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Biological Insights from Structures of Two-Component Proteins

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Abstract

Two-component signal transduction based on phosphotransfer from a histidine protein kinase to a response regulator protein is a prevalent strategy for coupling environmental stimuli to adaptive responses in bacteria. In both histidine kinases and response regulators, modular domains with conserved structures and biochemical activities adopt different conformational states in the presence of stimuli or upon phosphorylation, enabling a diverse array of regulatory mechanisms based on inhibitory and/or activating protein-protein interactions imparted by different domain arrangements. This review summarizes some of the recent structural work that has provided insight to the functioning of bacterial histidine kinases and response regulators. Particular emphasis is placed on identifying features that are expected to be conserved among different two-component proteins from those that are expected to differ, with the goal of defining the extent to which knowledge of previously characterized two-component proteins can be applied to newly discovered systems.

Keywords

histidine kinase; response regulator; signal transduction; phosphotransfer; phosphorylation

INTRODUCTION

Bacteria commonly use two-component signal transduction pathways to couple environmental stimuli to adaptive responses (for reviews see 45, 85). Most species contain more than a dozen two-component systems (TCSs) that regulate a wide variety of behaviors including fundamental processes such as metabolism and motility in addition to specialized processes such as virulence and development. TCSs are the most abundant multi-step signaling pathways in nature and have been extensively studied since their discovery ~20 years ago. Apart from their inherent interest as a fundamental signaling strategy, it is hoped that understanding these systems might be useful for development of antibiotics that disrupt essential systems in pathogenic organisms or might be exploited for the beneficial use of bacteria to enhance agriculture or environmental remediation.

The core of TCS pathways is a phosphotransfer reaction between two conserved components, a histidine kinase (HK) and a response regulator (RR) (Figure 1*a*). The HK is typically the “input” component of the pathway, designed to sense stimuli and correspondingly regulate the signaling pathway. The RR is typically the “output” of the system, regulated by the HK and effecting the cellular response. The HK autophosphorylates at a His residue, creating a high energy phosphoryl group that is transferred to an Asp residue of the RR, inducing a conformational change that activates the RR to elicit the response. The autophosphorylation and/or RR phosphatase activities of the HK control the level of RR phosphorylation, and hence the output response. Auxiliary proteins that regulate

the activities of the HK or that influence the stability of RR phosphorylation can augment this basic scheme, and many "two-component" pathways consist of more than two proteins. Additionally, the phosphotransfer pathway can be expanded into phosphorelay pathways, with two or more phosphotransfers between multiple His- and Asp-containing proteins (Figure 1*b*). Greater numbers of proteins within a pathway provide more loci for complex regulation.

Biochemical activities and three-dimensional structures are known for representatives of all conserved core domains of HKs and RRs, as well as for several variable input domains of HKs and for most of the subfamilies of output domains of RRs. However, TCS proteins are modular and extremely versatile, likely reflecting their widespread abundance in bacterial signaling. For every general feature of HKs and RRs, even the nature of the conserved His and Asp residues that constitute the definitive His-Asp phosphotransfer reaction, exceptions have been found, and the list of variations will undoubtedly increase as more systems are characterized.

With ~50,000 TCS proteins identified from genome sequences and >200 structures of two-component proteins determined, it is unrealistic either to identify and describe a single representative system or to provide a comprehensive catalog of structure/function relationships in HKs and RRs within the scope of this review. Instead, we will focus on identifying the similarities and differences that are expected to be observed among two-component proteins and a basic theme will emerge. For the most part, structural and functional features intrinsic to individual domains of HKs and RRs are conserved. In contrast, the ways in which domains interact with each other and the regulatory mechanisms resulting from these domain arrangements often differ. The length of this review precludes comprehensive coverage of the field and citation of the voluminous literature. Emphasis will be placed on recent advances rather than on historical studies that laid the foundation to the field and have been reviewed previously.

HISTIDINE KINASES

Domain Architecture

Similar to most signaling proteins, the HK superfamily has a modular architecture with diverse input domains linked to a conserved catalytic core. Such design allows the coupling of a wide variety of input signals to proper output responses through a conserved autophosphorylation and phosphotransfer pathway. In prototypical HKs, the cytoplasmic kinase core consists of two distinct domains: a well-conserved C-terminal catalytic and ATP-binding (CA) domain, also known as HATPase_c in the Pfam database (33); and a less-conserved dimerization and histidine phosphotransfer (DHp) domain, referred to as the His Kinase A domain clan in Pfam (Figure 2). The DHp domain contains the conserved His residue for phosphorylation and the CA domain carries the catalytic activity of transferring a phosphoryl group from ATP to the His residue. A different organization of the kinase core domains is observed in approximately 5% of ~10,000 HK sequences in the SMART database (54) in which the phosphorylation site is located on a distant histidine phosphotransfer (HPt) domain and a separate domain is responsible for dimerization. Almost all these HKs are predicted to function in chemotaxis-like systems and are closely related to the well-studied cytoplasmic HK CheA, which is extensively reviewed elsewhere (6, 12). Aside from the basic His-Asp scheme, nearly 25% of HKs incorporate a receiver (REC) domain of RRs to form hybrid kinases. These proteins are believed to function in concert with individual or linked HPt domains to employ a more elaborate and sophisticated scheme of His-Asp-His-Asp phosphorelay.

Kinase/Phosphatase Activities

The distinguishing feature of HKs is the formation of phosphoramidates instead of phosphoesters seen in Ser/Thr/Tyr protein kinases. The high energy N-P bond between the phosphoryl group and the imidazole ring of His is relatively unstable, making phosphoHis residues more suitable as phosphotransfer intermediates rather than as stoichiometrically phosphorylated sites for protein recognition. Not surprisingly, in addition to HK phosphorylation, phosphoHis has also been discovered in intermediates of enzymes such as succinyl-CoA-synthetase and nucleoside diphosphate kinase. Despite the absence of HKs in animals, mammalian kinases with His phosphorylation have been identified and the functional roles of the His phosphorylation are actively pursued (9, 83).

The chemical instability of phosphoHis hinders the detection of HK phosphorylation by conventional phosphoamino acid analysis at acidic pH because phosphoHis is labile to acid, though resistant to base treatment. The kinase activities of HKs are commonly assayed in vitro by purifying relevant domains and incubating with [γ-^{32}P]ATP. Phosphorylation of HKs usually occurs spontaneously in kinase core domains and the phosphoryl group can be transferred to the cognate RR if present. In contrast to unregulated phosphorylation in truncated proteins, it has been demonstrated that kinase activities can be either enhanced or inhibited by stimuli in several reconstituted full length HKs (23, 34, 92). Additional methods using in vivo ^{32}P labeling or a specific phosphate-binding tag have also been developed for direct detection of phosphoHis and phosphoAsp from cell lysates (7, 102), which could facilitate the understanding of HK regulation in vivo.

