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Molecular mechanisms of two-component signal transduction

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Abstract

Two-component systems comprising sensor histidine kinases and response regulator proteins are among the most important players in bacterial and archaeal signal transduction and also occur in reduced numbers in some eukaryotic organisms. Given their importance to cellular survival, virulence and cellular development these systems are among the most scrutinized bacterial proteins. In recent years a flurry of bioinformatics, genetic, biochemical and structural studies have provided detailed insights into many of the molecular mechanisms that underlie the detection of signals and the generation of the appropriate response by two-component systems. Importantly, it has become clear that there is significant diversity in the mechanisms employed by individual systems. This review discusses the current knowledge on common themes and divergences from the paradigm of two-component system signaling. An emphasis is on the information gained by a flurry of recent structural and bioinformatics studies.

Graphical Abstract

Keywords

signal transduction; two-component system; sensor histidine kinase; response regulator

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Introduction

The proteins comprising the bacterial two-component system (TCS) are the sensor histidine kinase (HK) and the response regulator (RR) (Fig. 1A.). These two factors are among the most abundant proteins in the sequence databases, owing to a wide distribution across the bacterial and archaeal kingdom and owing to significant amplification within bacterial and archaeal genomes [1, 2]. Further, TCS are also found in some eukaryotic organisms but are notably absent from genomes of the animal kingdom. Three decades' worth of genetic and molecular microbiology studies on these systems have elucidated the individual roles and importance to cellular survival for many of these systems in diverse bacteria. Full structural characterization of these proteins in contrast has initially lagged despite some early individual successes. However, in the past decade increased structural and bioinformatics efforts have closed the gap between our functional and mechanistic understanding of these system, in part due to a significant number of structurally characterized TCS protein domains.

In the prototypical TCS the HK and RR serve to connect the detection of an environmental or cellular signal with an appropriate cellular response. Communication between the proteins occurs via phosphoryl-group transfer from a histidine of the HK to an aspartate of the RR. Some HK also function as phosphatase for their respective RR under non inducing conditions [3]. Owing to the diversities of input signals and the cellular responses, significant variety exists in input and output domains (Fig. 1.). In contrast, the structures of the catalytic and regulatory domains of the two proteins are largely conserved; nonetheless recent studies have revealed diversity on the detailed molecular mechanisms by which these proteins phosphorylate and communicate.

In this review we divide the signal transduction cascade into four main focus areas: (1) signal detection, (2) kinase activation, (3) phosphotransfer and (4) response generation. Numerous excellent review articles in recent years have focused on individual aspects of the signal cascade and we refer the reader to these articles for some additional details and reference to specific studies that could not all be featured in a broad review [4–8].

Signal Detection and Transmission

Signal detection and transmission to the catalytic core of the kinase is mediated by modular and variable domains typically at the N-terminus of the HK [4, 7, 8]. We note that the catalytic core of the kinase has sometimes been referred to as transmitter-domain. In this article we refer to transmission as the ligand dependent conformational changes that ultimately lead to stimulation of kinase activity. Since the prototypical sensor kinase is a transmembrane protein, signal detection and transmission domains are localized to all three compartments of the cell, i.e. the extracytoplasmic space, the membrane and the cytoplasm (Fig. 1.). Experimental structures have been elucidated for a variety of domains and molecular insights about signal detection and transmission have been gained from these structures (Fig. 1B.). The remarkable diversity of signals, both chemical as well as physical detected by HK are evident from a few examples.

For instance, the *Bacillus subtilis* DesK kinase responds to temperature changes by detecting membrane fluidity utilizing its transmembrane domains [9]. Light-sensing PAS and GAF domains have been identified that utilize FMN or Biliverdin cofactors [10–13]. Small ligand nutrients such as amino acids and carboxylic acids are detected directly by a wide variety of domains in the extracytoplasmic space, for instance by the PAS domains of CitA (citrate ligand) [14] and the tandem PAS domain of KinD (pyruvate ligand) [15] or the all α -helical domain of NarX (nitrate ligand) [16]. The *Salmonella typhimurium* PhoQ HK utilizes a PAS domain to detect small antimicrobial peptides and Mg^{2+} -ions [17]. The NreB kinase detects oxygen concentration directly by utilizing a cytoplasmic PAS domain with an $[4Fe-4S]^{2+}$ cluster [18]. Other kinases have been shown to interact with proteins. This includes the quorum sensing LuxQ tandem PAS domain, which does not bind a signal directly but interacts with a periplasmic protein LuxP, which in turn interacts with a small auto-inducer ligand [19]. Activity of the essential WalK kinase of *B. subtilis* is regulated by interaction with transmembrane helices of WalH and WalI but no small molecule ligands are currently known [20]. There are numerous other examples in the literature with known ligands or signals, but the fact of the matter is that even for the most scrutinized signaling system the identity of the molecular ligand or signal often remains unknown (Fig. 1B. and 1D.).

Extracytoplasmic sensing domains and signal transmission

Although there are soluble kinases that do not span the membrane and so-called intramembrane sensing kinases that lack extensive extracytoplasmic domains [7, 21], the majority of kinases has one or more extra-cytoplasmic domains. Since these domains share little sequence identity, sequence characterization into Pfam domains had been lacking until a flurry of individual structures has identified the most common domain elements found in this cellular compartment.

Extracytoplasmic PAS aka PDC domains

Perhaps the most common extra-cytoplasmic domain is the PAS-like (PER-ARNT-SIM) domain that is more readily identified as a cytoplasmic signaling domain and found in a variety of signaling proteins, not just in HK [22–24]. The periplasmic PAS-like domain has also been referred to as a PDC domain (named according to its first three structural members PhoQ, DcuS, CitA), owing to structural differences with cytoplasmic PAS domains in their N- and C-terminal α -helical elements [25]. The domain was first observed in sensing domain structures of the CitA citrate sensing kinase of *Klebsiella pneumoniae* [14, 26] and the DcuS fumarate sensor of *Escherichia coli* [27] (Fig. 1B.). These two examples are PAS-domains that bind their respective ligand in a ligand-binding pocket. A divergent PAS-like domain not featuring a ligand-binding pocket was also described in the PhoQ sensor of *S. typhimurium*, which detects antimicrobial peptides [17]. This domain uses its membrane exposed surface to bind to and detect its ligands. The PhoR extra-cytoplasmic domain of *B. subtilis* shows a rudimentary PAS domain without a ligand-binding pocket [28], consistent with experimental studies that demonstrated the domain is not needed to induce the phosphate starvation response mediated by this HK [29, 30].

Tandem-PAS-like domains have also been described, featuring two consecutive extracytoplasmic PAS domains. One example is the LuxQ quorum sensor domain, which

does not directly bind a ligand but instead detects the presence of an auto-inducer signal bound LuxP protein. LuxP features a periplasmic solute binding domain fold (PBP) [31]. Direct small molecule detection by tandem-PAS domains has also been observed. One example is the sporulation kinase KinD, which was crystalized with a pyruvate ligand and shown to interact with several monocarboxylic acids [15]. In all known instances of tandem-PAS domains with small molecule ligand pockets, the ligands are detected by the membrane distal PAS domain. The function of the membrane proximal PAS domain however remains unknown (Fig. 1B.).

All α -helical sensing domains

Another common class of sensing domains found in the extra-cytoplasmic portion of HK are the all α -helical domains first observed in the chemotaxis receptors Tar and Tsr and known to bind amino acids [32]. Distinct but topologically similar all α -helical domains have since been described in the HK NarX, which directly binds and responds to nitrate/nitrite ligands [16]. The ligand binding sites differ between NarX and Tsr. NarX has a single binding site located at the interface between two monomers forming a dimer, whereas in Tsr each monomer of the dimer contributes an individual binding site [16, 33]. The TorS sensor kinase also features an all α -helical sensing domain of similar topology [34]. In contrast to the other domains and in analogy to LuxQ, TorS does not directly detect its stimulus ligand. Instead, it binds the protein TorT, which harbors the ligand-binding pocket for the alternative electron acceptor trimethylamine-N-oxide [34]. In analogy to LuxP this domain features the PBP fold. Thus, both, PAS as well as all α -helical signaling domains share the propensity to interact with and to transmit signals in response to PBP binding partners (Fig. 1B.).

