Protein Dynamics in Phosphoryl-transfer Signaling Mediated by Two-component Systems

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Running head: Phosphoryl-transfer and protein dynamics in TCS

Abstract

The ability to perceive the environment, an essential attribute in living organisms, is linked to the evolution of signalling proteins that recognize specific signals and execute predetermined responses. Such proteins constitute concerted systems that can be as simple as a unique protein, able to recognize a ligand and exert a phenotypic change, or extremely complex pathways engaging dozens of different proteins which act in coordination with feedback loops and signal modulation. To understand how cells sense their surroundings and mount specific adaptive responses, we need to decipher the molecular workings of signal recognition, internalization, transfer and conversion into chemical changes inside the cell. Protein allostery and dynamics play a central role. Here, we review recent progress on the study of two-component systems, important signalling machineries of prokaryotes and lower eukaryotes. Such systems implicate a sensory histidine-kinase and a separate response regulator protein. Both components exploit protein flexibility to effect specific conformational rearrangements, modulating protein:protein interactions, and ultimately transmitting information accurately. Recent work has revealed how histidine-kinases switch between discrete functional states according to the presence or absence of the signal, shifting key amino acid positions that

define their catalytic activity. In concert with the cognate response regulator's allosteric changes, the phosphoryl-transfer flow during the signalling process is exquisitely fine-tuned for proper specificity, efficiency and directionality.

Keywords: bacterial signalling, protein phosphorylation, allostery, histidine kinase, response regulator

1 Introduction

A vast number of organisms use two-component systems (TCSs) as an efficient means of sensing, transmitting and processing information, ultimately ensuring cellular homeostasis. Almost ubiquitous in bacteria, also present in fungi and plants, the simplest TCSs work by a concerted action of two protein components: a sensory histidine kinase (HK) and a response regulator (RR). These two components communicate by transferring a phosphoryl group from a particular histidine residue on the HK to a specific aspartate on the RR (Figure 1a). This phospho-transfer reaction can take place once the His residue is phosphorylated via an auto-phosphorylation reaction catalysed by the HK itself, using ATP as the phospho-donor substrate. The auto-kinase and phospho-transferase reactions are typically coupled, and correspond to a particular structure/functional state(s) of the HK defined as 'switched on', or 'kinase-active'. At some point the phosphorylated RR (P~RR) species is eventually dephosphorylated by hydrolysis, rendering inorganic phosphate (P_i). The latter reaction is frequently accelerated by the specific HK partner when switched off. In this kinase-inactive state, the HK acts as a phosphatase, accelerating P~RR dephosphorylation in a specific way: only the cognate HK partner and not other HKs can catalyse the dephosphorylation [1]. HKs thus constitute a fascinating example of enzymes with paradoxical activities [2], in that they catalyse reactions with opposite outcomes: the phosphorylation of its specific substrate, as well as its dephosphorylation, according to the signalling state of the pathway. Overall, a fundamental aspect behind the cascade of TCSs-mediated post-translational modifications, is that the P~RR active species, is functionally distinct from the inactive unphosphorylated

species. The biological functions engaged by the phosphorylated form of the RR, constitute the very output of the signalling cascade, ultimately orchestrating an adaptive response. The reactions involved in such cascades are tightly regulated, ensuring an accurate transmission of information. Thus, the evolution of mechanisms that minimise signal-independent activations and futile cycles has been critically important. Futile cycles could arise by uncontrolled phosphoryl-transfer and dephosphorylation of the RR (both catalysed by the same HK enzyme), which can be overcome by molecular means that separate such reactions efficiently in time.

A minimalistic two-component organization, with one HK and one RR (Figure 1a), is frequently observed in many TCSs, involved in signalling pathways that respond to a broad range of signals such as cell quorum, osmolarity, temperature or antibiotics among many others [3]. However, TCSs have also evolved in some cases into more complex linear or branched cascade systems, called phosphorelays, where additional domains and/or proteins integrate the phosphoryl-transfer circuits between the upstream sensory HK(s) and the output RR(s) (Figure 1b). Phosphorelay intermediary proteins comprise variants of the same type of domains found in simple HK:RR TCSs, often also including ***H***istidine ***P***hospho-***t***ransfer (HPt) proteins that harbour a phosphorylatable histidine residue, albeit structurally different from HKs and unable to auto-phosphorylate. Nonetheless, the mechanistic workings of TCSs and phosphorelays are thought to be similar at the molecular level, given the structural resemblance of the different modules that participate in signal transmission.

