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Two-Component Sensing and Regulation: How Do Histidine Kinases Talk with Response Regulators at the Molecular Level?

Alejandro Buschiazzo[1,2] and Felipe Trajtenberg[1]

[1] Laboratory of Molecular and Structural Microbiology, Institut Pasteur de Montevideo, Montevideo 11400, Uruguay; email: alebus@pasteur.edu.uy, felipet@pasteur.edu.uy

[2] Integrative Microbiology of Zoonotic Agents, Department of Microbiology, Institut Pasteur, Paris 75015, France

alebus@pasteur.edu.uy ORCID # 0000-0002-2509-6526 (AB)
felipet@pasteur.edu.uy ORCID # 0000-0003-0427-5549 (FT)

Running Title
Kinase/regulator interactions in signaling

Corresponding Authors
Alejandro Buschiazzo (alebus@pasteur.edu.uy) Institut Pasteur de Montevideo, Mataojo 2020, Montevideo 11400, Uruguay
Felipe Trajtenberg (felipet@pasteur.edu.uy) Institut Pasteur de Montevideo, Mataojo 2020, Montevideo 11400, Uruguay

Abstract

Perceiving environmental and internal information and reacting in adaptive ways are essential attributes of living organisms. Two-component systems are relevant protein machineries from prokaryotes and lower eukaryotes that enable cells to sense and process signals. Implicating sensory histidine kinases and response regulator proteins, both components take advantage of protein phosphorylation and flexibility to switch conformations in a signal-dependent way. Dozens of two-component systems act simultaneously in any given cell, challenging our understanding about the means that ensure proper connectivity. This review dives into the molecular level, attempting to summarize an emerging picture of how histidine kinases and cognate response regulators achieve required efficiency, specificity, and directionality of signaling pathways, properties that rely on protein:protein interactions. α helices that carry information through long distances, the fine combination of loose and specific kinase/regulator interactions, and malleable reaction centers built when the two components meet emerge as relevant universal principles.

Keywords

bacterial signaling / information metabolism / protein phosphorylation / allosteric regulation / protein:protein interactions / macromolecular structure

1. INTRODUCTION

Perception of the environment and homeostatic regulation are essential properties of living organisms. Signaling systems enable cells to recognize ambient and intracellular alterations and trigger adaptive responses. Major advances have been achieved in deciphering the molecular workings of such information-processing networks, facilitating the manipulation, and de novo creation of synthetic pathways for biotechnological applications (72, 108).

Biologic signaling machineries are different from electrical circuits and electronics, yet they share analogies that grasp key functional properties: modular nanodevices (in living systems these are proteins, protein domains, DNA and RNA segments, etc.) that connect input signals to output effects, through orderly organized pathways analogous to wired circuits. The biologic modules perform specific tasks in a similar way as do electronic switches, insulators, amplifiers, etc. Analogous to signal-carrier electrons that travel through a continuous wire in electric circuits, in biologic systems the signal is transmitted through specific protein-mediated interactions. Connectivity is thus intrinsically discontinuous in biology, given that protein:protein interactions are transient, which underscores the importance of second messengers and signal-triggered protein modifications to ensure transmission. As in other forms of networked architectures, emergent properties arise such as signal integration, robustness, graded or bistable responses, evolvability, adaptation, and sensitivity enhancement (9, 10, 53, 59, 70–72, 123).

At the very core of cell machineries, proteins are molecules that carry out work and execute cell functions. Information metabolism (signal sensing and processing) is typically orchestrated by protein components that transmit information by (a) allosterically regulated structural rearrangements; (b) posttranslational modifications, ensuring that the allosteric rearrangements are not short-lived; and (c) protein:protein and protein:DNA binding, according to a rich specificity code that transmits the information to the right recipient.

Two-component systems (TCSs) are one of the best studied models of signaling pathways (44). Almost ubiquitous in bacteria and also present in fungi and plants, TCSs in their most simple and frequent organization comprise a sensor histidine kinase (HK) acting in concert with a cognate response regulator (RR) (Figure 1). TCSs depend on phosphoryl-transfer reactions as a means of conveying information. Phosphorylations select (93) and stabilize discrete 3D conformations