Other sensing domains

Some HK proteins utilize PBP directly as signal binding domains. Another domain found in some kinases is the metal binding NIT domain [35]. Neither of these domains has received much experimental attention probably because of their scarcity. Given the vastness of the microbial sequence space other signal domain folds are bound to be discovered, however it appears that the most common domains are now known [4].

The signal transmission mechanism

With the diversity of signal detection domains, the question arises as to whether signal transmission through the membrane to the cytoplasm is conserved across these different kinases. Bhate *et al.* observed that most sensor domains have in common an extended helix at the dimer interface that is proximal to the ligand-binding domain and connects the former to the transmembrane helices [8]. They termed this helix the p(eriplasmic)-helix and suggested that ligand binding exerts a conformational change onto this helix which in turn effects the structure of the transmembrane region. Interestingly, in some structures this helix connects to the N-terminal transmembrane helix (e.g. PhoQ) and in others to the C-terminal transmembrane helix (e.g., NarX).

Several models for signal transmission have been described. Studies focusing on the chemotaxis receptor Tar utilizing disulfide cross-linking approaches suggest an asymmetric piston shift model, whereby one of the two p-helices shifts into the cytoplasm [36, 37].

Structures of various sensing domains in apo- and ligand-bound states have also suggested two other possible modes for signal transmission, namely scissoring or helical rotation of the two p-helices. The scissoring mechanism has been proposed based on experimental evidence for PhoQ [38], and the heparin binding kinase BT4663 [39]. Helical rotation appears evident in LuxQ, which upon binding of ligand occupied LuxP transitions into an asymmetric complex. This requires rotation of the transmembrane helices [19]. Helical rotation has also been implied for the chemotaxis receptor McpB of *B. subtilis*, which features a tandem-PAS sensing domain like LuxQ [40, 41].

A detailed analysis of structures of PhoQ and Tsr ligand binding domains in apo- and ligand-bound form suggests that piston shift and scissoring mechanism are not mutually exclusive and that at least for these two proteins conformational changes in the p-helix are observed consistent with a combination of these two modes [8]. Consistent with disulfide crosslinking studies a piston shift is more pronounced for Tsr than for PhoQ, perhaps reflecting the different architecture of the two sensing domains. It would thus seem that differing conformational changes can result in the same net output, modulation of cytoplasmic kinase activity. One general feature might be that the transition from apo- to ligand-bound state requires a transition between symmetric and asymmetric states. This has now been described in structures for Tsr, TorS, LuxQ and DctB binding domains and perhaps others [19, 33, 34, 42].

Transmembrane helical segments

It is clear that any extracellular signal detection has to be transduced to the cytoplasm via the transmembrane helical segment. Most transmembrane sensor kinases have two helices in the monomer and based on disulfide crosslinking studies it is believed that within the dimer this segment is organized as a four-helix bundle [8]. Of note, more extensive transmembrane helical segments are also evident in several kinases including the temperature sensing DesK kinase and the sporulation kinase KinB [9, 43, 44]. To date, crystal structures of this segment are still elusive and NMR structures of a series of HK proteins solved in micelles were monomeric, giving little information about the structure of these segments in an intact protein [45].

In lieu of dimeric structures, molecular dynamics simulations coupled with experimental techniques such as site directed mutagenesis and/or disulfide cross linking have been used in order to model transmembrane segments of, e.g. PhoQ and WalK [46–48]. For PhoQ these studies suggested that a water pocket created by a polar residue at the core of the transmembrane domain is important for signal transduction. Disulfide cross-linking data on this segment was consistent with a two state model for the transmembrane segment involving diagonal displacement of the transmembrane helices [38]. Of note, the WalK kinase was shown to be regulated through transmembrane helical interactions with its auxiliary proteins WalH and WalI [20, 48].

A structural model for the transmembrane segment of WalK could be assembled utilizing replica exchange molecular dynamics simulations. Addition of WalI or WalH transmembrane segments in these simulations resulted in a significant conformational change of the segment, similar to what is observed for PhoQ [46]. Site directed mutagenesis

studies proved consistent with WalK-Wall interaction, which is primarily mediated by conserved hydrophilic residues at the interface. Thus, the transmembrane segment not only serves as a signal transducer but can also receive signal input through interactions with other transmembrane proteins utilizing polar residues.

Another extensively studied transmembrane segment is that of DesK, a thermal sensor activated once temperatures drop below 30°C. DesK has five transmembrane helices per monomer and the dimer thus features a ten transmembrane helical segment [49]. Systematic reduction of this element showed that a single transmembrane helix is sufficient and identified three hydrophilic residues at the membrane/water interface to be crucial for temperature sensing [50]. In vitro studies utilizing artificial membranes of various thickness demonstrated that DesK detects temperature by measuring membrane thickness [50].

These three specific studies demonstrate that signal transduction and signal detection by the transmembrane segment commonly involves polar residues. It remains to be seen if this is a common feature among many HK.

Cytoplasmic signal transduction and signal detection elements

Many HK feature one or multiple domains as direct C-terminal extensions of the transmembrane helical elements. These either serve to transmit the N-terminal signal to the C-terminal catalytic domains or serve as additional signal input domains. The best studied of these is the HAMP domain found in roughly 30% of all HK. Additional elements discussed here are the PAS, the GAF and the newly identified STAC domain (Fig. 1.).

The HAMP domain

HAMP (histidine kinases, adenylyl cyclases, methylaccepting proteins, and other prokaryotic signaling proteins) domains in HK proteins are typically found as immediate C-terminal extension of the C-terminal transmembrane helix [51]. Thus any conformational change in the transmembrane helical segment directly arrives at this domain and needs to be propagated towards the C-terminus. Multiple HAMP domain structures have been solved and reveal a dimeric parallel four-helix bundle architecture, where helices one and two of each monomer are connected via a loop region [52] (Fig. 1C.).

Several models have been proposed for the signal transduction mechanism through the HAMP domain. The gearbox rotation model stems from the first HAMP domain structure [53]. This structure featured an unusual knobs-to-knobs coiled-coil packing and the authors proposed that a second conformational state of this domain might exist, in which a knobs-into-holes mode more typically seen for homotetrameric coiled-coils would be observed [53].

Additional structures and disulfide cross-linking studies have led to an alternative scissoring model, which suggests that the domain shuttles between two alternative states observed in crystal structures, one where the helices are tightly packed and another where helices move out at the C-terminus for a looser packed bundle [54]. The physiological relevance of these conformational states was supported by the fact that introduction of helical cross-links

resulted in constitutive off or on mutants, depending on the location of the cross-link [55, 56].

A third model proposes that the HAMP domain transitions between dynamic and static states, whereby the static, tightly packed state keeps the kinase in an inactive form and the dynamic more unstructured state allows for activation of the kinase [57, 58]. Such unstructured states have been observed in a HAMP domain associated with adenylate cyclases but thus far not for HK HAMP domains [59].

All three models have in common that the HAMP domain exists in two conformational states and that transition between the conformations is crucial for kinase activation. The HAMP domain is typically a feature of transmembrane HK and is crucial for signaling in those proteins where it has been studied. It would seem reasonable to expect that similar elements are necessary for the function of all transmembrane kinases. However, this is clearly not the case, raising the question why some HK do and others don't require a HAMP domain for proper functioning. There does not seem to be a clear correlation between the presence of HAMP domains and the presence or absence of other extra-cytoplasmic or cytoplasmic sensing domains. One clue as to why some HK use a HAMP domain and others do not, comes from the structure of CpxA. Here contacts between the HAMP domain and the catalytic kinase domain can be observed, locking the kinase in an inactive conformation [60]. Whether such interactions are physiologically relevant and representative for other HAMP containing kinases remains to be seen.