More often than not, signal sensation triggers HK activation. The opposite holds in some cases, where the signal turns off an otherwise constitutive auto-kinase activity, such as in CheA-regulated chemotaxis [4], among several other pathways [5,6]. HK ‘on/off’ switching is one of the key elements in defining the outcome of the TCS pathway, especially considering that most HKs can act both as a kinase and as a phosphatase of their cognate RR partners. Signal-sensing is known to modulate the on/off switching transition in HKs [7], a key step in TCS signalling that this review will focus on. Recent progress on understanding the mechanisms whereby activation switching is also engaged in controlling the specific

association between HKs and RRs, will also be elaborated. We shall see that HK and HK:RR functional transitions rely on the modulation of dynamic features of the proteins. Additional signalling steps such as HK autophosphorylation [8,9] and RR activation [10], are also critically dependent on protein flexibility, but will not be reviewed here.

2. Key structural features of TCS proteins

Currently over 600 three-dimensional structures of separate HKs and RRs have been reported, most of them determined by X-ray crystallography, and also by NMR [11,12]. Both TCS proteins display modular architectures, including domains belonging to a number of different classes which appear with varying frequencies in different HKs and RRs. However, few specific domains define whether a given protein is an HK or an RR (Figure 1c). Response regulators always comprise at least one receiver domain (REC), which harbours the phosphorylatable aspartate residue within a conserved α/β Rossmann-like topology fold. RRs can be single-domain proteins, or may comprise additional domains, such as DNA-binding or enzymatic modules, among many others [13]. HKs are defined by two distinct domains (Figure 1c): i) a centrally localised DHp (***D***imerization and ***H***istidine-***p***hosphotransfer) domain, which is typically an elongated all-helical module engaged in homodimerisation and includes the phosphorylatable histidine residue; and ii) a CA (***C***atalytic and ***A***TP-binding) domain, which is a globular α/β module belonging to the Bergerat fold of ATPases [14]. CA domains bind ATP and exert a slow ATPase activity. Many HKs comprise additional modular domains, for example sensory domains, trans-membrane regions, HAMP (for ***H***istidine kinase, ***A***denylyl cyclase, ***M***ethyl-accepting chemotaxis protein, and ***P***hosphatase), PAS (for ***P***er-***A***rnt-***S***im) or GAF (for c***G***MP-specific phosphodiesterases, ***A***denylyl cyclases and ***F***hlA) domains (see [11] for a review). There are also more complex hybrid architectures that include REC, DHp and CA domains in the same polypeptide [15,3]. Such hybrid TCS proteins are classified as hybrid-RRs or hybrid-HKs if the REC domain is respectively placed N- or C-terminal to the HK modules [16].

Structures of full-length RRs have been solved in active and inactive conformations, including some in complex with their cognate DNA [17,18]. In contrast, no structures of full-length trans-membrane HKs have yet been determined. The most complete picture so far corresponds to the high-resolution images of a portion of NarQ from *E. coli* [19], comprising the periplasmic sensory domain, the TM region and the first signal transmission domain (a HAMP) on the cytoplasmic portion of this HK. Comparison of apo and signal-bound crystal structures [19] revealed the workings of a signal-triggered activation mechanism. However, downstream signal transmission to the catalytic region (DHp and CA domains) remains hypothetical, as the entire intracytoplasmic region was lacking from the crystallized constructs. On the other hand, a few crystal structures of the whole catalytic portion of HKs have been determined (DHp+CA modules, sometimes with additional intra-cytoplasmic domains) [20,9,21], and in one case different conformations corresponding to distinct kinase/phosphatase functional states were captured [22]. By combining information from both sensory/transmembrane and catalytic intracytoplasmic snapshots, a common picture starts to arise which will be elaborated further below.

3. Classification of HKs in families

HKs were discovered in the 1980's [23] as enzymes that catalyse auto-phosphorylation on a conserved His residue, using ATP as the phospho-donor substrate [24]. The Bergerat fold of HK CA domains, different from Walker ATPases, is shared with other, distantly related, slow ATPases (DNA ***g***yrase, ***H***sp90 and Mut***L)*** constituting the so called GHKL superfamily [25]. Early sequence alignments revealed the essential features distinguishing HK subclasses [26]. In particular, those comparative studies identified a marked correlation of particular HK classes to RR classes, confirming that these signalling systems tend to function as two-component pairs or dyads [27]. Current HK classifications have been simplified to a fewer number of groups, notably using hidden Markov profile approaches [28], with all the HK sequences comprised within the single Pfam clan His_Kinase_A (CL0025). The classification of HKs in different families [29,30] appears to have relevant mechanistic implications.

Within the His_Kinase_A clan four different families currently classify HKs according to DHp domain sequences: i) HisKA (PFAM family PF00512), ii) HisKA_2 (PF07568), iii) HisKA_3 (PF07730) and iv) HWE_HK (PF07536). Additionally, HK can be classified according to their CA domain: HATPase_c (PF02518), HATPase_c2 (PF13581) and HATPase_c5 (PF14501), the latter including examples of HKs with novel secondary structure elements [31] compared to the most populated family, the HATPase_c.