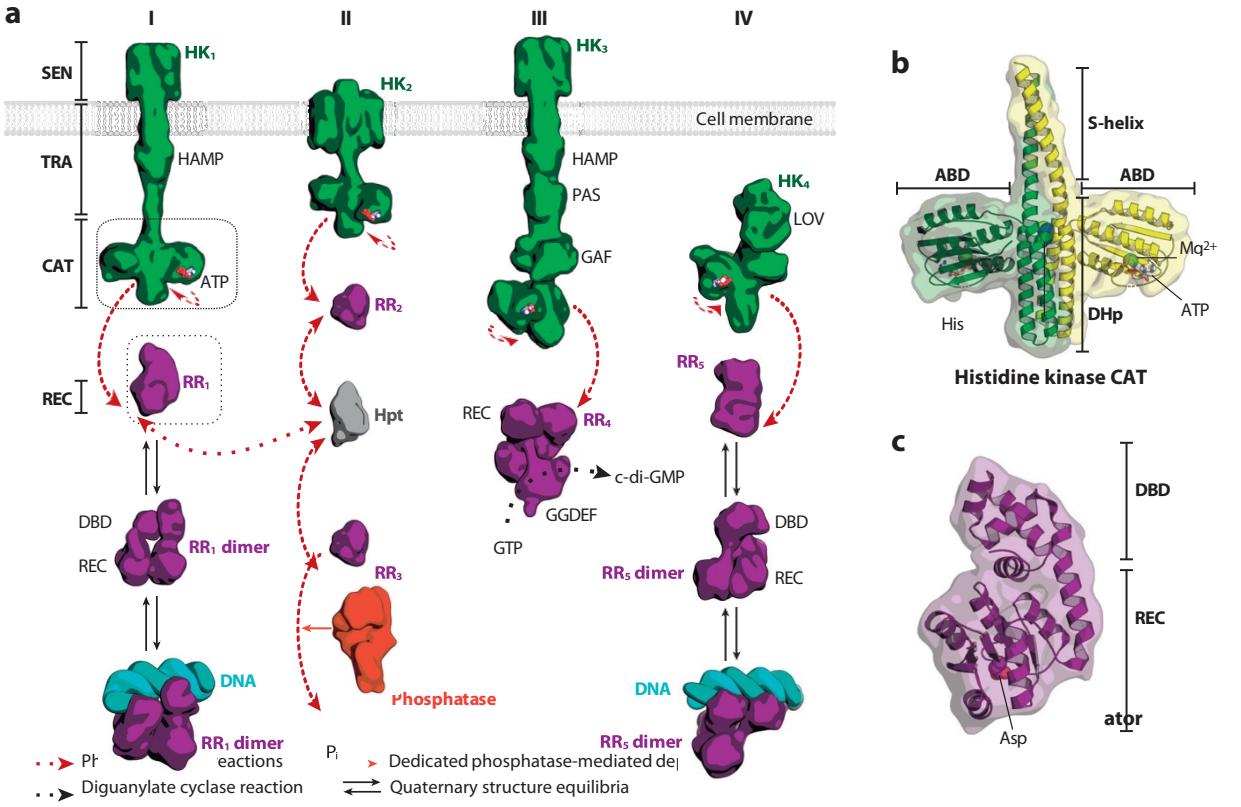

Figure 1. Two-component signaling pathways. (*a*) Molecular architectures of common TCS pathways. Pathway I: a prototypical HK:RR engaging a transmembrane HK. Pathway II: a phosphorelay with intervening REC and HPt proteins. Pathway III: a more complex HK architecture with a longer transmission module. Pathway IV: implicates a soluble HK. Molecular surfaces respect proper scales (models were built from experimental atomic coordinates). Structure/functional modules are indicated (SEN, TRA, CAT, REC). Different types of His-kinases (HK_{1-4}) and response regulators (RR_{1-5}) are distinguished. Recurring domains are labeled (HAMP, PAS, etc.). Dedicated phosphatases can dephosphorylate specific RRs (e.g., pathway II); most frequently, however, the cognate HK acts as a phosphatase when the pathway is off (not shown, for clarity). (*b*) The HK CAT module highlighting secondary structure elements, covered by a transparent view of the molecular surface (PDB 5IUN). The two protomers within the homodimer are distinguished with colors (*green* and *yellow*). Structural domains are labeled. The phosphorylatable His is depicted in spheres; in this case it is not solvent-exposed, as it corresponds to the phosphatase (kinase-off) state. ATP-Mg^{2+} moieties bound within the ATP-binding pocket are shown as sticks. (*c*) A monomeric RR displayed with its molecular surface transparent (PDB 4GVP). This particular RR possesses a REC domain and a DBD as an output effector. The phosphorylatable Asp is shown as spheres sitting on the Rossmann-like fold β sheet toward the C termini of the strands (not visible from this perspective). Abbreviations: ABD, ATP-binding domain; DBD, DNA-binding domain; DHp, dimerization and histidine-phosphotransfer domain; CAT, catalytic module; GAF, cGMP-specific phosphodiesterases/adenylyl cyclases/FhlA domain; HAMP, histidine kinases/adenylate cyclases/methyl-accepting proteins/phosphatases domain; HK, histidine kinase; HPt, histidine phosphotransfer domain; LOV, light-oxygen-voltage sensing domain; REC, receiver domain; RR, response regulator; SEN, sensor module; TRA, transmission/transduction module; PAS, Per-Arnt-Sim domain.