Besides kinase activity, many HKs possess a phosphatase activity toward their cognate phosphorylated RRs. One notable exception is the chemotaxis HK CheA, which has a different kinase domain organization and appears to be monofunctional with only a kinase activity, and instead, a separate CheZ protein is dedicated to RR dephosphorylation. The capability of decreasing the level of RR phosphorylation through rapid dephosphorylation provides a swift mechanism to shut down the signaling pathway and is suggested to be important for suppressing cross-talk from non-specific phosphorylation (1, 51). Considering that phosphatase activity might be overlooked in phosphorylation assays with truncated proteins lacking regulation from sensory domains, it seems a general design that most prototypical HKs are bifunctional. The phosphatase activity resides in the DHp domain and interaction between the DHp and CA domains greatly affects the activity. As shown in the *Escherichia coli* osmosensor HK EnvZ, the conserved His residue plays an important role in phosphatase activity and one potential dephosphorylation mechanism is through a reverse transfer of the phosphoryl group from RR to HK (28, 109). However, such reverse transfer is not readily observed in all HKs and some HK mutants that lack the conserved His still retain phosphatase activity (18, 19, 46). Therefore, multiple mechanisms might have evolved for phosphatase activities and different regulatory strategies are utilized by individual HKs. Since the RR phosphorylation level ultimately determines the output response in TCS pathways, signal-induced adjustment of RR phosphorylation is achieved by regulating the balance of the kinase and phosphatase activities of HKs. Signal perception that regulates primarily the kinase (e.g. CpxA and LuxN) (34, 92), primarily the phosphatase (e.g. KdpD) (16) or both (e.g. NtrB and PhoQ) (19, 48) have all been reported, indicating diverse regulatory mechanisms in HK signal transmission.

Structure/Function of the Kinase Core

Several characteristic sequence motifs are conserved across the kinase core domains of HKs. Based on specific sequences, they are termed the H, N, G1, F and G2 boxes with the H box containing the phosphorylation site in the DHp domain and the rest located in the CA domain. The structural and functional roles of these homology boxes were not fully

understood until the structures of individual DHp/CA domains (DHp: EnvZ; CA: EnvZ, NtrB, PhoQ and CheA) and entire kinase cores (*Thermotoga maritima* HK853 and *Geobacillus stearothermophilus* KinB) became available in recent years (10, 11, 13, 57, 58, 82, 89, 93).

The CA domain alone exists as a monomer in solution and is capable of binding ATP and transferring phosphoryl groups to the dimeric DHp domain. The α/β sandwich fold of the CA domain (Figure 2*a*) is distinct from any known Ser/Thr/Tyr kinases but similar to the ATPase domains of the GHL ATPase superfamily, whose members include GyrB, Hsp90 and MutL (29). A highly conserved ATP binding cavity is defined by the conserved residues in the N, G1, F and G2 boxes. Between the F and G2 boxes, a flexible region named the ATP lid can adopt different conformations upon nucleotide binding. In structures of PhoQ and CheA with bound ATP analogs (13, 57), the ATP lid is relatively ordered and covers the nucleotide, while it is highly mobile in nucleotide-free structures of CheA and NtrB (11, 82). Therefore, the conformational changes of the ATP-lid are proposed to couple the ATP-binding to alterations of interdomain or protein-protein interactions for regulation, as seen in other GHL ATPase family members (69). Interestingly, radicicol, one of the inhibitors targeting the GHL family member Hsp90, also binds with low affinity to the CA domain of the HK PhoQ and inhibits the autokinase activity (41), representing a potential lead for the development of HK inhibitors.

HKs function as dimers and a *trans*-phosphorylation mechanism has been demonstrated in which the CA domain from one subunit of the dimer phosphorylates the specific His residue on the DHp domain from the other subunit. The DHp domain mediates dimerization of the kinase core. DHp domain structures of EnvZ, HK853 and KinB exhibit a four-helix bundle formed by a pair of two antiparallel helices (Figure 2*b*), with the conserved H-box His positioned midway on the exposed surface of helix I (58, 93). In the HK853 structure, the connections between two antiparallel helices are crossed within the bundle, which could allow contact of the CA and DHp domains from different subunits, consistent with a *trans*-phosphorylation mechanism.

HKs possess multiple enzyme activities including autokinase, phosphotransfer and phosphatase activities. Alternating between these activities requires different conformational states of individual CA and DHp domains and, most importantly, corresponding three-dimensional arrangements of these domains (Figure 3). The structure of the entire cytoplasmic domain of HK853 provided the first structural insight into the inter-domain contacts of the prototypical kinase core (58). A substantial interface is observed between the CA and DHp domains. This interaction is believed to keep the HK in a state with predominant phosphotransferase and phosphatase activities, while disruption of the interaction upon signal perception leads to autophosphorylation. Stabilizing or destabilizing mutations at the interface diminished or enhanced the kinase activity, respectively. A coiled-coil region immediately preceding the DHp helix I is proposed to transduce the signal from the transmembrane (TM) region into the kinase core domains, affecting the interdomain interactions or the disposition of the His residue to regulate HK activities. Certainly, HKs might have evolved diverse strategies optimized for individual systems to regulate kinase/phosphatase activities. As seen in the cytoplasmic HK NtrB where transmembrane signaling is not involved, a unique β hairpin in the CA domain is presumed to bind to the cytoplasmic signal transduction PII protein for HK regulation (82).

Diversity of Sensory Domains

The modular kinase core domains are coupled to an overwhelmingly diverse array of sensory domains, enabling HKs to sense a wide variety of stimuli, including small molecules, light, turgor pressure, cell envelope stress, redox potential, electrochemical

gradients, etc. The sensory domains are usually located N-terminal to the kinase core and share little primary sequence similarity. Stimuli are sensed by these domains either directly or indirectly through protein-protein interactions with auxiliary signal transduction proteins. Despite great advancements in structural characterization of signal perception mechanisms in several HKs in recent years (reviewed in 60, 87), the structures of sensory domains and the identity of exact stimuli still remain unknown for the majority of HKs.

Nevertheless, a few common topologies have been observed in sensory domains and HKs are classified into three major groups based on these membrane topologies (60). The largest group is represented by the classical HKs characterized by an extracytoplasmic sensory domain residing between two TM helices. The extracytoplasmic sensory domain includes various domain families and perception of extracellular stimuli is transduced across the membrane to regulate the kinase/phosphatase activities. The second group has multiple (2–20) membrane-spanning helices but does not contain an apparent extracellular domain. The stimuli sensed by these HKs are believed to be membrane-associated (e.g. cell envelope integrity) stimuli derived from membrane-integral components and ion gradients. The third group of HKs features a cytoplasmic sensory domain responsible for sensing diffused and internal stimuli. Clearly, some HKs may combine multiple features of these topologies, integrating signals from different input domains, extracellular or cytoplasmic, for a concerted response to complex environments.

Due to the great sequence variability of extracytoplasmic sensing domains, only a limited number of HKs contain well-identified periplasmic domain families, such as the periplasmic solute-binding PBPb domain, CACHE and a series of CHASE domains. In contrast, many HKs share a few common cytoplasmic sensory and signaling domains. Nearly 33% of HKs contain at least one PAS domain, which can bind small ligands and sense changes in light, oxygen, redox potential and the overall energy level of a cell (90). Another family of small-molecule binding domains, the GAF domain (4), is present in ~9% of all HKs. Both PAS and GAF domains have rather high sequence variability and structural plasticity, making them versatile signaling domains for not only stimuli recognition but also signal transduction, due to their propensity for protein-protein interactions. HAMP domains, present in ~31% of HKs, usually occur immediately following TM regions and are central to signal transmission. Recently, a common functional motif, the signaling helix, has also been identified in HKs, connecting sensory and kinase domains (3) and presumably playing a central role in transduction.