HisKA (covering ~80% of all DHp-containing HKs) and HisKA_3 (~15%) DHp domains, as all DHp sequences, are predicted to contain two antiparallel α-helices forming a helical hairpin that, when dimerized, form a 4-helical bundle. Very few examples of monomeric HKs have been reported, corresponding exclusively to members of the peculiar HisKA_2/HWE_HK group [32]. DHp helix α1 tends to be longer than α2, hence the N-terminal portion of the typically dimeric DHp results in two parallel helices, with predicted tendency to form a left-handed coiled-coil. The conservation of hydrophobic residues in positions *a* and *d* within the coiled-coil heptad repeat pattern (or equivalent hydrophobic-core positions in undecad repeats identified in certain HKs), is far from ideal: those key positions are often occupied by polar residues instead, which reduce the energetic stability of an otherwise tight helix-to-helix binding. Early sequence alignment studies and prediction of expected helical packing behaviours [33,34] proposed a regulatory function for such marginally stable coiled-coils in the helical domain that precedes the His-containing phosphorylation site in HKs.

4. HK conformational switch: the signal regulates kinase/phosphatase activities

The proposed regulatory function of the coiled-coil region in the DHp domain of HKs was observed directly for the first time in the crystal structures of DesK from *B. subtilis* [22] (Figure 2), a HK that belongs to the HisKA_3 family. The kinase-off phosphatase state comprises a well formed left-handed coiled-coil arrangement, whereas the kinase-on states, pre- and post-phosphorylation, reveal a disrupted coiled-coil whereby the DHp helices α1 of both protomers dissociate from each other [35,22]. These crystallographic studies comparing

wild-type species and point-mutants that trap functionally relevant conformational states, showcased a successful approach to study such dynamic systems [22,36,35]. In summary, HK active states are associated with higher inter-domain flexibility and a disrupted DHp coiled-coil segment, which are in turn linked to asymmetric dimer assemblies. An inverse picture correlates to kinase-inactive, phosphatase states of HKs. Such a switching scheme based on HK flexibility regulation and substantial DHp/CA interdomain rearrangements is consistent with data gathered from several TCSs and HK families, using a variety of different approaches, among others, differential proteolysis sensitivity [37], cysteine engineering and crosslinking analyses [38,39], hydrogen/deuterium exchange assessed by mass spectrometry [40], NMR [41] and other spectroscopic techniques like electron spin resonance [42]. Crystallization of the sensory and transmembrane portion of the nitrate-sensing HK NarQ [19], including the first intracytoplasmic signal-transmission module (a HAMP domain), was a major achievement leading to the currently only available 3D structure of a HK encompassing its trans-membrane segment, detail which is key for understanding signal transduction. These crystal structures of apo and nitrate-bound NarQ consistently showcase the relevance of the DHp coiled-coil breaking/making mechanism (Figure 2), and for the first time revealed that small signal-triggered piston-like movements within the sensory domain are amplified and transduced into a large scissoring motion of the HAMP module, leading directly to DHp domain coiled-coil disruption [19,43].

The DHp conformational rearrangements that drive coiled-coil assembly and disruption are also coupled to CA domain mobility control. Indeed, major reorganization of the CA domains relative to the central helical DHp along the signalling cycle appears to be a universal feature in the regulation of all HKs, even though variations in the details of the effecting mechanism appear to exist. As mentioned above, in HisKA_3 HKs such as NarQ, DesK or LiaS among others, the DHp helices rotate as a coupled movement to the coiled-coil folding/unfolding switch. Rotational rearrangements in HKs' signalling helices had been proposed in HK activation in quorum sensing [6] and light perception [44] pathways, but had yet to be verified by structural interrogation of both conformations. A first observation was achieved in *B.*

subtilis DesK [22] and then confirmed in several other cases such as NarX [45] and NsaS [46]. Such findings pinpoint the crucial importance of the phosphorylatable histidine, which occupies a special skip position within the DHp coiled-coil heptad repeat pattern of HisKA_3 HKs, allowing for a dramatic rotational shift during activation. This rotational shift is maximal in the region surrounding the HK's phosphorylation site [22], resulting in exposure or burying of the key His to the solvent [35]. Consequently, this shift generates or obliterates a docking surface for the CA domains (Figure 2). A large DHp-CA interface burying approximately 1200 $Å^2$, is observed in the phosphatase state, leading to a symmetrically open, more rigid, butterfly-shaped structure [22]. This rigid configuration minimizes the likelihood of CA-bound ATP moieties reaching the reactive His on the central DHp. In contrast, the kinase/phosphotransferase state, with substantially reduced or no inter-domain interface, results in liberated CA domains, poised to engage in auto-phosphorylation reactions, or recruitment and phosphotransfer to the cognate RR. Such HK active states typically display asymmetric structures (Figure 2).