STAC domain

Another possibility for the absence of HAMP domains in most transmembrane HK is that other kinases feature distinct domains with similar functions. In this light the discovery of the STAC (SLC and TCST-Associated Component) domain family detected in the HK CbrA of *Pseudomonas* [61, 62] might be of interest. STAC domains occur in the same general position as HAMP domains as C-terminal extensions of a transmembrane helix. Unlike the HAMP domain, the STAC domain is a monomeric antiparallel four-helix bundle. It commonly connects a multi-transmembrane region identified as solute carrier 5(SCL5)-like domain to a coiled-coil region in HK proteins [61]. At the moment it is not clear if this domain serves a similar function to the HAMP domains in their respective proteins. The sole available structure of this domain however does not show a groove or cleft that would suggest small molecule binding [61]. This recent discovery demonstrates that other domains of low sequence conservation connecting transmembrane and cytoplasmic domains of HK might still await discovery.

Cytoplasmic Sensing Domains PAS & GAF

C-terminal of the transmembrane, and HAMP elements, many kinases feature domains with the potential to integrate additional cytoplasmic signals. Most common are the cytoplasmic PAS domain and the GAF domain (c-GMP-specific and c-GMP-stimulated phosphodiesterases, *Anabaena* adenylate cyclases and *E. coli* FhlA), which respectively comprise a five or six stranded anti-parallel β -sheet of similar topology. Although nearly 40% of all sensor kinases contain either one or both cytoplasmic domains (PAS and/or

GAF), the roles for most of these domains remain unresolved (Fig. 1D.). In those instances where a role has been identified, these domains are commonly occupied by a co-factor and detect intracellular stimuli such as gases and light directly [4].

O₂ represents a substrate that is involved in many molecular pathways. Therefore, many aerobic, anaerobic and facultative anaerobic bacteria harbor O₂ measuring HK proteins [63]. For example, the cytoplasmic sensor kinase NreB from *Staphylococcus carnosus* responds directly to O₂ and controls together with the RR NreC the expression of genes of nitrate/nitrite respiration [64]. NreB harbors a PAS domain featuring four conserved Cys residues which form an [4Fe-4S]²⁺ cluster. In the presence of O₂ the [4Fe-4S]²⁺ cluster degrades to the unstable [2Fe-2S]²⁺ cluster. The lack of the [4Fe-4S]²⁺ cluster leads to reduced kinase activity. However, the molecular signal transduction mechanism remains unresolved as there are no available crystal structures [18, 64, 65].

The PAS domain of NreB is strikingly similar to the heme-binding PAS domain of the cytoplasmic sensor kinase FixL of *Bradyrhizobium japonicum* (BjFixLH). The FixL/FixJ TCS controls the expression of N₂-fixation genes in response to oxygen [66]. In the absence of the O₂ ligand the heme-binding domain permits kinase activity and in the presence the kinase is inactivated [67].

From structures of this domain in apo- or O₂-bound form, conformational changes are evident. These are mainly localized to the heme-coordinating and/or ligand-stabilizing residues [67–69]. Long-range conformational changes are observed in the protein, driven by relaxation of steric interactions between the bound ligand and the amino acid side chains and/or changes in heme stereochemistry [66]. However, a well understood signal transduction mechanism from the N-terminal heme-bound PAS domain to the C-terminal HK domain has not been forthcoming from these studies.

The signal transduction through PAS domains is mostly based on speculations. While it is not clear whether all PAS domains induce similar structural changes to modulate kinase activity, recent data points to conserved mechanisms since often sensor and catalytic domain are interchangeable. The group of Möglich was capable to replace the N-terminal oxygen-sensitive PAS-B domain of the FixL sensor kinase with the flavin-mononucleotide (FMN)-binding light-oxygen-voltage (LOV) photosensor domain from *B. subtilis* YtvA. In the dark, the engineered fusion kinase YF1 is capable of phosphorylating the RR FixJ to an extent similar to the native kinase FixL. A blue-light absorption causes an enhanced YF1 phosphatase activity, which results in a more than 1000-fold decrease of phosphorylation of RR FixJ [10, 11]. Chimeric HK, e.g. a Tar-EnvZ chimera and a NarX-Tar chimera, with altered signaling properties have been constructed before [70, 71]. The study of Möglich proves that the general approach is also applicable on cytoplasmic signaling domains.

Helical linkers

An important structural feature for signal transmission from the sensing domains to the catalytic core of the proteins is the short helical linker connecting input and catalytic domains. A first observation about this region came from Möglich *et al.* who observed that this region has a typical length of multiples of seven residues and utilized this knowledge to

generate the above mentioned hybrid protein [10]. The structure of CovS provides structural insights into this region and it is evident that the linker might serve to translate symmetric conformational changes in N-terminal signaling domains such as PAS and HAMP to asymmetry in the activated catalytic core [72]. Bhate *et al.* observed that unlike the dimeric interfaces in PAS and HAMP domains, the linker regions are often rich in polar residues, buried at the dimer interface [8]. They speculated that this feature of the linker introduces tension; in turn no symmetric conformation is significantly stabilized allowing the linker helices to bend asymmetrically. This can be observed in structures of DesK and an artificial HAMP-EnvZ chimera [9, 73]. Consistent with that notion is the observation that mutations in this region greatly affect kinase signaling [8]. A recent study on DesK utilizing computational modeling and simulations and structure-guided mutagenesis suggests that a coiled-coil stabilization/destabilization mechanism mediated through helix stretching and rotation leads to an asymmetric kinase competent state [74].

Kinase activation

The HK catalytic core comprises two domains, the so-called dimerization and histidine containing domain DHp and the ATP-binding domain known as the CA domain (Fig. 1D.). The latter is described by a single Pfam protein family, the HATPase_c domain family also found in other enzymes. This domain features several conserved residues that are involved in ATP and metal ion coordination. The overall fold is a five-stranded β -sheet flanked by three parallel helices on the side of the ATP-pocket. The DHp domain can be subdivided into multiple Pfam sequence models, captured either by the dominant HisKA domain and the less frequent HisKA_2, HisKA_3 and his_kinase domains. These sequence families identify most but not all HK. Experimental structures exist for three of the four subfamilies revealing a similar fold, with monomers forming two antiparallel helices connected by a loop region. The DHp domain typically forms a stable homodimer revealing a four-helix bundle architecture; to the contrary the single existing HisKA_2 structure reveals a functional monomeric architecture [75]. Despite the similarities in structure we caution that based on current evidence the separation into different sequence families appears to reflect differences in mechanism of autophosphorylation as discussed below. This aspect of HK diversity is often overlooked in the analysis of available data and structures.

Autophosphorylation mechanism

The above-described input domains serve the purpose to transition the HK between active and inactive states. To understand structurally the different conformational states of the kinase is at the heart of understanding the signal transduction mechanism. The initial catalytic step in two-component signal transduction is the phosphorylation of the Ne atom of a conserved histidine in the DHp domain by the γ -phosphoryl group of ATP. Experimental structures of the kinase competent state had long been elusive and in the absence of such structures, bioinformatics and molecular dynamic simulation studies combined with cross-linking and mutagenesis was originally employed to deduce this conformation for HK853 as a representative for HisKA-type [76] and DesK as a representative for HisKA_3-type kinases [77]. Dago *et al.* utilized a bioinformatics approach termed direct coupling analysis (DCA) [76]. This analysis is capable of identifying co-evolving contact residue pairs

between interacting proteins and protein domains from vast sequence alignments [78]. Applying this technology to HisKA type HK, the authors identified residue contacts consistent with structures of kinase incompetent states and other contacts consistent with a putative kinase competent state, in which ATP and histidine could be simultaneously in phosphotransfer distance. Utilizing these sequence-derived contacts they employed MD simulations to model the active conformation of the kinase and supported their model by repairing a non-functional DHP-CA hybrid kinase. Subsequent experimental structures of CovS and VicK trapped in the kinase competent state confirmed this initial model to be remarkably accurate with all DCA derived contacts made in the crystal structures [72, 76]. Since these contacts were derived from sequences of all HisKA type HK, it can be concluded that the available structures are representative for all kinases of this class.