of the intervening signaling proteins (47, 62), such that the resulting distinct structures favor disparate molecular functions (enzymatic activities, binding properties, etc.) and, ultimately, signaling outputs. Excellent reviews have covered several facets of TCS biology (15, 45, 48, 50, 61, 75, 110, 151). Knowledge gaps remain in understanding the molecular details of an utmost critical step: the connection between sensory and output devices. Likely due to the transient nature of their association, high-resolution images of HKs in complex with RRs are still extremely scarce (25, 77, 101, 131, 146). Only three different systems have currently been solved, two involving HisKA[1] HKs from the extremophile *Thermotoga maritima* and the other one corresponding to complexes of HisKA_3 HKs from the mesophile *Bacillus subtilis*.

A given signal must be unequivocally linked to a specific response for signaling to be effective. TCSs have evolved to ensure efficiency, specificity, and proper directionality. This review focuses on the molecular means by which TCS proteins bring about these three properties. Efficiency is defined here as the amount of signal-dependent work effectively achieved, with a given quantity of expended energy. A more efficient system is thus one that turns on in tight correlation to the presence (or absence) of the signal and subsequently performs work losing the least energy throughout transmission. Specificity and directionality must be encoded within signaling proteins to ensure proper connectivity, but with the challenge of allowing for evolution to produce novel combinations. First, we introduce basic structural concepts, important for later elaboration on the mechanisms.

2. FAMILY MATTERS: TCS SEQUENCES AND 3D STRUCTURES

2.1. Histidine Kinases

HKs are typically homodimeric proteins (Figure 1a,b), with other organizations remaining exceptional (98, 107, 139). HKs bind ATP, transferring its γ-phosphoryl group to a conserved histidine. Phosphorylated HKs (P∼HK) then transfer the phosphoryl to a conserved aspartate of cognate RRs. Finally, P∼RR dephosphorylation, critical in determining steady-state levels of active P∼RR (46), results from: (a) spontaneous hydrolysis; (b) dedicated phosphatases; or, in most cases, (c) the phosphatase action of the cognate HK in its kinase-off state.

Dozens of different HKs are coexpressed in a given cell, constituting a large group of paralogous proteins with shared 3D structures and catalytic/regulatory mechanisms. However, important variations have also evolved, and excellent reviews further elaborate on the link between HK families and mechanisms (15, 151). Briefly, HKs are grouped in three major classes, I–III, on the basis of their modular design (2). The vast majority belong to class I, organized in three structure/functional regions: (*a*) a sensor module (SEN), (*b*) a transmission/transduction module (TRA), and (*c*) a catalytic module (CAT) (Figure 1*a*). The reader should be aware that within the CAT module, we are including the ATP-binding domain (ABD), also known as catalytic and ATP binding (CA). Avoiding possible confusion between CAT and CA acronyms, we will further elaborate on the arguments behind the moduli denominations in the next subsection.

2.1.1. The sensor, transmission, and catalytic modules. HKs may bear a SEN module at the N-terminal, harboring one or more sensory domains.The latter are highly diverse, consistent with the large variety of detected signals (see reviews 28, 68, and 83 for further details). Connecting the sensor and catalytic modules, HKs frequently include a TRA module consisting of two or r

[1]HisKA, HisKA_2, HisKA_3, and others are the families into which HKs are currently classified. See Section 2 for further details.

transmission domains, such as HAMP (histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein, and phosphatase), PAS (contained in Per-Arnt-Sim proteins), or GAF (cGMP-specific phospho- diesterases, adenylyl cyclases, and FhlA), among many others (15).