In the case of the HisKA family of HKs, snapshots of the same kinase in different functional states are still missing. Nevertheless, from available structures [8,20,9], key conformational rearrangements appear to be shared between HisKA and HisKA_3 proteins. In HisKA HKs the coiled-coil sequence pattern is slightly different, suggesting that the phosphorylatable His will not swing between exposed and buried conformations as in HisKA_3 kinases. Nevertheless, comparing the structures of *Thermotoga maritima* HK853 [47,20], and CpxA [9] and EnvZ [8] from *E. coli*, suggests that a transition from a symmetric to an asymmetric configuration is indeed a hallmark of HK activation.

Switching implies substantial protein flexibility, but in such a way as to populate distinct states: a form of modulated flexibility, enabling a single sequence to adopt at least two (or more) different 3D structures. This situation is different from a continuum (*e.g.* an unstructured protein), allowing specific distinct states to be defined as either active or inactive. Certainly, each one of such alternative states can also display flexibility on their own, but not confounding active and inactive species. Experimental evidence supporting

dynamic switching has mostly come from NMR studies of response regulators [10,48], for which the inactive state appears to be much more conformationally dynamic compared to the Asp-phosphorylated species. The latter is the active form, competent to initiate a response, typically acquiring a more rigid, unique 3D structure. More work is needed in this area to understand HK activation switching [49], with a seemingly opposite scenario to RRs: more rigid inactive HKs switching to flexible and partially unfolded forms in the active state. NMR-based approaches face a challenging goal since HKs are typically larger proteins compared to their RR partners, and frequently comprise transmembrane segments.

Once the HK is activated, and in the presence of physiologic high cellular ATP concentrations, HKs catalyse the autophosphorylation reaction, which is typically an asymmetric process: one His in the dimer is more rapidly/efficiently phosphorylated than the other [50,51]. This asymmetry is also consistent with the molecular features of crystal structures of HKs trapped in Michaelis complexes with ATP *en transfer* [8,9].

5. HK-RR complexes

For historic reasons the two components, HKs and RRs, have largely been studied separately. Transiently associated protein:protein complexes, such as those formed by HK:RR binding, present huge technical challenges given the spatial/temporal resolution required to study their structural and dynamic features. The 3D structures of associated His- and Asp-containing TCS proteins in binary complexes have been determined mostly for phosphorelay pathways [52-57]. The His-containing proteins of such complexes are intermediary components with no auto-kinase activity (hence not *bona fide* HKs), functioning only as carriers of phosphoryl moieties between upstream and downstream Asp-containing receiver domains. The reason for this bias in structural determination of phosphorelay complexes is not clear, and is likely related to lower intrinsic flexibility of phosphotransfer proteins compared to HKs. Although limited in number, experimental 3D structures of *bona fide* HK:RR complexes have provided extremely relevant information [47,35,58], notably showing that the reaction centres of the

phosphotransferase and phosphatase reactions are assembled with residues from both protein partners [35].

Early on, it became clear that some sort of code dictating pairwise interactions between specific HKs and RRs ("specificity code") was biologically relevant, given that dozens of highly similar RRs are being co-expressed at any given time in the cell, and that cross-talk among "non-cognate" HKs and RRs is not usually observed [59-62]. Over the past decade, important contributions have allowed specificity determinants to be restricted to a small subset of defined amino acid positions in the HKs' DHp domain [63,64], revealing that this interface is extremely plastic and tolerant to natural mutations [65]. Crystal structures of HK:RR complexes accurately explain those observations, revealing low surface complementarity and very few protein:protein contacts [35,47,58], just enough to retain affinity between the specific (or cognate) partners. The TCS DesK:DesR is currently the only system for which crystal structures of the HK:RR complex have been determined in both phosphatase and phosphotransferase states [35] using X-ray diffraction (Figure 3). Structure-based point-mutants that trap DesK in the phosphatase state (DHp coiled-coil hyperstabilisation), or that mimic its phosphotransferase form (a glutamate substituting the phosphorylatable histidine), were demonstrated to be important to stabilize the complex in solution [66]. The HK recognizes its cognate RR largely using the same interface in both states; the outcome of the pathway is dictated by the positioning of a few key residues from the HK partner in interaction with the RR's active site. Such subtle but decisive dissimilarity, explains why phosphotransfer and dephosphorylation reactions cannot be catalysed simultaneously, inhibiting futile cycles [67,35].