Signaling Mechanisms

Dimerization appears obligatory for HK functions, as it is required for *trans*-phosphorylation between DHp and CA domains. However, unlike the regulatory mechanisms of many eukaryotic receptor families, HK signaling is not through signal-mediated dimerization of kinase domains. Rather, it is believed that perception of stimuli causes alteration of protein-protein interactions within the preformed HK dimer surface and these perturbations are relayed to the kinase core domains. In recent years, structural details have been elucidated for a handful of HKs with known ligands, revealing diverse ligand binding modes and the overall folds of both extracytoplasmic and cytoplasmic sensory domains. A few classes of similar folds seem to be shared despite low sequence homology and common features in transmembrane signaling mechanisms are beginning to emerge.

Transmembrane signaling in the chemotaxis aspartate receptor Tar represents the earliest HK-related mechanism illustrated in structural detail (Figure 3*a*), albeit Tar interacts intermolecularly with the non-canonical HK CheA for regulation (32). Functional chimeric proteins of Tar and various HKs, e.g. EnvZ and NarX, have been successfully constructed with interchanged periplasmic sensory domains to explore the potential shared

transmembrane signaling mechanism (97, 104). Interestingly, the periplasmic sensory domain of the *E. coli* HK NarX has been shown to have a similar four-helix bundle structure as Tar, although the nitrate/nitrite ligand binds to a single site at the dimer interface instead of to two negatively-cooperative sites as in Tar dimers. Ligand binding results in a symmetrical piston-like displacement of two N-terminal helices toward the TM region in NarX and an asymmetrical displacement of one helix in Tar. Presumably these piston-like movements are transmitted through the TM helices and reach the cytoplasmic domains, while the exact conformational changes that occur there remain unknown.

A distinct family of folds is observed in the periplasmic domains of LuxQ, DctB, CitA, DcuS and PhoQ (21, 31, 62, 63, 67, 73, 78, 108). These sensory domains feature a PAS-like fold with flanking α helices on both sides of a central β sheet in contrast to the all α-helical structures in Tar and NarX. The quorum-sensing HK LuxQ from *Vibrio harveyi* indirectly senses the quorum signal AI-2 through interactions with the periplasmic AI-2 binding protein LuxP. Structures of $(LuxPQ_p)_2$ complexes with or without the ligand AI-2 indicate little difference in the conformations of the tandem PAS-like folds of the LuxQ periplasmic domains ($LuxQ_p$) (63). Instead, ligand binding causes the formation of an asymmetrical dimer and the asymmetrical positioning of individual proteins is thought to be transduced into the cytoplasm, disrupting the symmetrical dimer of kinase core domains, thereby shutting off kinase activity (Figure 3*e*).

Unlike the indirect sensing mechanism of LuxQ, *E. coli* and *Salmonella enterica* PhoQ proteins directly sense divalent cations, e.g. Mg^{2+}, and antimicrobial peptides while the sensory domains of *Klebsiella pneumoniae* CitA, *Sinorhizobium meliloti* DctB, *Vibrio cholerae* DctB and *E. coli* DcuS bind to small metabolic molecules citrate or C_4-dicarboxylates such as succinate, fumarate and malate. The overall structure of these domains is also named the PDC sensor fold (PhoQ, DcuS and CitA), due to the distinctive positioning of the α helices relative to PAS domains (20, 21). Isolated PDC domains have very low intrinsic affinity for dimerization and the plasticity of the PAS-like fold gives rise to different orientations of dimer subunits in different crystal lattices, making it difficult to distinguish the physiologically relevant quaternary structure. On the other hand, these different dimeric arrangements might also reflect different signaling states captured in different experiments that are worth further investigation, as seen in structures of *E. coli* and *S. enterica* PhoQ proteins or *K. pneumoniae* CitA (20, 22, 39, 73, 78). Moreover, some PDC structures suggest a common feature of the close proximity of the N and C termini for these periplasmic sensory domains (20, 39, 78), which places the TM helices at positions compatible with a four-helix bundle similar to those of Tar and NarX. Therefore, it is tempting to speculate whether common signaling mechanisms might be conserved in these proteins. Intriguingly, citrate binding to CitA results in a contraction of the sensory domain, consistent with a piston-type movement of TM helices (78).

The highly flexible HAMP domain is usually located C terminal to the last TM segment and plays an important role in relaying signals from TM regions to the kinase core. The NMR solution structure of a HAMP domain from a hyperthermophile showed a dimeric architecture and a parallel four-helical coiled-coil with each monomer contributing two parallel helices connected by a long loop (Figure 3*b*) (47). Furthermore, the coiled-coils adopted an unusual knobs-to-knobs packing, deviating from the conventional knobs-to-holes packing that can be achieved by helix rotation (Figure 3*f*). Hence, a rotary mechanism has been suggested for the signaling of HAMP domains, however, it is not known whether this helical rotation mechanism is generally conserved for all proteins containing HAMP domains and how the transmembrane signaling events, sometimes piston-like movements, are converted to helix rotation in HAMP domains.

Structures of individual cytoplasmic sensory domains have also been determined for a few HKs, such as the PAS domains from both *Bacillus subtilis* KinA (52) and the heme-based oxygen sensor FixL in Rhizobia (40, 43), the GAF domains of the O_2/NO sensors DevS/DosT from Mycobacterium (53, 72) and the chromophore-binding bacteriophytochromes containing PAS, GAF and PHY domains (30, 103). Binding of ligands and cofactors causes conformational changes in specific regions of these similar structures, yet it remains unknown how these changes are coupled to kinase activity until the spatial arrangements of the kinase and sensory domains are characterized. Certainly, without the barrier of the plasma membrane, signaling can be executed through signal-mediated direct contacts with the kinase as well as quaternary structural changes due to the general plasticity of these domains. A structure of the full photosensory core domain from *Pseudomonas aeruginosa* bacteriophytochrome provides an interesting insight into the spatial organization of multiple sensory domains (103). A long continuous helix directly connects the GAF and PHY domains and forms a parallel dimer with individual domains resembling gall-like extrusions from the central helical stem (Figure 3*c*). Long coiled-coils have also been identified immediately preceding the kinase domain extending into the four-helix bundle of the DHp domain (Figure 3*d*). Such long helices are commonly found in various prokaryotic signaling proteins. Termed the signaling helix, this motif joins a wide variety of signaling domains, such as PAS, GAF, HAMP, TM helices, DHp, REC, GGDEF, EAL and Ser/Thr/Tyr kinases (3). The wide distribution of all these signaling domains across different prokaryotic signaling systems raises the interesting question of whether one or a few signaling mechanisms observed in HKs might also be conserved in other prokaryotic signaling systems.