For DesK a docking approach using Haddock software was utilized to deduce the active state conformation [77]. The resulting structure is remarkably different from that predicted and later experimentally confirmed for HisKA-type HK. In essence, the CA domain in this structure rotates almost 180 degrees in respect to the DHP domain in the proposed model. The model was experimentally supported by mutagenesis and cross-linking approaches. An experimental structure trapped in the phosphorylation competent state would truly clarify the models accuracy. In addition, more structures of HisKA_3-type HK are needed to clarify if the proposed model is representative for this class of HK proteins.

Cis vs trans

When autophosphorylation as a feature of TCS HK was first discovered in stable dimeric HK proteins, an initial question asked was whether these proteins phosphorylate in cis (i.e. each monomer phosphorylates itself) or in trans (i.e. each monomer in the dimer, phosphorylates the other). Elegant studies by Ninfa and colleagues for the nitrogen sensor NtrB demonstrated that this reaction occurs in trans [79]. Similar studies on the structurally distinct CheA chemotaxis kinase also demonstrated a trans-mechanism supporting the notion that perhaps all HK phosphorylate in trans [80]. This notion was not challenged until several laboratories showed cis phosphorylation in HK, e.g., the structurally resolved *Thermotoga maritima* kinase HK853 [81] and *E.coli* kinase ArcB [82].

As mentioned, the bioinformatic contact analysis DCA suggested a generic autophosphorylation mechanism for all HisKA-type HK. Then how can both, cis and trans mechanism be accommodated by these kinases? The answer stems from comparison of the four-helix bundle architecture of the DHP domains of EnvZ and HK853 that show a reversal in rotation of the four-helix bundle [83]. This observation has led to the conclusion that kinases with a left-handed four-helix bundle phosphorylate in cis and those with a right-handed fourhelix bundle phosphorylate in trans (Fig. 2A.). Thus the determinant for cis and trans phosphorylation is the DHP loop that connects the two individual helices of the domain. In support of this notion engineered kinases with altered loop handedness show reversal of the phosphorylation mechanism from cis to trans and vice versa [84, 85].

One example often ignored that does not fit the right handed versus left handed scheme is DesK, which is believed to phosphorylate in trans [77], yet has a left-handed four-helix DHP bundle. As explained earlier however, DesK contains a HisKA_3 type DHP domain and it is

possible that the differing autophosphorylation mechanism is a feature of this subclass of HK (Fig. 2C.). In fact, the above-mentioned model for the DesK phosphorylation competent state, with a 180 degrees rotated CA domain [77] is entirely consistent with this notion. It is possible that HisKA_3 type HK have a kinase competent state distinct from that of the now wellappreciated HisKA-type kinase state (Fig. 2C.).

Monomeric vs dimeric activation

To date all studied examples of HK of the large class of HisKA kinases as well as the HisKA_3-type kinase DesK have been demonstrated to exist as stable homodimers. In these HK signal dependent activation occurs through conformational changes within the homodimer. In contrast, a recent study demonstrated that the HisKA_2-type kinase EL346 from *Erythrobacter litoralis* exists as a monomer and that signal detection and signal mediated activation does not require dimerization [75]. Instead the structure of this protein suggests an activation mechanism where a blue light sensitive LOV domain competes with the CA domain for the phosphorylatable histidine residue on the DHp domain. The proposed model is that the light signal alters the affinity of the LOV domain for the DHp domain and thereby activates or inactivates the kinase.

At this point it remains unknown whether this is an oddity for this particular kinase or whether perhaps all HisKA_2-type HK follow a new paradigm of monomeric signaling and activation. Since this protein is a monomer, naturally, the phosphorylation occurs in cis (Fig. 2B.), but a kinase competent state has not yet been modeled or proposed for this kinase. It is thus not clear whether it would look similar to that observed for HisKA-type kinases or not. To summarize the above paragraphs, it appears that classification of DHp domains into various Pfam-domains captures important functional features related to autophosphorylation mechanisms rather than being physiologically inconsequent.

Asymmetry in kinase signaling

While the initial in silico derived model for HisKA autophosphorylation [76] proved consistent with subsequent experimental structures it failed to capture what is now considered an important aspect of kinase activation, the transition between inactive symmetric and active asymmetric states. Bhate *et al.* describe a detailed analysis of more than 20 available DHp-CA structures and find that kinase-incompetent conformations or RR bound kinases are typically symmetric, whereas activated versions of kinases, and particularly those that are in a kinase competent state are highly asymmetric [8]. It appears to be a feature that a kinase active conformation with close proximity of ATP and histidine is only observed in one of the two subunits of the homodimer. The latter observation is consistent with biochemical studies for NRII and HK853 that showed accumulation of hemi-phosphorylated dimers [85, 86] (Fig. 3A.).

Phosphotransfer

One of the most intriguing features of two component signaling is the communication that has to occur between HK and RR proteins to translate a signal into the appropriate response. This interaction has the added requirement to provide specificity to exclusively connect

correct signal and response pairs in the light of heavily amplified protein folds (reviewed in [5, 87]). Kinetic preference for paired partners has been observed in extensive biochemical phosphorylation studies [88, 89] and a significant body of literature on the existence and relevance of cross-talk between systems has been reviewed elsewhere [90].

For phosphoryl group transfer the RR docks onto the DHp domain and catalyzes its own aspartyl phosphorylation, utilizing the phospho-histidine as a substrate (Fig. 3D.). As a side note, since the RR itself is an enzyme that catalyzes its phosphorylation it might not come as a surprise that some RR are capable of utilizing acetyl-phosphate as a phosphor-donor as well. Cases for physiological relevance of acetyl-phosphate based phosphorylation have been documented and reviewed elsewhere [91].

Histidine kinase – response regulator interaction

Identification of the crucial interaction between HK and RR in two-component signal transduction was first attempted by alanine scanning mutagenesis of the surface of the Spo0F RR, thus identifying the protein surface crucial for the interaction with its kinases [92]. A first structural view of this interaction consistent with the alanine scanning mutagenesis was obtained in the form of the *B. subtilis* Spo0F/Spo0B complex structure [93]. Spo0B itself is not a HK and instead serves as an intermediary phosphotransfer protein shuttling a phosphoryl group from RR Spo0F to Spo0A [94]. Nonetheless, the structure of Spo0B is remarkably similar to HK proteins featuring both a DHp type four-helix bundle and a rudimentary CA domain that has lost the ability to bind ATP [95]. The notion of Spo0B/Spo0F providing a structural representative for HK/RR interaction was later confirmed and refined when the first true HK/RR structure of *T. maritima* proteins HK853 and RR468 was published [96]. Both structures featured identical orientations of DHp and RR domain with significant contacts involving helix $\alpha 1$ of the kinase and helix $\alpha 1$ and the $\beta 5$ - $\alpha 5$ loop of the RR (Fig. 3.). The latter structure also displayed additional contacts between the helix $\alpha 2$ in the DHp domain and helix $\alpha 1$ of the RR. These contacts are not realized in the Spo0B/Spo0F structure due to a slightly different orientation of this helix in Spo0B.