The phosphatase reaction. Besides the universal phosphotransferase activity that catalyses phosphoryl-transfer from the P~HK to the inactive state of the RR, most DHp-containing HKs (*i.e.* excluding CheA/CheA-like HKs) are able to accelerate the dephosphorylation of their specific P~RR partner(s) when the HK itself is not kinase-active [67]. High resolution crystal structures representing snapshots of the phosphatase state just prior to dephosphorylation [35], uncovered a highly symmetric organization of the phosphorylated

form of DesR (mimicked by covalently bound BeF_3^- to the reactive aspartate) in complex with DesK, a HisKA_3 HK. A balanced stoichiometry of two RR moieties bound to the two HK protomers, is coherent with a strongly symmetric butterfly-shaped HK dimer. This trend has also been observed HisKA HKs: the crystal structure of *T. maritima* HK853:RR468 complex reveals a strongly symmetric organization and was posited to be a snapshot of the phosphatase state [47].

A glutamine, just over one helical turn C-terminal to the phosphorylatable His, is highly conserved in HisKA_3 HKs (in some kinases substituted by an Asn, or even a Thr in the equivalent position of HisKA HKs). The polar residue in this position is proposed to facilitate the correct positioning of a hydroxyl anion, which performs the nucleophilic attack on the Asp-bound phosphate of the RR [68,35,69]. The key role of residues other than the phosphorylatable histidine for the phosphatase function of the HK is consistent with early reports [70] demonstrating that the dephosphorylation reaction is not the reverse of phosphoryl-transfer, as later confirmed [67]. The HK is not back-phosphorylated to take off the RR's phosphate. Instead, hydrolysis produces inorganic ortho-phosphate. Reversed phosphotransfer was initially proposed to be relevant in DHp-containing HKs such as EnvZ [71], but later proved to be the likely result of not using the full-length form of the HK under *in vitro* conditions [72]. Genuine reversed phosphoryl-transfer reactions have been reported in CheA-like pathways [73] and phosphorelay systems [74,75], a biologically relevant reaction course, yet distinct from HK-mediated P~RR dephosphorylation (see below). Although the role of HKs acting as P~RR phosphatases is often difficult to quantify *in vivo* [76], the physiological importance of such activity is now widely accepted [1], ensuring robustness with respect to ATP and TCS protein concentrations [2,77], and allowing for minimization of cross-talk between different TCS pathways [59,78]. The phosphatase activity of HKs has been shown to depend on the presence of ATP (or ADP) in some cases, a link that seems to hold for HisKA and not HisKA_3 HKs. The reason for this difference might be due to the ATP-lid, a loop of variable length in different HK sub-classes, juxtaposed to the ATP-binding pocket, and which often becomes ordered upon ATP binding and Mg^{2+} coordination. The

ATP-lid makes direct contacts with the P~RR partner in HisKA-containing complexes [47], but not in HisKA_3-containing ones [35], consistent with the fact that HisKA_3 HKs have evolved to include substantially shorter ATP-lid loops in (Figure 3).

The phosphotransferase reaction. In contrast to the phosphatase state, the phosphotransferase complex of DesK:DesR is strongly asymmetric, not only in the organization of the HK itself, but also in the 1:2 RR:HK stoichiometry (Figure 3). At the reaction centre, the activation-switched rotational shift of the reactive histidine region places this residue perfectly in line for the RR's aspartate to perform a nucleophilic attack on the His-bound phosphate group. For reasons that are not yet clear, while one CA domain remains freely mobile on the side that engages in HK:RR association, the other CA remains bound to the DHp through a different, and smaller, interface than the one observed in the phosphatase complex. This has been confirmed in different crystal forms under variable crystal packing environments (PDBs 3GIF, 5IUM, 5IUJ), supporting its biological relevance.

A 3D structure corresponding to a HisKA HK in complex with its RR in the phosphotransferase state is not yet available. However, it is predicted to be asymmetric [79], as observed for the HisKA HKs CpxA and EnvZ captured in nucleotide-bound Michaelis complexes poised for auto-phosphorylation [8,9]. It is clear that more complete and direct structural data of HisKA HK phosphotransferase complexes are needed. The rotational motion implicating the HK phosphorylation site and neighbouring residues in HisKA_3 HKs will likely mean that it will not be possible to switch from phosphatase to phosphotransferase states due to the different positioning of the reactive His in the coiled-coil heptad repeat register of HisKA kinases. Albeit less dramatic, a change in the relative position of the His, modulating its precise location within the active site (closer or farther away from the RR aspartate), should not be ruled out.

7. Conclusions and perspectives

Great progress is being achieved in the structural biology of TCSs. The importance of protein dynamics in populating distinct conformations, essential for the function of histidine kinases,

is thus being highlighted. The controlled flexibility within and among their modular domains, underlies the regulation of their auto-kinase activity, as well as the way they interact with their cognate response regulator partners, dictating further catalytic roles as phosphotransferases or as phosphatases. Mechanistic insights about HK activation converge toward a scenario where the CA domains' mobility is regulated by a conformational rearrangement of the DHp domain, itself triggered by the sensory region through disruption or assembly of the connecting coiled-coil.