Phosphotransfer Specificity

Due to the great versatility of TCSs, most sequenced bacterial genomes usually encode dozens of two-component proteins. The presence of multiple paralogous HK/RR proteins makes it possible for cross-phosphorylation between highly similar DHp and REC domains. This is distinct from inherent multiple cognate pairs where one-to-many, many-to-one and many-to-many relationships occur between HKs and RRs. For example, the chemotaxis HK CheA can phosphorylate both CheY and CheB while the phosphorylation level of the *B. subtilis* RR Spo0F is under the control of 4 HKs: KinA-KinD, and the nitrate/nitrite responsive HKs NarQ and NarX regulate both NarL and NarP phosphorylation. In contrast, the unwanted cross-phosphorylation, or cross-talk, between different pathways could be detrimental to signaling specificity and fidelity. A few examples of cross-talk do exist (reviewed in 51) however a strong kinetic preference for cognate HK-RR phosphorylation has been demonstrated by system-wide phosphotransfer profiling (81), indicating specific interaction between cognate HKs and RRs. A computational method was recently used to identify the co-variant residues in large numbers of cognate HK-RR pairs (80). In HKs, two clusters of co-varying residues have been recognized, one above the phosphorylation site His residue and the other near the bottom of the four-helix bundle of the DHp domain. Replacing the segment containing the lower cluster with the one from another HK or mutating the specific residues successfully converted the phosphotransfer specificity to the targeted non-cognate RR (80). Future structural characterization of HK-RR complexes may provide contact details of these specificity-determining residues.

HISTIDINE-CONTAINING PHOSPHOTRANSFER PROTEINS

TCSs involve two types of phosphoamino acids, phosphoHis and phosphoAsp. Both have a high free energy of hydrolysis, making them ideal candidates for generating a flux of phosphoryl groups from phosphoHis to phosphoAsp in the basic HK-RR scheme as well as a reverse transfer from phosphoAsp to phosphoHis in a multi-step phosphorelay system. Nearly 25% of all HKs carry an additional REC domain, which potentially could be

phosphorylated by the endogenous kinase domain. Subsequently, a His-containing phosphotransfer domain, usually an HPt domain, can receive the phosphoryl group from the REC domain and relay the phosphorylation to a cognate RR for output responses. This HPt domain can be directly linked to an HK, such as the *E. coli* redox sensor ArcB, an independent protein, e.g. YPD1 in the yeast SLN1-YPD1-Ssk1 pathway, or part of another membrane protein, such as RcsD (also named YojN) in the *E. coli* Rcs signaling system. HPt domains do not have kinase or phosphatase activities hence the multistep His-Asp phosphorylation cascade likely does not serve to amplify signals as seen in common eukaryotic phosphorylation cascades. Rather, the involvement of multiple components in the phosphorelay scheme allows extra regulatory checkpoints along the lengthened pathway. The intermediate REC or HPt domains can be targeted by specific phosphatases for additional regulation (66, 70). Aside from their roles in phosphorelays, HPt domains can also be indispensable components of kinase function, replacing DHp domains in non-prototypical HKs exemplified by CheA.

Structures of several HPt domains (*E. coli* ArcB and *Saccharomyces cerevisiae*YPD1) have a similar four-helix bundle architecture as DHp domains except that all four helices belong to an HPt monomer instead of the dimer of DHp domains (49, 101). Again the active site His is similarly positioned near the middle of the solvent-exposed helical face. YPD1 functions as a monomer and the structure of a YPD1-SLN1 REC domain complex has defined interactions important for phosphotransfer (107). Despite the overall fold conservation, individual HPt domains show different lengths and/or orientations of helices or contain additional structural elements, possibly reflecting specific features in their individual signaling tasks. The phosphotransfer function is not restricted to HPt domains. Spo0B, the phosphotransfer protein in *B. subtilis* sporulation regulation (KinA-Spo0F-Spo0B-Spo0A), one of the earliest-identified phosphorelay schemes, does not share significant sequence similarity with HPt domains. The structure of the Spo0B dimer resembles the dimeric structure of the HK kinase core with the central His-containing four-helix bundle and a CA-like domain that cannot bind ATP (96). Such close resemblance also provides insights into HK-RR interactions from structures of the Spo0B-Spo0F complex (105).

RESPONSE REGULATORS

Domain Architecture

The RR superfamily has a modular architecture and is defined by a conserved regulatory or receiver (REC) domain referred to as the Response_reg domain in the Pfam database. The prototypical RR contains two domains, an N-terminal REC domain linked to a variable effector domain, however, many different domain architectures have been observed. The REC domain participates in catalysis of phosphoryl transfer from the HK to itself and regulates the activity of the effector domain in a phosphorylation-dependent manner. The effector domain elicits the output response, with the diversity of effector domains allowing for an enormous array of responses that are regulated through two-component signaling. Most effector domain folds have been observed to exist independently of RR proteins, either as isolated domains or integrated with other regulatory domains, emphasizing their modular nature. Approximately 17% of RRs exist as stand-alone REC domains. The majority of these correspond to CheY proteins encoded in chemotaxis operons, many of which regulate motility by intermolecular interactions with motor proteins. Other stand-alone REC domains, such as *B. subtilis* Spo0F (95), function as phosphorylated intermediates in phosphorelay pathways. These REC domains do not directly regulate output responses and, in this regard, are functionally analogous to REC domains located within hybrid HKs.

Receiver Domain Activities

The REC domain functions as a phosphorylation-activated switch. REC domains exist in equilibrium between two predominant conformations, designated inactive and active. NMR dynamics studies indicate that an unphosphorylated REC domain exists primarily in the inactive conformation, but continuously samples both states on a microseccond timescale and that phosphorylation shifts the equilibrium, stabilizing the active conformation (38). Through inhibitory and/or activating interactions specific to each RR, the inactive and active conformations of the REC domain control effector domain activity.

Phosphorylation occurs at a conserved Asp residue, generating a high energy acyl phosphate. By analogy with P-type ion-translocating ATPases (reviwed in 86), it is thought that the acyl phosphate provides energy coupling to drive a conformational change in the REC domain. In this sense, the phosphate of the REC domain phosphoAsp is both chemically and functionally more similar to the γphosphate of a tightly bound GTP in Ras-family small GTPase switch proteins than to phosphoSer/Thr/Tyr residues that promote their effects primarily through altered local electrostatics.

The REC domain has enzymatic activity, catalyzing both phosphoryl transfer and autodephosphorylation. Phosphotransfer from the HK to the RR involves a protein complex in which both proteins contribute to the active site. The catalytic contributions of the RR are illustrated by the ability of most RRs to autophosphorylate using high-energy small molecule phosphodonors such as phosphoramidate, acetyl phosphate, carbamyl phosphate, and phosphoimidazole (56). The K_m values for these substrates are very high and rates of phosphotransfer are typically ~one hundred fold slower than those observed with cognate HKs as donors (24). However, due to the complexities of using purified transmembrane HKs as phosphodonors, much in vitro analysis of RR phosphorylation has been conducted with small molecule donors. Interactions that stabilize the active conformation of the REC domain, such as binding of OmpR to DNA (2) or CheY to the flagellar motor (77), have been shown to accelerate phosphotransfer from small molecule phosphodonors. Likewise, interactions that stabilize the inactive conformation, such as an interface with an effector domain (35), decrease rates of phosphotransfer. Whether these effects occur during phosphotransfer from HKs and are of physiological significance is unknown. Indeed, under some physiological circumstances, intracellular acetyl phosphate influences RR phosphorylation (reviewed in 100), but its contribution is typically minimal compared to that of cognate HKs.