The Spo0B/Spo0F co-crystal structure allowed for investigation of conservation or variability of interaction surface residue positions; it became clear that many contacting residues at the interface are correlated but variable to give rise to specificity of the HK/RR interaction [96]. This initial observation along with rapidly expanding sequence databases captured the interest of bioinformaticians to deduce if specificity determining residue positions can be extracted from sequence alignments. Initial efforts employed local covariance measures utilizing alignments of functional HK and RR pairs [97–100]. It became clear that such local measures can identify some of the interacting residue positions pairs but that they are also quite noisy. Many residue pairs that are not at the interaction surface also show high correlation. Information from structures and covariance analysis was however sufficient to change specificity of HK and RR to non-native interaction partners [101, 102].

From a structural perspective the accuracy of local covariance approaches in deducing protein interaction parameters however was disappointing. Weigt *et al.* hypothesized that this

inaccuracy stems from cumulative (and thus indirect) correlations of jointly interacting residues along correlation chains and applied a statistical inference step to eliminate such indirect correlations [78, 103] (Fig. 3C.). This resulted in a highly successful sequence based protein interaction prediction algorithm termed direct coupling analysis (DCA). Applied to the HK-RR complex, it perfectly captures interaction residue pairs (Fig. 3.) and Schug *et al.* showed that such information was sufficient to recapitulate the HK-RR complex from individual proteins [104, 105]. The same approach was later utilized to deduce the first accurate kinase competent structural model for HK, as mentioned above [76, 106]. While first developed on and applied to two-component signaling proteins, DCA and its iterations have proven to be an immensely useful technique for the determination of unknown protein structures and protein interactions [107–110].

The correlation analysis was only possible since most HK-RR pairs are organized in chromosomally adjacent operons and functional pairs can hence be easily deduced. A significant fraction of RR and HK however are so-called orphans without partner proteins in chromosomal proximity. Predicting the correct pairs is a significant problem for complex bacteria such as *Myxococcus xanthus*. Several co-variance and DCA-based approaches have been published aimed at predicting orphan interaction partners bioinformatically utilizing the sequences of chromosomally adjacent HK/RR pairs as training sets [98, 99, 111]. In particular, DCA-based approaches prove highly accurate in deducing chromosomally adjacent partners not included in the training set. However, as noted by Burger and van Nimwegen many orphan HK and RR are statistically distinct from the paired proteins making prediction algorithms trained on the sequences of chromosomally adjacent HK/RR pairs only of limited value [99]. The prediction of RR-HK orphan partners is still an outstanding problem.

Response regulator phosphorylation by S/T kinases

In addition to the paradigm histidine-aspartate phosphotransfer reaction, recent studies have identified serine/threonine kinases capable of phosphorylating RR proteins (reviewed in [112, 113]). Initial in vitro identification of potential phosphorylation sites of *Staphylococcus aureus* GraR [114] and VraR [115], *Mycobacterium tuberculosis* DosR and Rv2175c [116–118], *Streptococcus pneumoniae* RitR [119] and RR06 [120] and Group A and B *Streptococci* CovR [121] have been followed by physiological studies of RR06 [120], CovR [122] and *B. subtilis* WalR [123]. The molecular mechanism of these reactions remains poorly understood. Some molecular insights stem from studies of WalR. This essential RR involved in cell wall homeostasis regulation was shown to be subject to phosphorylation on threonine residue 101 by the S/T kinase PrkC [123]. Position T101 is a moderately conserved residue position among RR proteins. Nonetheless, the authors were able to show that PrkC exhibits strict specificity for WalR. The determinants for this specificity remain unknown. Future work will need to aim to understand the mechanism of this phosphorylation and the interplay of aspartyl and threonine phosphorylation on RR proteins. In fact, unlike aspartyl phosphorylation that involves the same residue position on all RR protein, S/T phosphorylation appears to occur at different sites depending on which kinase and RR is involved. It seems plausible that there is no conserved outcome of S/T phosphorylation. For WalR it appears that T101 phosphorylation results in RR activation.

Since this site is localized to the dimerization interface, it is plausible that T101 phosphorylation stabilizes the active dimer conformation of this protein [123].

Response Generation

The RR protein is the key element to execute the specific cellular output in response to the input detected by the HK. By simply exploring the vast number of annotated RR among all sequenced genomes (currently there are more than 80,000 annotated RR according to the prokaryotic TCS database [2]), one quickly stumbles over the fundamental question: ‘What are the molecular determinants for the specificity of the response mediated by the RR?’. It was suggested that only a modular architecture of the RR can guarantee to combine a specific stimulus to a specific cellular response. Indeed, early studies on the RR superfamily revealed a modular architecture of the typical RR, usually comprising two domains. The prototypical RR contains a conserved N-terminal receiver domain (REC), which is connected to a highly diverse C-terminal effector domain by a variable linker. The REC domain of the RR protein catalyzes the transfer of the phosphoryl group from the associated HK onto itself. This results in self-activation in a phosphorylation-dependent manner. The activated effector domain of the RR in turn triggers the specific cellular output response. The modular architecture of the RR is observed for the vast amount of known RR, combining any imaginable signal to a cellular response. Therefore, the RR is one of the most intriguing and intensively studied bacterial proteins [124].

We will focus on the molecular determinants for the output specificity of prototypical RR proteins, i.e. those that are modular and phosphorylation dependent. However, it should be mentioned that a variety of atypical RR exist. Some so-called stand-alone RR are not modular but utilize the REC domain for both, input and output. One example is CheY. CheY is involved in chemotaxis and modulates motility by direct protein-protein interaction with flagellar motor switch proteins. [125]. Other atypical RR can function as phosphorylated intermediates in a phosphorelay pathway, either as single domain RR such as Spo0F in the sporulation pathway from *B. subtilis* or as a hybrid HK such as the heparin binding kinase BT4663 from *Bacteroides thetaiotaomicron* [39]. Furthermore, it should be noted that some RR function phosphorylation- and kinase-independent. For example, the production of the antibiotic Jadomycin B in *Streptomyces venezuelae* involves the atypical RR JadR1. Presumably Jadomycin B directly binds to the N-terminal REC domain of JadR1 leading to inactivation and dissociation of JadR1 from its target promoters [126].

Structural insights into the prototypical receiver Domain (REC)

Since publication of the first structure of a RR a common theme for its activation/inactivation has emerged. Nearly 20 years ago the crystal structure of the stand-alone RR proteins CheY and Spo0F allowed to describe in detail the molecular mechanism for the activation of the RR superfamily [127]. Indeed, several years later the same mechanism was observed for the prototypical RR PhoB and NarL from *E. coli* that consists of two domains, an N-terminal REC domain and a C-terminal DNA-binding effector domain [128, 129].

Conserved residues and activation of the receiver domain

The typical RR REC domain consists of roughly 120 amino acids and exhibits a doubly wound α/β fold with a five-stranded parallel β -sheet encompassed by two α -helices on one and three on the other side (Fig. 4A.). An alignment of all known RR regulators revealed a rather low sequence similarity of $< 25\%$ between single RR. Only a few residues are highly conserved within the RR superfamily. This core set of residues is involved in RR activation through phosphorylation accompanied by rearrangement of key structural elements of the RR. The active site of all common RR is comprised of three signature residues. An aspartic acid residue at the end of the third β -strand receives the phosphoryl group from the conserved histidine residue of the respective HK. Additionally, two acidic residues, usually an aspartate/glutamate and aspartate within the loop that connects $\beta 1$ and $\alpha 1$ are involved in Mg^{2+} -ion binding. This is necessary to coordinate the phosphorylation of the conserved aspartic acid (Fig. 4B.).