The molecular details of HK:RR complexes have also started to reveal reaction directionality features. As stated above, more often than not *bona fide* TCSs catalyse highly irreversible P~HK→RR phosphoryl-transfer reactions (Figure 1a). Interestingly, phosphorelay pathways display many examples of reversible reactions from, and to, phosphorylatable Asp/His residues (Figure 1b). This intriguing divergence may well find its molecular basis on the distance between the phosphorylatable histidine and aspartate residues and, directly correlated with this distance, on how symmetrically the RR-bound Mg^{2+} cation sits with respect to both reactive amino acid side chains. An unexpectedly large distance (>7.5Å) was observed between the phosphorylatable residues in the DesK:DesR phosphotransferase complex [35], compatible with a nucleophilic substitution with significant dissociative character. The Mg^{2+} cation, essential for the phosphoryl-transfer reactions, is an appealing candidate to stabilize the transition state, *en transfer* phosphoryl anion: a symmetric Mg^{2+} position would allow for transfer to occur in both directions. In support for such hypothesis, available structures of phosphorelay TCS complexes reveal significantly shorter distances between the reactive His and Asp (<5.5Å), anticipating an associative nucleophilic substitution correlated with a more symmetric position of the Mg^{2+} cation. Ongoing and future work shall verify whether this mechanism underlies directionality control.

Additional structures of HK:RR complexes in different signalling states, and from different HK and RR protein families, are needed to confirm and generalize the molecular mechanisms that govern TCS signalling in bacteria. For instance, the *T. maritima* HK853:RR468 complex was proposed to represent the phosphatase state of the pathway [47] displaying a highly

symmetrical organization with a 2:2 stoichiometry and the CA domains rigidly packed against the central DHp. It is worth noting that the relative orientation of the CA domains is strikingly different compared to the phosphatase DesK:DesR complex (Figure 3) which could reflect an authentic difference between HisKA and HisKA_3 families, with still unknown functional consequences. Further structural data, exploiting techniques in addition to X-ray crystallography, are required to complete the full picture of such dynamic protein systems. Comparison of the *T. maritima* phosphatase complex with other HisKA HK autophosphorylation snapshots reveal that the location of the CA domains in the former complex are intriguingly close to the position they will likely adopt in the kinase-active state [8,9]. Furthermore, structural comparison of free HK853 [20] with the HK853:RR468 complex [47] discloses a more flexible coiled-coil region towards the N-terminus of the DHp α1 helices in the complex, supporting the hypothesis that the complex captures the phosphotransferase state or a phosphotransferase-like intermediate form.

Cryo-electron microscopy and tomography, as well as ever more powerful NMR approaches, are anticipated to play key roles in structural determination of full-length HKs and HK:RR complexes with high resolution, and unveil detailed pictures of their dynamic behaviours.

In analogy with electric engineering, an input signal (*e.g.* an antibiotic, temperature, salt, quorum, etc.) triggers a deviation from cellular homeostasis due to physical or chemical effects on the cells' components and/or metabolic status. By means of a suitable TCS, that particular signal can be detected, transmitted and processed into a 'control' output, ultimately re-establishing homeostasis, a fascinating example of exquisite biological regulation. Suitability in this context means that: i) the particular TCS's HK is able to detect the specific signal (input sensitivity); ii) the HK is able to catalyse ATP-dependent auto-phosphorylation in a regulated manner, allosterically modulated by its sensory status (HK auto-kinase on/off switching); iii) the P~HK associates to the correct RR, selecting it out from dozens of simultaneously co-expressed RRs (specificity code/proper wire connectivity); iv) phosphatase *vs* phosphotransferase activities are tightly regulated such that futile cycles and P_i loss are minimised (efficiency/lossless signal transmission); v) reversible *vs* irreversible

phosphotransfer reactions among reactive histidine- and aspartate-containing protein domains are properly modulated according to the needs of the pathway (signal transmission directionality); and, vi) the P~RR output affinity is modulated to effect the output response, be it DNA-binding, protein:protein association, and/or enzymatic catalysis of downstream substrates (output device on/off switching). The complex puzzle of TCS mechanistic regulation is just starting to be solved. The complete understanding of how signals are efficiently transmitted by TCSs will be instrumental for pathway engineering and synthetic biology approaches.