The level of RR phosphorylation is determined by the relative rates of phosphorylation and dephosphorylation. Several different factors influence dephosphorylation. Acyl phosphates are inherently unstable, with half-lives of ~6 h under ambient conditions. RRs also catalyze autodephosphorylation, with rates ranging over four orders of magnitude, corresponding to half-lives of seconds to hours. In many TCSs, phosphatase activities of HKs and/or auxiliary phosphatases, such as the chemotaxis protein CheZ (106) and the Rap, Spo0E, YnzD and YisI phosphatases involved in *B. subtilis* sporulation (70, 71), regulate RR dephosphorylation in vivo. The lability of phosphoAsp complicates analysis of phosphorylated RRs and prohibits the use of analytical methods common for phosphoSer/Thr/Tyr-containing proteins. A recently developed phosphate chelator, Phos-tag, has provided alternative strategies for detecting phosphoAsp in RRs and has enabled quantitation of RR phosphorylation in vivo (7).

Structure/Function of Receiver Domains

The REC domain is an ~120-residue doubly-wound α/β fold, with 21345 topology of the five-stranded parallel β sheet (Figure 4*a*). REC domains exhibit an average of 26% sequence

identity with a small set of highly conserved residues corresponding to active site and "switch" residues. In addition to the site of phosphorylation, an Asp in the loop following β3, the active site contains two additional Asp/Glu residues that coordinate an essential Mg^{2+} and a conserved Lys residue that forms a salt bridge with the phosphate in the activated domain. Phosphotransfer and phosphatase mechanisms are assumed to involve a similar pentavalent phosphorus intermediate stabilized by the active site residues and divalent metal ion, with the nucleophilic carboxyl oxygen of the phosphorylation site Asp (phosphotransfer) or water (phosphatase) at the axial positions (84). Specific active site residues have been found to influence rates of dephosphorylation in some RRs, however the determinants of phosphoryl stability appear to be complex and are not predictable from sequence analysis (91).

The conformational differences that distinguish inactive and active REC domains have been defined by structural analyses. Originally, a variety of strategies were employed to trap REC domains in their active conformations (15, 42, 50, 55). Recently, structural studies have relied almost exclusively on beryllofluoride (BeF_3^-), which serves as a good mimic of a phosphoryl group, stabilizing the active conformation of RRs by non-covalent binding to the phosphorylation site Asp (99). The inactive and active conformations are very similar, typically involving backbone displacements of <1 Å and functionally significant perturbations of the molecular surface at the α4-β5-α5 face (Figure 4*a*). However, both the range and magnitude of structural differences differ among RRs; perturbations extend to α1 (15) and α3-β4 (44) and larger displacement and rotation of α4 occurs in some REC domains (5, 44).

The most readily distinguishable feature of the two conformations is the distinct rotameric states of two highly conserved "switch" residues: Ser/Thr on β4 and Phe/Tyr on β5. In inactive REC domains, the side chains of these residues are oriented away from the active site with the Phe/Tyr exposed on the domain surface, where in many RRs, it interacts specifically with residues in associated effector domains. In the active conformation, the side chains of the switch residues are oriented toward the active site, with the hydroxyl of the Ser/Thr forming a hydrogen bond with a phosphate oxygen and the aromatic ring of the Phe/Tyr buried in the space vacated by the repositioned Ser/Thr. This conserved mechanism couples phosphorylation at the active site to structural perturbations at the distal α4-β5-α5 surface.

Diversity of Effector Domains

The great diversity of attached or independent effector domains creates an almost limitless variety of output responses that can be controlled through RRs (36). In prokaryotes, RRs are usually the last components of signaling pathways, directly effecting the responses, while in eukaryotic TCSs, RRs are often intermediates, interfacing with proteins linked to common eukaryotic strategies such as MAP kinase cascades or cyclic nucleotide second messengers (76). As noted previously, ~17% of prokaryotic RRs exist as stand-alone CheY-like REC domains. Most of the remaining RRs contain domains that can be categorized into a relatively small set of nucleic acid binding, enzymatic, and protein/ligand binding domains (Figure 4*b*).

The majority of RRs (63%) contain DNA-binding effector domains, dominated by a small number of structural families named after extensively characterized members. These subfamilies include the OmpR/PhoB winged-helix domain (30% of all RRs) (59), the NarL/FixJ four-helix helix-turn-helix domain (17%) (61), the NtrC/DctD AAA^+ ATPase domain fused to a factor of inversion (Fis)-type helix-turn-helix domain (10%) (8) and the recently characterized LytTR domain with an unusual, predominantly β fold (3%) (79). RNA-

binding domains are found in only ~1% of RRs and are mostly of the ANTAR family, functioning as anti-termination factors (65).

Enzymatic domains are found in ~13% of RRs. The majority of these RRs (6% of all RRs) are involved in regulation of c-di-GMP (75). Enzymatic domains within these response regulators include GGDEF diguanylate cyclase domains and/or phosphodiesterase domains of the EAL or HD-GYP families. The remaining enzymatic subfamilies, in order of prevalence, are chemotaxis methylesterase CheB domains; HK domains (often coupled with additional PAS and/or GAF domains), presumed to be output domains because of their location C terminal to REC domains and the absence of sensing domains; and protein phosphatase domains of the PP2C family. Many additional enzymatic domains occur infrequently in RRs.

A small and diverse group of effector domains that mediate interactions with other proteins or ligands occur in 3% of RRs. Approximately half of these RRs correspond to chemotaxis CheV-like proteins. Additional subfamilies include tetratricopeptide repeat (TPR), Hnr-like regulators of stress sigma factor RpoS, HPt, PAS, GAF, and cNMP binding domains.

Structures are available for representative members of most of these effector domains and conserved functional features such as the mode of interaction with DNA for transcription factors or the configuration of the active site in enzymes have been discerned and can be readily extrapolated to other subfamily members. However, this mechanistic description provides little insight to physiological function. Understanding RR transcription factors requires knowledge of the genes regulated by each, and this is known in only a very limited number of cases. RRs can function as activators, repressors, or both and the regulons of different RRs range from a single operon to hundreds of genes. Once defined, DNA recognition sequences of RRs are often very weakly conserved and require experimental rather than bioinformatic identification. Similar complexities exist for most enzymatic domains. For example, RRs containing diguanylate cyclase and phosphodiesterase domains regulate synthesis and degradation of c-di-GMP. The targets of this second messenger, and whether its effects are global or local are largely unknown (88). Furthermore, the presence of GGDEF and EAL domains (some vestigial and some presumably active) with potentially opposing activities in the same protein, makes it difficult to predict the role of each RR in determining levels of c-di-GMP. Predictions of protein and ligand interactions are even more challenging.

Regulatory Strategies

As a phosphorylation-activated switch, the REC domain exploits its ability to promote inhibitory and/or activating protein-protein interactions specific to its inactive or active conformations. This fundamental mechanism provides versatility, enabling a broad range of regulatory strategies. Given the great diversity of stimulus-response coupling mediated by TCSs, it is unsurprising that RRs have evolved diverse regulatory mechanisms that adapt each protein to the pathway in which it functions. As structures of multi-domain response regulators have accumulated, it has become apparent that conserved REC and effector domains often exhibit distinct arrangements in different RRs, even within a single subfamily, imparting different regulatory strategies to each.