Other key residues are the highly conserved threonine/serine (T/S) and phenylalanine/tyrosine (F/Y) switch residues at the end of $\beta 4$ -strand and the middle of $\beta 5$ -strand, respectively. A highly conserved lysine residue at the end of $\beta 5$ -strand is important for phosphorylation dependent structural rearrangements of the REC domain leading to activation of the RR. The phosphorylation and accompanied conformational changes of the RR are in principle similar for all RR, but the magnitude of structural changes differs. The phosphorylation of the conserved aspartic acid residue leads to perturbation of the molecular surface at the $\alpha 3$ - $\beta 4$ - $\alpha 5$ face of the REC domain triggered by rearrangement of the T/S and F/Y switch residues. The principal difference between the active and inactive conformation of the REC domain is the distinct rotameric state of these two switch residues. In the active and inactive conformations, the side chains of these residues face either away from or point towards the active center, respectively. This conserved mechanism links phosphorylation on one site of the RR to structural perturbation on the distal $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface of the respective RR [124] (Fig. 4C.).

Other key residues are of structural nature and include a highly conserved proline in the loop connecting $\beta 3$ and $\alpha 3$ as well as two conserved glycine residues, one located at the N-terminus of $\alpha 3$ and the other within the loop connecting $\alpha 4$ and $\beta 5$ [130, 131].

Dimerization modes for receiver domains

Phosphorylation of the RR REC domain primes many RR for protein-protein interactions – in particular homo-multimer or dimer formation. These protein interactions are typically required for output generation. Structural elements of the REC domain that exhibit the largest structural rearrangement upon phosphorylation/activation are typically involved in dimerization of the RR. In particular, it was shown that most of the studied RR have a dimer interface that at least partially involves the $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface of the respective RR. The significance of this surface for dimerization is supported by the fact, that even some RR that work independently of phosphorylation dimerize within the $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface [132]. Many reoccurring dimerization modes have been characterized. There are two dimerization modes that involve the complete $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface (‘4-5-5’ dimer). The ‘4-5-5’ dimer displays a two-fold rotational symmetry of the $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface, which is typical and conserved

across the OmpR/PhoB RR superfamily. Within the $\alpha 4$ - $\beta 5$ - $\alpha 5$ dimer the two C-termini connecting to the effector domain face the same direction allowing dimerization or close proximity of the latter to execute the response. Furthermore, some single-domain RR exhibit an $\alpha 4$ - $\beta 5$ - $\alpha 5$ dimer such as the phytochrome-associated RR from *Cyanobacteria* [133, 134] (Fig. 5.).

Some RR dimer interfaces do not encompass the complete $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface. Such dimerization modes include the $\alpha 5$ - $\beta 5$ dimer of HupR [135], the $\alpha 4$ - $\beta 5$ dimer of FixJ and NtrX [136, 137] and the $\alpha 4$ - $\beta 5$ - $\beta 6$ dimer of spr1814 [138]. The latter might however be a crystallographic artifact as this author-identified dimer is inconsistent with computational predictions. According to protein database PISA software and supported by DCA correlation analysis (unpublished) the most likely physiologically relevant dimer involves an interface comprising of helices $\alpha 1$ and $\alpha 5$. The computational predicted interface of $\alpha 1$ and $\alpha 5$ was recently also found in DesR and VraR leading to the assumption that this mode of RR activation is representative for the NarL family [139–141] (Fig. 5.).

Interestingly some RR proteins alter their dimerization interface depending on the active/inactive conformation, respectively. For instance, some RR of the NtrC subfamily have an interchanging dimerization mode. The inactive/dephosphorylated conformation of the RR forms a $\alpha 4$ - $\beta 5$ - $\alpha 5$ dimer and the active/phosphorylated conformation is characterized by a $\alpha 4$ - $\beta 5$ dimer [142] (Fig. 5.).

In summary activation of the RR through phosphorylation provokes dimerization of the RR REC domain for many RR. However, some RR do not homo-dimerize but instead undergo heterodimer formation with other proteins. One of the best-studied examples for heterodimerization is the single-domain RR CheY that binds upon activation the flagellar motor switch protein FliM at the center of its $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface [143]. This is not a complete list of the up till now identified dimerization modes of the RR REC domain. Despite of the large amount of uncharacterized RR it is most likely that in the future other dimerization modes will be identified and perhaps reshape some of our knowledge about RR activation.

The above-mentioned structural insights into the prototypical REC domain is a summary of the most common themes of activation and oligomerization found in the most extensively studied RR. In recent years many studies revealed deviations that do not follow these general mechanisms. Spe1814, a member of the NarL subfamily RR of *S. pneumoniae* exhibits a rather unusual location of the conserved switch residues threonine/serine (T/S) and coliphenylalanine/tyrosine (F/Y). The switch residues threonine and phenylalanine are both located at the end of $\beta 4$ in contrast to the typical position of the threonine/serine (T/S) and phenylalanine/tyrosine (F/Y) switch residues at the end of $\beta 4$ and the middle of $\beta 5$, respectively. The physiological relevance of the relocation of the switch residues is still unknown. However, the mechanism that links phosphorylation on one site of the RR to structural perturbation on the distal surface of the respective RR appears to be similar to prototypical REC domains [138]. Another recent study challenges the switch residue mechanism for the activation of the RR NtrC. Based on NMR relaxation dispersion experiments and molecular dynamics simulations it was shown that the Y-T switch of RR activation is not valid for NtrC. In detail the motion of the moderately conserved Y residue

in the middle of $\beta 5$ is not needed for the stabilization of the active conformation of NtrC. Thereby the authors challenge the conventional believe that the tyrosine residue is involved in the structural rearrangements that links phosphorylation on one site of the RR to structural perturbation on the distal surface of the RR. Moreover, this residue is most likely involved in key interactions with downstream partners, such as the RNA polymerase-holoenzyme [144].

The molecular dynamic that describes the transition from the inactive to the active conformation of the REC domain has been studied extensively since publication of the first crystal structure of the active and inactive conformation. Initially the structural changes of the REC domain were considered as 'on' and 'off' states best described by allosteric conformational change. Later it was shown that phosphorylation of the REC domain stabilizes a pre-existing active conformation of the REC, thus changing the equilibrium between inactive and active conformations of the single domain RR proteins CheY and Spo0F [145–147]. For NtrX it was also shown that phosphorylation stabilizes a preexisting fraction of the REC domain population that samples the active conformation. Furthermore, it was demonstrated that Mg^{2+} binding and phosphorylation of the EL-LovR RR dramatically stabilizes the REC domain fold. EL-LovR is able to adopt a stable active conformation fold only in the metal bound phosphorylated state. Whether these regulatory mechanisms are representative for many RR remains to be seen. The above examples point to a continuous rather than a simple on/off mechanism for the transition from the inactive to the active conformation of the RR [137, 148].

Response regulator dephosphorylation

Dephosphorylation of the REC domain is a critical aspect of signal transduction to terminate a specific response and allow for adaptation. Dephosphorylation is typically guided by a nucleophilic attack of a water molecule on the phosphoryl group in the active center of the RR. Intrinsic dephosphorylation rates vary by several orders of magnitude for different RR, a necessity due to the various different RR roles. Intrinsic dephosphorylation rates are altered due to differing accessibilities of the active site for a water molecule. In particular, the amino acid choice at two residue positions C-terminal to the phosphorylatable aspartate and two positions C-terminal to the conserved threonine residue are crucial to block the nucleophilic attack of water on the phosphoryl group. Structural changes of the RR during the transition from the active to the inactive conformation of the REC domain also influences the dephosphorylation rate of the RR [149, 150]. Dephosphorylation of some RR is subject to further acceleration by dedicated phosphatases or by phosphatase activity of the associated HK in the kinase off state.