The CAT module constitutes the minimal structural core shared by all class I HKs, comprising two domains: a dimerization and histidine-phosphotransfer domain (DHp) and an ABD (Figure 1b). The DHp is elongated, formed by a two-α-helix hairpin that drives homodimerization. Once the dimer is formed the DHp thus constitutes an antiparallel four-helix bundle, which becomes a parallel two-helix structure by way of N-terminal extensions of helices α1. The phosphorylatable histidine is located within a conserved sequence motif (H-box) on α1, approximately in the middle of the DHp (81, 126). The ABD displays a Bergerat fold: a globular α/β domain with a core of three helices packed on one side against a five-stranded β sheet (Figure 1b). This fold is present in the GHKL protein superfamily (DNA gyrase, Hsp90, histidine kinase, and MutL), all of which exhibit slow ATPase activities (35). ABDs bind ATP-Mg+2 (130), engaging amino acids that define conserved motifs (N-, G1-, F-, G2-, and G3-boxes). A loop that includes the F-box acts as a true ATP cover (ATP-lid), shaping the nucleotide-binding pocket and displaying highly variable lengths in different families.

2.1.2. Histidine kinase families and architectures. Early HK sequence classifications (49) have been updated into fewer groups using hidden Markov profile alignments (40). The most useful categorization is based on DHp sequences, including the phosphorylatable histidine. Four families are currently recognized: HisKA (Pfam family PF00512), HisKA_3 (PF07730), HisKA_2 (PF07568), and HWE_HK (PF07536). The former two represent respectively ∼80% and ∼15% of all DHp-containing HKs.

Besides the simple [SEN/TRA/]CAT organization (pathway I, Figure 1a), more complex architectures are also found. Some include receiver (REC) domain(s) in the same HK polypeptide (30, 120), named hybrid HKs (HHKs) (143). HHKs are frequently involved in TCS phospho-relays, which include additional intermediate proteins composed of the same type of CAT and REC domains found in simple TCSs. Phosphorelays often include intermediate phosphotransfer proteins, which can be of two types: DHp-containing ones that are very similar to bona fide HKs, or instead proteins that bear histidine phosphotransfer (HPt) domains (37, 120) (pathway II, Figure 1a), which are structurally different yet fulfill analogous roles. Both types of intermediate phosphotransferases possess a phosphorylatable histidine but are not able to autophosphorylate.

2.1.3. Coiled-coil segments in Histidine kinases. HK sequences predict a coiled-coil structure along the DHp four-helix bundle and through the N-terminal two-helix extension (114) (see the Sidebar titled Coiled Coils and Signaling in Biology). The latter connects to additional N-terminal domains when present. A precise boundary delimiting the two-helix coiled-coil segment [also named helix Jα (88) or the S-helix (6, 114)] from the DHp domain is somewhat arbitrary. It seems convenient to include the S-helix, always present and juxtaposed to the DHp, as part of the CAT module (Figure 1b). When longer TRA modules are present (pathways I and III, Figure 1a), additional S-helices connect the intervening domains (76, 119, 134). S-helices display nonideal heptads, with specific coiled-coil core positions occasionally occupied by polar residues, reducing the coiled-coil's energetic stability (hence prone to conformational switching). Sequence variations within coiled coils from different HKs encode a variable number and position of heptad

deviations (skips, stutters, or stammers) in the vicinity of the reactive histidine, especially N-terminal to it. Defining heptad positions in each HK family for comparative purposes seems unfeasible (and likely misleading), due to variations of insertion positions and accommodation lengths, even when informed by structural analysis. The precise location of heptad insertions in a given HK is bound to change according to the protein's on/off state, which has been confirmed experimentally in structures for which more than one state is known (4, 111).

2.2. Response Regulators

RRs always include a REC domain, which harbors the phosphorylatable aspartate residue within a conserved α/β Rossmann-like fold (Figure 1c). RRs can be REC only or comprise additional do- mains, mostly DNA binding but also ligand binding, protein binding, with enzymatic functions, etc. (42). Activation of RRs is associated with their phosphorylation, with P∼RRs subsequently engaging in output effector responses (transcriptional regulation, allosteric modulation of partner proteins' activities, etc.). Seven REC-based families are currently recognized in Pfam, but one includes the vast majority of known RR sequences (Response_reg PF00072). On the other hand, analysis of output effector domains results in dozens of RR families revealing broad functional diversity (42). We do not elaborate upon RR 3D structures any further; several authoritative reviews are available (43, 44).