A common regulatory theme among RR transcription factors is phosphorylation-induced dimerization or higher order oligomerization, with additional regulation, most notably inhibition, often integrated into this basic scheme. Structures of four inactive OmpR/PhoB family members, *Thermotoga maritima* DrrB (74) and DrrD (17) and *Mycobacterium tuberculosis* PrrA (64) and MtrA (35), all exhibit a monomeric state with different domain arrangements (Figure 5*a*). Structures of isolated regulatory domains of *E. coli* PhoB (5) and

B. subtilis PhoP (14) show yet another variation, propensity for dimerization in distinct modes specific to the unphosphorylated proteins. DrrB, PrrA, and MtrA have extensive interfaces between REC and DNA-binding domains, while DrrD has none. The recognition helices of DrrD and DrrB are exposed, while those of PrrA and MtrA are occluded. These features impart different levels of inhibition to the inactive RRs. Upon phosphorylation, OmpR/PhoB REC domains form rotationally symmetric dimers mediated by an extensive network of salt bridges between highly and exclusively conserved residues on their α4-β5-α5 faces (5, 94). Dimerization of the REC domains brings the DNA-binding domains into close proximity, allowing them to bind the direct repeat half-sites that comprise the recognition sequences for most OmpR/PhoB RRs. Flexible linkers presumably connect the domains, allowing different dimer symmetries. The common structure of active OmpR/PhoB family members is unusual among RRs. In spite of the highly conserved α4-β5-α5 face, most OmpR/PhoB RRs dimerize with specificity. A few *E. coli* OmpR/PhoB RRs form heterodimers in vitro, but their physiological significance is unknown (37).

Diverse regulatory mechanisms also exist in the NtrC/DctD family of transcription factors that activate σ^{54} promoters. These RRs induce open complex formation in RNA polymerases by promoting assembly of AAA^{+} ATPase domains into hexameric or heptameric ring structures, competent for ATPase activity. Structures of *S. enterica* NtrC (25), *S. meliloti* DctD (68), and *Aquifex aeolicus* NtrC1 (27) and NtrC4 (8) reveal variations in domain arrangements and corresponding regulatory mechanisms, one based primarily on activation and the other on inhibition (Figure 5*b*). The ATPase of unphosphorylated NtrC is inactive. Phosphorylation of REC domains promotes their intermolecular association with neighboring ATPase domains resulting in ring assembly and ATPase activity. In contrast, the ATPase domains of unphosphorylated DctD and NtrC1, though intrinsically competent for ring assembly, are held in an inactive dimeric state by interactions of the unphosphorylated REC domains. Phosphorylation of the REC domains disrupts these interactions, allowing ring assembly and ATPase activity. NtrC4 is similar to NtrC1, but with different domain arrangements and weaker inhibition. Thus there appear to be two distinctly different mechanisms and variations in between, with the phosphorylated REC domain required for activation in one extreme, and mere removal of the inhibitory influence of the unphosphorylated REC domain sufficient for activation in the other.

Similar diversity is observed in RRs containing GGDEF domains with diguanylate cyclase activity. Synthesis of c-di-GMP requires the juxtaposition of two GGDEF domain active sites, each bound to GTP substrate. Additionally, product inhibition by c-di-GMP, which through its bidentate structure is optimal for cross-linking domains in nonproductive orientations, appears to be a common feature of diguanylate cyclase RRs and is postulated to be important for stringent regulation of c-di-GMP levels. However, the structural basis for these common functions differs in the two RRs that have been characterized to date (Figure 5*c*). *Caulobacter crescentus* PleD contains two REC domains and a cyclase domain. Subtly different intramolecular dimerization modes of the unphosphorylated and phosphorylated REC domains favor inactive PleD monomers or active PleD dimers, respectively (98). *P. aeruginosa* WspR contains a single REC domain linked by a long helical stalk to a cyclase domain. Inactive WspR exists as an elongated antiparallel dimer, while active WspR, reportedly independent of phosphorylation, exists as a compact parallel dimer, mediated by association of REC domains through their α4-β5-α5 faces (26).

A VERSATILE STRATEGY FOR SIGNALING

Structures of HKs and RRs have established the domain folds and active site geometries that provide the conserved activities of TCS proteins. The structures of multidomain HKs and RRs in different states of stimulation or activation have revealed great diversity in domain

arrangements, reflecting different regulatory mechanisms. The regulation of HKs and RRs is based on a single fundamental and extremely versatile strategy that allows each component to be optimized for the system in which it functions. Perception of stimuli by HK sensory domains or phosphorylation of RR REC domains subtly alters their molecular surfaces. These perturbations are exploited for inhibitory and/or activating protein-protein interactions that discriminate between the different states, favoring different domain arrangements that promote or suppress specific activities.

Recent structures of Hks and RRs have provided some examples of inhibitory and activating mechanisms that can be achieved by specific domain interactions. It is likely that the available repertoire of mechanisms is much greater, and perhaps not well represented by the current structural database, which is presumably biased toward compact and rigid structures that are more amenable to crystallization. It is clear that regulatory mechanisms are diverse, even within structurally conserved subfamilies, making elucidation of mechanisms in different TCS proteins a daunting task. The prediction of both conformational changes within domains and determinants of specificity that underlie specific protein-protein interactions responsible for overall domain arrangements remain major challenges for the future.

GLOSSARY AND ACRONYMS

TCS	two-component system
Phosphotransfer	transfer of a phosphoryl group -PO_3^{-2}), as occurs between histidine kinases and response regulators in two-component systems
HK	histidine protein kinase, input component of two-component signal transduction systems
RR	response regulator, output component of two-component signal transduction systems
CA	conserved catalytic and ATP-binding domain of histidine kinase proteins
DHp	conserved dimerization and histidine phosphotransfer domain of histidine kinase proteins
TM	transmembrane portion of an integral membrane protein
HAMP	helical signaling domain found in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases
PAS	sensory input domain named after eukaryotic Per, ARNT and SIM proteins
GAF	small ligand binding sensory input domain found in some cGMP-regulated phosphodiesterases, adenlyl cyclases, and transcription factor FhlA
HPt	histidine phosphotransfer domain, often a stand-alone domain in phosphorelays
REC	conserved receiver domain of response regulator proteins
Beryllofluoride	non-covalent complex of beryllium and fluoride, which, in the form of BeF_3^-, mimics a phosphoryl group, binding to the active site Asp of response regulator receiver domains

Winged-helix	DNA-binding domain that makes major groove contacts via a recognition helix and minor groove contacts via flanking wing-like regions
c-di-GMP	cyclic diguanylate, a bacterial intracellular signaling molecule
GGDEF	guanylate cyclase domain that catalyzes synthesis of c-di-GMP from two GTP molecules
EAL	phosphodiesterase domain that catalyzes the hydrolysis of c-di-GMP

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SUMMARY POINTS

1. Structural and functional features intrinsic to conserved domains are common to most HKs and RRs.
2. HK and RR proteins have modular architectures that allow great variation in primary and tertiary domain arrangements.
3. Although HKs and RRs have conserved sequences and structures, TCS proteins in a single organism typically exhibit specificity of intermolecular interactions (e.g. phosphotransfer from HKs to RRs and RR dimerization).
4. Both HKs and RRs use a fundamental strategy for regulation: signals (perception of stimuli by HKs or phosphorylation of RRs) stabilize alternate conformations, each favoring distinct intra- or intermolecular protein-protein interactions, promoting domain arrangements that inhibit or activate specific functions.
5. Different domain arrangements and thus different regulatory mechanisms occur in HKs and RRs, even among members of subfamilies with similar domain architecture.
6. Prediction of domain arrangements and regulatory mechanisms from sequence remains a challenge for the future.