Typically, dedicated RR phosphatases enhance the dephosphorylation rate of the REC domain by guiding the nucleophilic attack of a water molecule on the phosphoryl group in the active center of the RR (reviewed in [151]). For the RR/phosphatase pair CheY3/CheX of *Borrelia burgdorferi* and CheY/CheZ of *E. coli* the dephosphorylation rate in the presence of the phosphatases CheX and CheZ is ~40 and ~100 times enhanced, respectively [152, 153]. Currently there are at least four structurally distinct RR phosphatase families: CheC/CheX/FliY [154], CheZ [155], Rap [156] and Spo0E [157].

Structures of three of the four phosphatase families in complex with their target RR (CheX, CheZ and RapH in complex with the respective RR CheY3, CheY and Spo0F) have revealed how these proteins facilitate RR dephosphorylation. The molecular mechanisms of these reactions are remarkably similar even though the phosphatase families are structurally unrelated [152, 153, 158]. All three RR phosphatases insert an amino acid side chain into the active center of the RR REC domain to mediate the nucleophilic attack of a water molecule on the phosphoryl group. Typically, these side chains contain an amide (Gln and Asn) to provide an additional functional group to further enhance the autodephosphorylation rate of the respective RR [152, 153, 158]. In addition to the amide containing side chain, the phosphatases CheZ and CheX insert an acidic amino acid (Asp143 and Glu96, respectively) into the active center of the REC domain of the RR to form a salt bridge with the active site lysine residue. This salt bridge is required for phosphatase activity of the CheZ and CheC/CheX/FliY RR phosphatase families [153, 159].

Currently, there is no structure of the fourth phosphatase family, Spo0E, in complex with its RR. Nonetheless structural data on Spo0E alone, along with mutational data suggests that members of the Spo0E phosphatase family use a similar strategy as the other three families. Spo0E features the conserved amide and acidic residues pair and both are important for catalytic activity. In contrast to the other three families, the acidic residue appears more crucial for activity suggesting that the acid, not the amide residue of Spo0E facilitates the nucleophilic attack on the phosphoryl group [160, 161]. In summary, dedicated phosphatases guide a water molecule to the phosphoryl group thereby facilitating the nucleophilic attack and HK phosphatase activity likely functions similarly [153].

Response regulator diversity

The more than 80,000 annotated RR proteins in the databases show a significant diversity in type and structure of their effector domains, thus allowing to execute any imaginable cellular output in response to an input signal. The RR superfamily can be divided into five global classes according to the nature of how the effector domain exerts its response. These classes are DNA-binding, RNA-binding, enzymatically active, protein-binding and single domain RR proteins (Fig. 6.).

Roughly 70% of the classified RR contain a DNA-binding domain and serve as transcription factors. Among them are the well-studied RR of the OmpR, NarL, LytTR and NtrC subfamilies [124]. In contrast, only 1% of all classified RR have an RNA-binding effector domain. Nearly all of the studied RNA-binding RR belong to the AmiR subfamily. Examples of single domain RR are CheY and Spo0F. CheY is an effector in chemotaxis and Spo0F a phosphorelay protein in sporulation, respectively. Well-studied examples of enzymatically active RR proteins include the chemotaxis CheB methyltransferase and the developmentally important PleD diguanylate cyclase of *Caulobacter*.

Based on the fold of the effector domain at least 35 subclasses of RR ranging from single members up to 25,000 proteins have been characterized. These subclasses are predominately named according to their best-studied members. Owing to the ever expanding sequence space there are also increasing numbers of new subclasses of RR with no identified function.

DNA binding RR

Nearly 70% of all classified RR contain a DNA-binding domain and are generally assumed to function as transcriptional regulators. For this RR class phosphorylation of the REC domain generally results in homo-oligomer, most commonly dimer formation. Dimerization of the RR is typically accompanied with increased affinity of the RR ability for specific DNA binding motifs, which are commonly direct or inverted repeats. This principal mechanism has been observed for most DNA-binding RR subfamilies such as OmpR, NarL, LytTR and NtrC [128, 142, 162, 163].

OmpR and NarL represent the largest and second largest subfamily of all DNA-binding RR and display winged HTH and HTH effector domains, respectively. Phosphorylation activation and dimerization of these subgroups follows the paradigm discussed above. Details on how phosphorylation relates to increased DNA-binding affinity and promoter activation is beyond the scope of this broad review and we refer the reader to dedicated articles [164, 165].

The third largest group of DNA-binding RR is the NtrC subfamily. Typically, these transcription factors activate σ 54-dependent promoters. In their inactive conformation these RR form dimers. Upon phosphorylation/activation they form higher-order oligomeric states (hexamer or heptamer) triggered by oligomerization of a central σ 54 binding ATPase domain. These ring-like structures induce open complex formation of RNA polymerase [166].

The LytTR subfamily of RR contains a rather unusual DNA binding fold which has only been recently experimentally determined. This DNA-binding fold differs from the more typical HTH motifs and contains mostly β -strands. Residues within the loops that connect the single β -strands enable the RR to bind DNA (Fig. 7.). Binding of LytTR RR to DNA initiates bending of the respective DNA fragment. Most likely this mechanism is applied to modulate promoter-activity [163, 167, 168].

RNA-binding RR

RNA-binding RR are rare and represent about 1% of all classified RR. To date there are only two known RNA-binding domain folds associated with RR proteins. Most RNA-binding RR belong to the AmiR subfamily that features an ANTAR effector domain. Typically, these RR regulate transcription by inhibition of termination at Rho-independent terminators [169, 170]. A small fraction of the RNA-binding RR proteins contains a CsrA RNA-binding domain. This class of RR has not yet been functionally explored. Our knowledge of this domain stems from the effector domain protein CsrA, which is involved in mRNA decay of carbohydrate metabolism genes in *E. coli*. By extrapolation, it is expected that RR-CsrA homologs might also have a function in mRNA decay [171, 172].

RR with enzymatic activity

8% of the classified RR belong to a group that combine the REC domain with various enzymatic domains involved in signal transduction. Among them is the well-characterized chemotactic methylesterases (CheB). Phosphorylation of the CheB REC domain activates

the enzymatic domain [173, 174]. Other common enzymatic output domains in RR are involved in second messenger homeostasis such as c-di-GMP. The PleD RR subfamily contains a GGDEF-type output domain that has diguanylate cyclase activity. RR with phosphodiesterase activity typically contain an EAL or HD-GYP domain. They belong to the enzymatically active subfamilies VieA and RpfG, respectively [175–179]. Regulation of RR involved in c-di-GMP homeostasis is highly sophisticated. Typically, activation of these RR can be regulated by dimerization or a relief-of-inhibition mechanism. Diguanylate cyclases such as members of the PleD subfamily that combine the REC domain with a GGDEF output domain are activated by phosphorylation due to dimerization of the enzymatically active GGDEF domain [180, 181]. In contrast, EAL domain containing RR with c-di-GMP specific phosphodiesterase activity are active as monomers. Activation of these RR upon phosphorylation of the REC domain seems to be by a relief-of-inhibition mechanism [178, 182, 183].

Other enzymatically active RR combine a REC domain with a phosphatases domain. Among them are Ser/Thr and Asp specific protein phosphatases that are members of the RsbU and CheC subfamily, respectively [159, 184, 185].

Protein binding RR

In addition to the single domain RR proteins mentioned earlier that exert their function through protein-protein interactions, there are also two known RR subfamilies with dedicated protein-protein interaction effector domains. The larger subfamily includes the CheV chemotaxis protein known to play a role in adaptation in *B. subtilis* chemotaxis. CheV combines the REC domain with the protein binding CheW domain and interacts with and regulates the activity of chemotaxis receptor kinase complexes. Phosphorylation of the RR CheV is required for adaptation to attractants during *B. subtilis* chemotaxis [186]. *Vibrio cholera* VieB is a recently described subfamily of RR proteins with a predicted protein-binding effector domain. VieB interferes with the VieSA HK/RR phosphotransfer reaction by binding and inhibiting the VieS kinase [187].