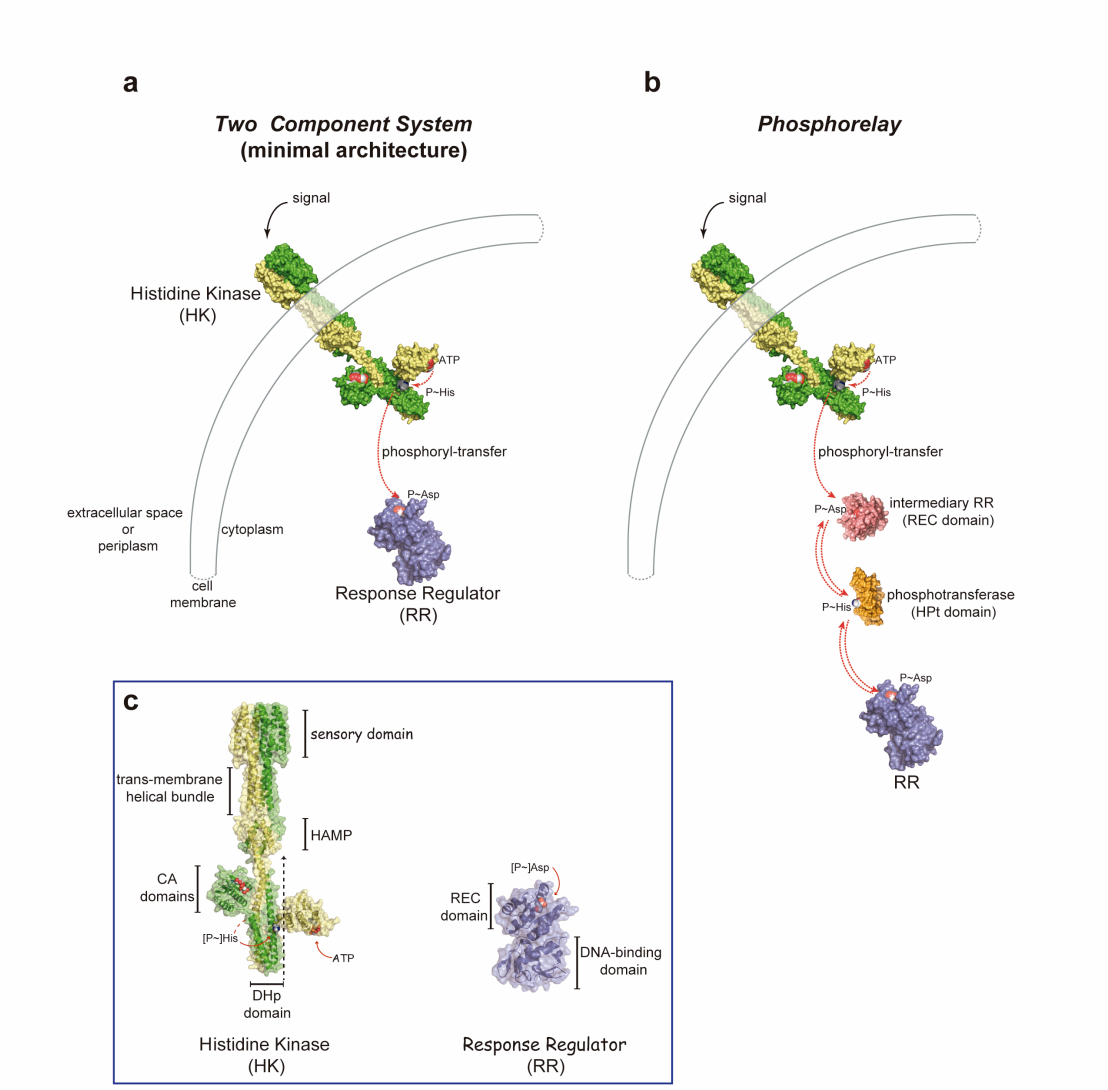

Figure 1. Two-component system pathways and protein domain architecture. (a) Minimal organization of a canonical TCS comprising a sensory protein (histidine kinase HK)

and an output effector one (response regulator RR). HKs are typically dimeric (each protomer distinguished with different colours), and although they can be cytoplasmic soluble proteins, a transmembrane representative has been chosen for this illustration. The phosphoryl-transfer is typically irreversible ($P{\sim}His_{HK} \rightarrow Asp_{RR}$) in simple TCSs. **(b)** A phosphorelay pathway is schematized, showing the role of intermediary proteins that receive and transfer the phosphoryl group, often through reversible reactions (double-headed arrows). **(c)** Modular domain organization of HKs and RRs. Both protein components can have a larger or smaller number of domains than drawn in the figure, but the minimal architecture that defines each component always includes at least a DHp and CA domains in the HK, and a REC domain in the RR. HAMP (signalling domain found in ***H***istidine kinases, ***A***denylyl cyclases, ***M***ethyl-accepting chemotaxis proteins, and ***P***hosphatases); DHp (***D***imerization and ***H***istidine ***p***hosphotransfer domain); CA (***C***atalytic and ***A***TP-binding domain); REC (***Rec***eiver domain).