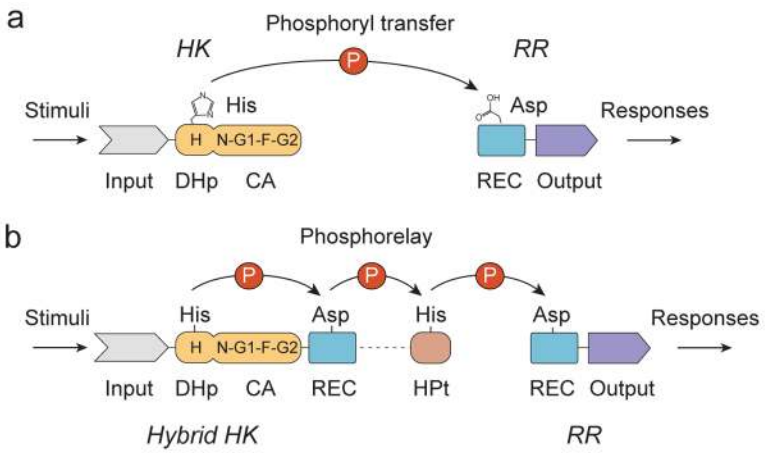

Figure 1.

Schematic diagram of two-component systems. (*a*) The prototypical TCS pathway features a conserved phosphoryl transfer between the highly conserved kinase core (DHp and CA) and REC domains to couple various input stimuli and output responses. The kinase core contains several homology boxes, H, N, G1, F and G2, which are conserved across HKs. (*b*) A phosphorelay scheme is utilized by hybrid HKs involving additional REC and HPt domains for multiple phosphotransfer events. The intermediate HPt domain can be either an independent protein or linked to the HK.

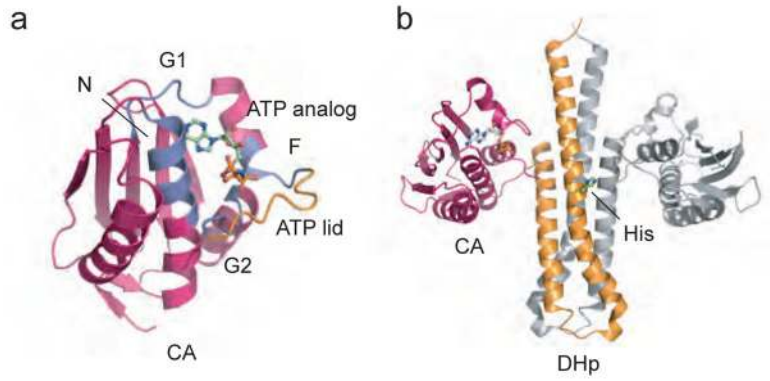

Figure 2.
Structures of the kinase core. Crystal structures of (*a*) the CA domain of *E. coli* PhoQ and (*b*) the entire kinase core of *T. maritima* HK853 are illustrated in ribbon diagrams (PDB IDs: 1ID0 and 2C2A). Homology boxes crucial for ATP binding are shown in blue in the PhoQ CA structure. A flexible ATP lid (orange) covers the bound ATP analog, AMPPNP. HK853 is dimeric with one monomer shown in color and the other in gray. The histidine-containing DHp domain is colored orange and the catalytic CA domain is colored pink.

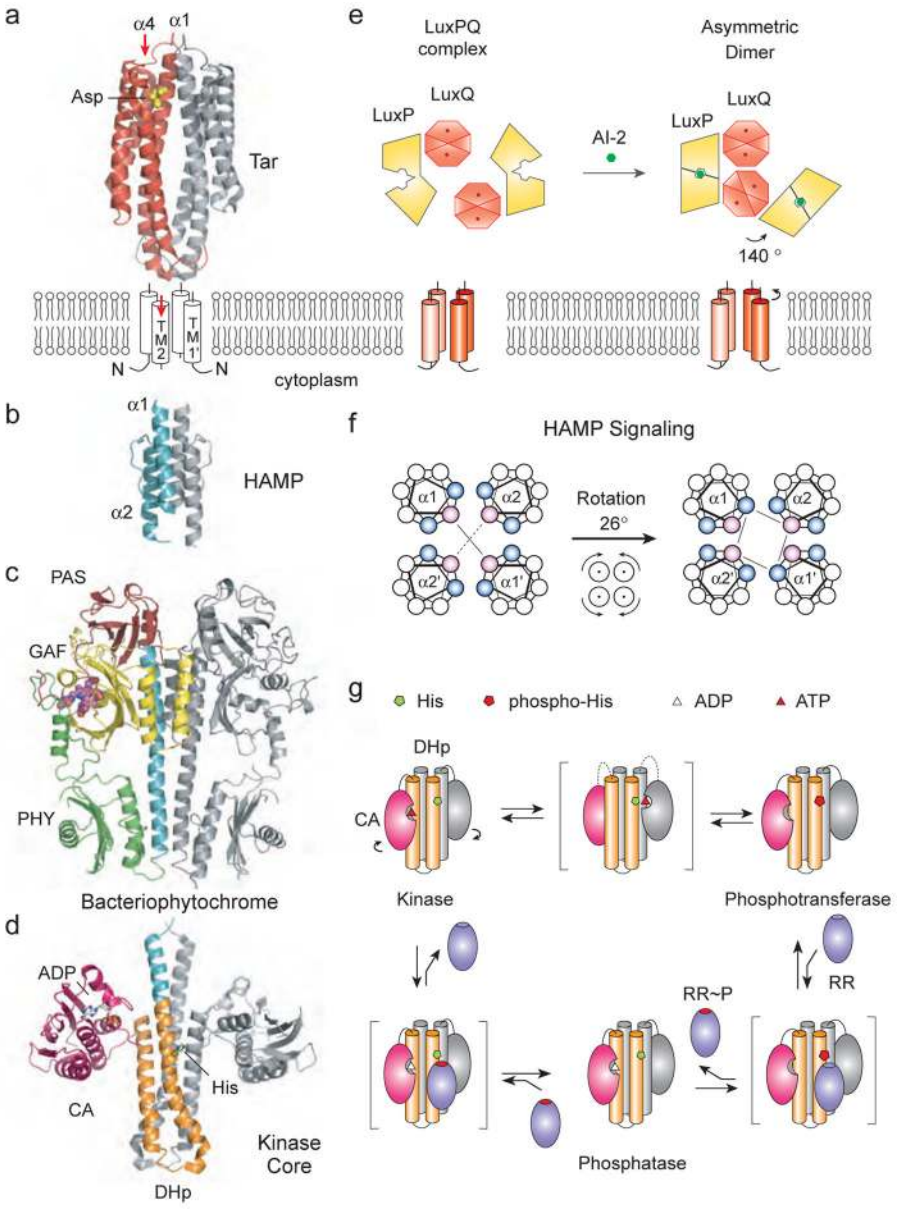

Figure 3.