Perspective

Much information has been gained in recent years from structural and bioinformatics analysis of TCS. In particular, the output generation and the communication between HK and RR are now well understood on a molecular level. The holy grail in the molecular characterization of two-component signal transduction remains the structural characterization of a full length membrane embedded HK in the absence and presence of ligand. Only once multiple such structures become available can common themes be deduced on the molecular nature of transmembrane dependent signal transmission. The discussed structures of individual domains and domain fusions however have shed light on and allowed for hypotheses that culminated in the current appreciation of symmetric and asymmetric fluctuations of segments in the HK. A currently unresolved challenge in the study of TCS that requires new techniques and increased emphasis is the identification of input signals, which for the vast majority of even well-studied systems remains undetermined. On the RR protein side recent structures of less-common subclasses have let

to the appreciation of different dimerization modes and activation mechanisms. However, many emerging subclasses of RR remain unexplored and we are certainly in for new surprising findings on the array of responses and their underlying mechanisms executed by these proteins.

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Abbreviations

HK	sensor histidine kinase
RR	response regulator
TCS	two-component system
REC	receiver

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Research Highlights

Two-component systems are the most prevalent signal transduction pathways in bacteria

A flurry of new structures have contributed to our understanding of these systems

This review discusses common features, paradigms and deviations from the paradigm

Fig. 1.

Domain architecture of the typical two-components system. A) Schematic view of a prototypical membrane-bound sensor histidine kinase, featuring domains for signal recognition, transmission and catalysis. B) Variable extra-cytoplasmic sensor domains (SD). The most common extra-cytoplasmic SD are PAS and α -helical domains. The citrate-bound periplasmic PAS domain of CitA (PDB ID: 2J80), the sensor domain of KinD (PDB ID: 4JGO) with tandem PAS domains and the α -helical extra-cytoplasmic SD of TorS (PDB ID: 3O1H) are illustrated on the left, middle and right respectively. Ligands are shown in green and red spheres. Only the N-terminal PAS domain of KinD is responsible for ligand binding. C) Signal transduction occurs via the cytoplasmic HAMP domain. The HAMP domain of Af1503 (PDB ID: 2L7H) is shown as a representative. D) The cytoplasmic SD are responsible for intracellular signals detection and transmission. GAF (PDB ID: 4G3K) and PAS (PDB ID: 4I5S) domains are shown. E) The conserved kinase core consists of the DHp and C-terminal ATP-binding catalytic domain. The core of the kinase HK853 is illustrated (PDB ID: 3DGE). The individual dimeric structure of DHp is illustrated in green and the CA domains in red. Location of ADP and phosphorylatable histidine are shown.

(a)

HisKA autophosphorylationCis-autophosphorylation

Examples: HK853, VicK, YF1, PhoR, ArcB

Trans-autophosphorylation

Examples: AgrC, AtoS, CpxA, EnvZ, NtrB

(b) HisKA_2 autophosphorylationCis-autophosphorylation

Example: EL346

(c) HisKA_3 autophosphorylationTrans-autophosphorylation

Example: DesK

Fig. 2.

Schematic view of the different autophosphorylation modes observed for histidine kinases. The orientation of the DHp helices determines whether phosphorylation is performed in cis (phosphorylation of the same subunit) or in trans (phosphorylation of the neighboring subunit). A) Cis (left) and trans (right) autophosphorylation for HisKA-type kinases. B) Cis autophosphorylation for the monomeric kinase EL346 is a representative for HisKA_2-type kinases. C) The sensor kinase DesK is a structural representative for HisKA_3-type kinases. Experimental evidence suggests that this kinase phosphorylates in trans, but its DHp domain is orientated as cis-phosphorylating HisKA-type SK. Examples for representative kinases of each mode are listed.

Fig. 3. Complex formation and phosphotransfer of the SK DHp and RR receiver domain. A) Structural comparison of complexes of sporulation phosphorelay proteins Spo0B/Spo0F and the SK/RR pair HK853/RR468 are depicted, showing a conserved interaction mode. DHp domains, catalytic domains and RR receiver domains are illustrated in green, orange and blue, respectively. B) The interface of HK853 and RR468. Residues involved in interface formation are shown in red (HK853) and orange (RR468) spheres. The His-Asp residues involved in phosphoryl group transfer are in yellow. C) Highly correlated interface contacts identified by DCA are shown for the same protein pair. Residues involved in direct protein-protein interaction are depicted as in B. D) Close up view at the phosphotransfer active site between HK853 and RR468. Phosphotransfer is guided by the close proximity of the conserved Asp53 (yellow) and His260 (red) after complex formation. PDB IDs 3DGE and 1F51 were used.

Fig. 4. Receiver domain secondary and tertiary structure. A) Secondary structure and annotation of the structural elements of a typical RR receiver domain. (blue β -strands and red α -helices.) The additional β -strand 6 and α -helix 6 of the NarL subfamily are indicated in yellow. B) Receiver domain crystal structure of PhoP, showing the alpha/beta fold $[(\beta\alpha)_5]$ with 5-stranded parallel beta-sheet encompassed by two alpha-helices on one side and three on the other side. C) Superimposition of the active (red) and inactive (blue) conformation of the PhoP receiver domain. This illustrates the slight conformational changes upon phosphorylation of the receiver domain. The two conformational states of the switch residues T79 and Y98 are depicted in black. Beryllium trifluoride that mimics

phosphorylation of the receiver domain is shown in cyan spheres with the conserved side of phosphorylation in yellow (Asp51). D) Close up view of the active center and switch residues of the well-studied RR PhoP, CheY and Spo0F illustrating a conserved switch mechanism for the typical receiver domain. Active conformation of the switch residues are depicted in red, the inactive state is shown in blue. The following PDB IDs were used to present the different RR receiver domains (PhoP: active [2PL1] vs. inactive [2PKX], CheY: active [1FQW] vs. inactive [2CHF] and Spo0F: active [1PUX] vs. inactive [1FSP]).

Fig. 5. Dimerization modes of the most prevalent RR subclasses. Among the most common dimerization modes are the $\alpha 1$ $\alpha 5$ interface of DesR, $\alpha 4$ $\beta 5$ $\alpha 5$ interface of PhoB, $\alpha 4$ $\beta 5$ $\alpha 5$ interface of ComE and $\beta 5$ $\alpha 5$ interface of HupR as representative for their respective RR-subfamily. Structural elements involved in interface formation are colored in red, green or yellow. The RR backbone is illustrated in blue. The same color code is used for the secondary structure of each dimerization mode. The following PDB IDs were used to

present the different RR receiver domains (DesR: 4LDZ, PhoB: 1ZES, ComE: 4CBV and HupR: 2JK1).

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Fig. 6.

Classification of the response regulator superfamily. Pie chart presenting the percentage distribution of the 5 RR families among all classified RR. Further classification in subclasses is presented in horizontal bars. The percentage distribution of the subclasses within the respective RR family is indicated below. Structures of representative members of the most studied subclasses are illustrated in ribbon diagrams. The following PDB IDs were used to present the single RR effector domains (CheV: 3UR1, AmiR: 1S8N, CheB: 3SFT, PleD:

3IGN, RpfG: 1YOY, OmpR: 2GWR, NarL: 4HYE, NtrC: 3DZD, LytTR: 4G4K and YesN: 3LSG).

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Fig. 7. Full-length structure of ComE, a member of the LytTR subfamily of DNA-binding RR. A) Secondary structure and annotation of the structural elements of the RR ComE. β -strands are in blue and α -helices in red. B) Structure of full length ComE (dark blue: receiver domain and pale blue: effector domain). The PDB ID 4CBV was used to illustrate full-length ComE.