αi from the other subunit. This has been discussed and analyzed thoroughly by Bhate et al. (9) and corroborated by the recent structure and modeled dynamics of WalK (35). The DHp domain of ShkA belongs to the major HisKA pfam family (36) that exhibits a HxxXP motif (Fig. 7B). Superposition of the C-terminal part of DHp α structures of this family (Fig. 7C) demonstrates kinking of αi with a pivot close to the proline and shows that the N-terminal parts of αi helices of autophosphorylation competent structures (CpxA, EnvZ, WalK) are tilted toward the helix bundle axis. A symmetric structure with both αi helices tilted inwards is sterically probably not feasible, which would thus be the basis for the asymmetry of the functional dimer.

The proposed asymmetric catalysis is not equivalent with half-of-sites modification and is, therefore, not at odds with our finding of complete modification at high ATP concentration (Fig. 5E). Disengagement of the CA domain after the first phosphorylation event could easily allow the other CA domain to approach the second histidine for modification (compare states 1 and 2 in Fig. 7A). Intriguingly, however, CpxA shows a hemiphosphorylated catalytic structure in the presence of ATP (33), although the non-phosphorylated histidine seems perfectly poised to react with CA-bound ATP in the observed canonical kinase competent arrangement. Based on our thermodynamic results on ShkA, we predict that CpxA behaves similarly, with the equilibrium of the reversible autophosphorylation reaction being on the side of the reactants (ATP, His). This would explain why the substrate and not the product complex was observed, since the products (ADP, P–His) would react back and ADP would stay in the active site assuming strong crystal lattice constraints.

Our analyses provide mechanistic and kinetic insight into how c-di-GMP competes with a protein–protein interaction to activate an HHK, and how ADP affects the autophosphorylation reaction. For a full mechanistic understanding of HHKs, more investigations are required to decipher the kinetics of the phosphotransfer and autodephosphorylation steps and to unravel the physiological advantage of combining HK core and cognate receiver domain on 1 polypeptide.

Materials and Methods

A detailed description of the materials and methods used in this study is provided in SI Appendix, including the following: Cloning, expression and protein purification, crystallization, data collection and X-ray structure determination, NMR experiments, ITC and FBC assay, SV-AUC, β-Gal translocation assay, radiometric phosphorylation assay, and nucleotide quantification by oIEC. Also given are the thermodynamic equations for reversible autophosphorylation and the LCCE model used for data fitting.

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