Signaling mechanisms of HKs. Structures of common HK domains are represented by (*a*) the periplasmic aspartate-sensing domain of *S. enterica* chemoreceptor Tar, (*b*) the cytoplasmic HAMP domain of Af1503 from *Archaeoglobus fulgidus*, (*c*) the cytoplasmic photoactive bacteriophytochrome sensory domains from *P. aeruginosa* and (*d*) the kinase core domains of *T. maritima* HK853 (PDB IDs: 2LIG, 2ASW, 3C2W and 2C2A). They all have a dimeric structure and individual domains are shown in different colors. Binding of aspartate to Tar is believed to cause a piston-like movement of one helix toward the TM region (highlighted in red arrows). Central coiled-coils (cyan) in cytoplasmic HK domains are suggested to be important for signal transmission to the kinase core. (*e*) Schematic representation of signaling in LuxPQ. In the absence of the quorum signal molecule, autoinducer 2 (AI-2), the periplasmic binding protein LuxP (yellow) in an open conformation forms a complex with the periplasmic domain of the HK LuxQ (orange) and there is no association of LuxQ periplasmic domains. Upon binding AI-2 (green), LuxP

adopts a closed conformation, promoting formation of an asymmetric dimer and inhibiting kinase activity. Individual $LuxPQ_p$ subunits are related to one another by an ~140° rotation. This symmetry-breaking rotation presumably repositions the LuxQ TM helices for signal transduction to the cytoplasmic domains. (*f*) Schematic representation of two packing modes of the HAMP domain. Residues at different positions of the four-helix bundle interface are illustrated in blue and pink. These two packing modes are interchangeable by rotating adjacent helices in opposite directions. (*g*) Schematic diagram of HK catalytic activities. Dimeric DHp domains are shown as a four-helix bundle. The conserved His residue (green pentagon) is poised for phosphorylation once a suitable orientation of CA (red) and DHp domains is achieved to bring the bound ATP close to the His residue. Upon autophosphorylation, the kinase is relaxed to a conformation that allows access of the RR (violet) to the phosphorylation site. Phosphotransfer occurs when the RR binds to the DHp domain. Phosphatase activity could be dominant in yet another orientation, with an ADP bound CA domain and an unphosphorylated DHp domain.

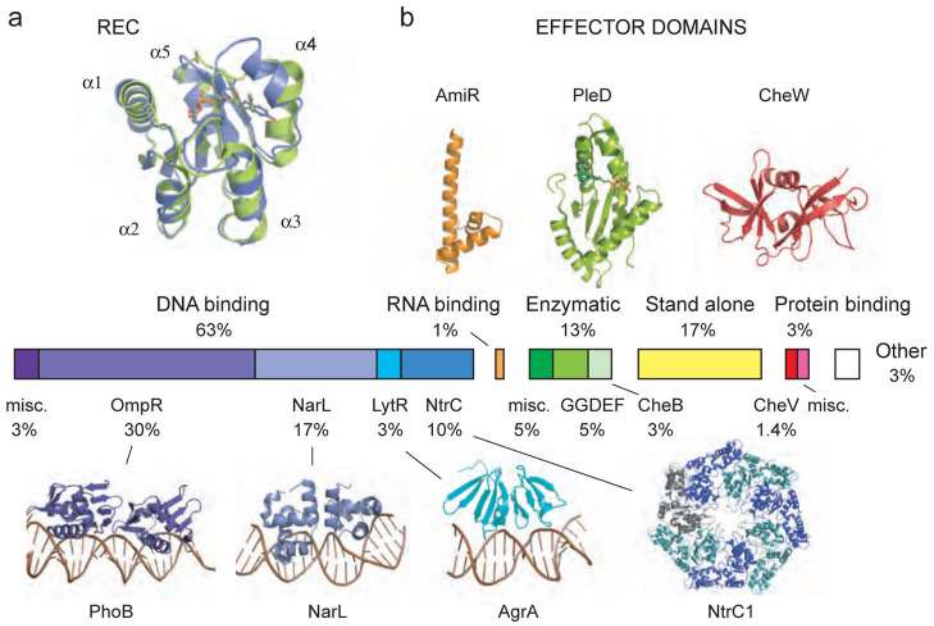

Figure 4.

Structures of RR domains. (*a*) The structures of inactive (blue) and active (green) PhoB REC domains are superimposed to illustrate the subtle conformational differences (PDB IDs: 1B00 and 1ZES). "Switch" residues are shown in ball-and-stick mode and beryllofluoride is colored orange. (*b*) Distribution of diverse effector domains is represented by the horizontal bar with different RR families grouped according to their functions. Percentile distribution of functional classes is indicated above the bar and distribution of RR subfamilies is shown beneath the bar. A representative of individual subfamily effector domain folds is illustrated in ribbon diagrams (PDB IDs: AmiR, 1QO0; PleD, 2V0N; CheW, 2CH4; PhoB, 1GXP; NarL, 1JE8; AgrA, 3BS1; NtrC1, 1NY6).

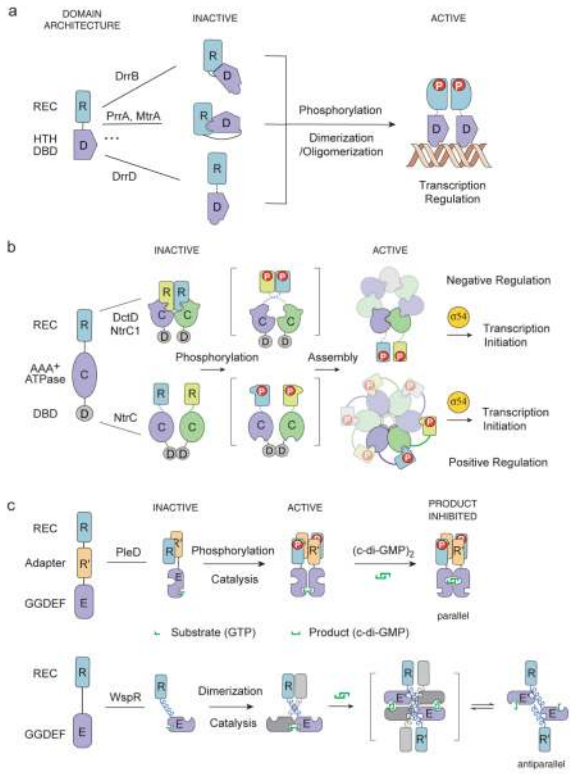

Figure 5.

Schematic diagrams of diverse regulatory mechanisms in RR subfamilies: (*a*) the OmpR/PhoB subfamily, (*b*) the NtrC/DctD subfamily and (*c*) RRs with GGDEF domains. REC domains are colored blue and different effector domains are colored violet. Mechanisms are described in the text.