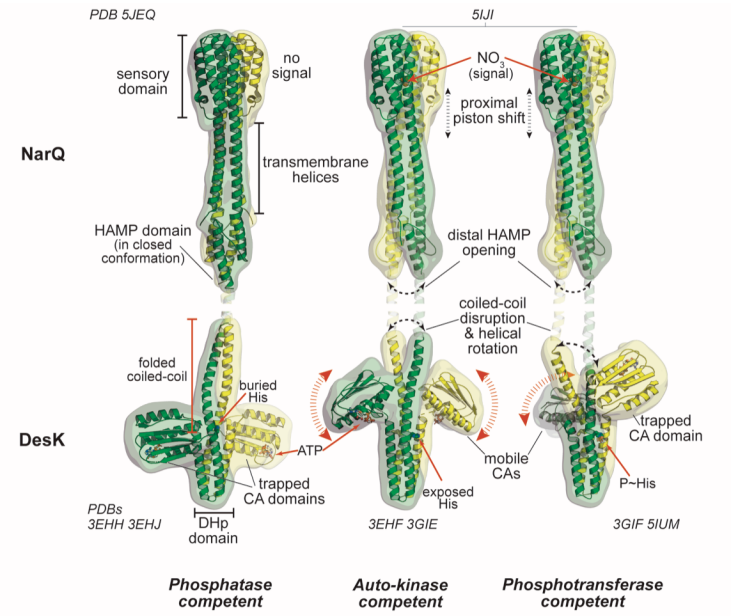

Figure 2. Protein dynamics in histidine kinase activation switching. Integrating data from two different HisKA_3 histidine kinases, NarQ (top row) [19] and DesK (bottom) [22,35], an overall picture of HK activation has been uncovered. The two protomers of the HKs' dimers

are coloured green and yellow. The structures are shown as cartoons with overlaid semi-transparent solvent-accessible surfaces. The connection between both is disrupted to illustrate it is hypothetical, as no 3D structure of a full-length transmembrane HK has yet been determined. PDB accession codes are shown for each depicted structure. An elaborated description is given in the text but note the general trend of symmetric to asymmetric organization when going from the phosphatase- to the phosphotransferase-competent states. DHp domain coiled-coil disruption and coupled helical rotational motion in the active state correlate with freely mobile CA domains, poised for auto-phosphorylation. Note the added regulatory effect of position rearrangement of the phosphorylatable His, swinging from a buried location within the DHp 4-helix bundle (phosphatase state) to a solvent-exposed one (kinase-active and phosphotransferase states).

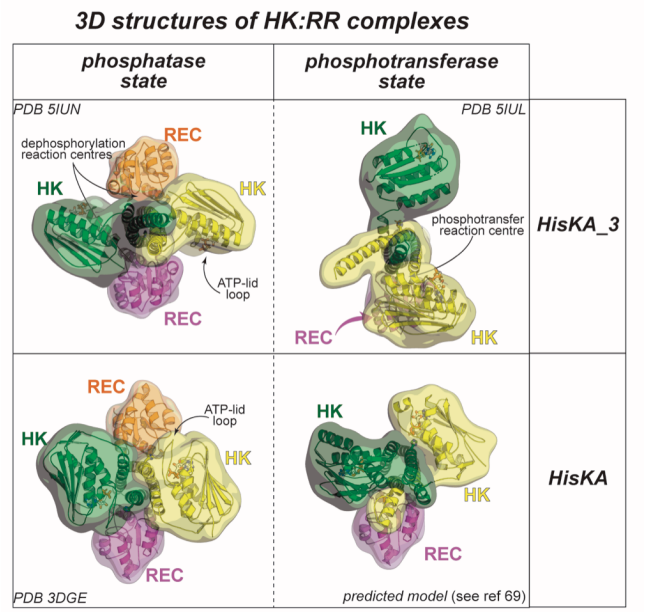

Figure 3. 3D structures of His-kinases in complex with their response regulators. Overall 3D configuration of TCS complexes, representing snapshots of the P~RR dephosphorylation reaction (phosphatase state) and the phosphoryl-transfer to RR (phosphotransferase state). Colouring scheme and model representations are the same as in Figure 2, with the receiver

domain (REC) of bound RRs coloured orange and violet. The perspectives have been chosen by maximizing the structural superposition of the central DHp domain helices, which remains similar in all the panels. PDB accession codes or relevant references are indicated. ***(Top row)*** Both states have been captured in a HisKA_3 HK TCS (DesK:DesR). HK DHp coiled-coil disruption and CA domain reorganization, complex asymmetry are hallmarks of the phosphotransferase form. Subtle but critical repositioning of key amino acid residues in both HK and RR are not seen in this perspective (see ref [35] for details). ***(Bottom row)*** The analysis of complexes engaging HKs that belong to the HisKA family allows identification of common features. The HisKA phosphotransferase state has not yet been observed but has been proposed on the basis of the autophosphorylating, active states of two different HisKA HKs.

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