3. EFFICIENCY REGULATION IN TCSs

It is difficult to quantify signal-triggered effector activities (e.g., gene transcription, flagellar motor direction switching) as the output of a precise number of ATP molecules used to phosphorylate the corresponding sensory HK and maintain the pathway in an on state for the right time. However, two means of controlling efficiency in TCSs do emerge from analyzing experimental evidence:

(a) tight signal-dependent control of the pathway's on/off switching and (b) minimization of in- formation loss along the pathway.

3.1. Controlling Signal-Dependent Activation of TCS Proteins

Signals allosterically modulate HK activities (4, 26, 64), effectively switching the TCS pathway on and off. HKs are implicated in three phosphoryl-transfer reactions along the signaling cascade that we here denominate functional states: (a) autokinase (autophosphorylation), (b) phosphotransferase (P∼HK→RR phosphoryl-transfer), and (c) phosphatase (HK-mediated P∼RR dephosphorylation) states. The former 2 states are active, engaged when the pathway is on, while the phosphatase corresponds to the inactive state of the HK, shutting off the pathway. Molecular details are needed to understand how HKs prevent signal-independent activation.

Structural approaches that image ground states of HKs and HK:RR complexes at high resolution contribute with valuable molecular insights about on/off switching. A complete picture awaits the disclosure of 3D structures and functional assignments of all relevant signaling states of any single TCS, a challenge that has not yet been overcome. When currently available data are integrated, switching seems to be steered by a coiled-coil rearrangement mechanism that translates along the central helical spine in all HKs (Figure 2), with family-specific variations in line with this general scheme.

Several studies of HisKA_3 TCSs, using a variety of structural, biochemical, and even

bacterial physiology approaches, have uncovered detailed features of the phosphatase/phosphotransferase switch (4, 51, 57, 109, 131). HisKA structures, on the other hand, have beautifully captured the autokinase state (24, 85, 134) and also provided snapshots that have been interpreted as phosphotransferase-active (81, 86) or phosphatase-trapped configurations (25). Further confirmation of HisKA functional assignments will be instrumental, especially when structural details are consistent with alternative states. Additional functional insights have also been supplied from a number of studies on signal-triggered switching focused on a variety of TCSs, including HKs from other families besides HisKA and HisKA_3 (13, 33, 34, 38, 76, 88, 89, 133). In the following paragraphs within this subsection we attempt to summarize what we have learned from all these studies, distinguishing whenever possible universal mechanisms from family-specific variations. The final paragraph will very briefly touch upon the activation of the RR partners.

The S-helix is the means to transmit positional information downstream, by helical rotation and helix axis translation following coiled-coil rearrangements (Figure 2a,b). Such movements are difficult to predict on the basis of sequence alone, since they rely on the position of heptad insertions and local accommodation regions of the coiled-coil hydrophobic core. 3D structures are valuable in showing precisely where such rearrangements take place. The vicinity of the reactive histidine always hosts such alterations, accentuating the shifting of residues in both DHp helices around that region. Two general effects derive from such signal-triggered switching via coiled-coil rearrangements: (a) ABD mobility and orientation are strongly affected and (b) the position of the reactive His is modulated, either strongly (HisKA3) or more subtly (HisKA). A body of coherent findings endorses this general view, as we will now elaborate with greater detail.

High-resolution structures of Escherichia coli NarQ (51) provide with the first images of a trans- membrane HK, including the entire SEN and most of the TRA modules. Nitrate sets up a piston motion in the SEN module (27, 51), directly transferred to the transmembrane domain, which then transduces it into a scissoring rigid-body motion of the two halves of the intracellular HAMP (Figure 2a). The S-helix coiled coils are absent from the crystallized construct, but the C-terminal ends of the HAMP readily predict coiled-coil formation in the kinase-off state (no signal), versus coiled-coil disruption in the kinase-on state (with nitrate). Consistently, coiled-coil stabilization also takes place in the phosphatase state of B. subtilis DesK (Figure 2a), the entire CAT mod- ule of which was crystallized in both states (4, 131). DesK's S-helix drives coiled-coil composite movements that translate directly to the phosphorylation site (Figure 2b).

Autophosphorylation is thus inhibited when the pathway is off because of the modifications at the DHp. The shifts near and on the reactive His produce two effects. (a) They modulate the DHp surface to establish strong interactions with the ABD C-terminal helix (also known as the gripper); ABDs are thus rigidly held in open position, which also depends on the ABD-DHp interdomain linker, key in allowing the ABD to approach the DHp (sometimes through

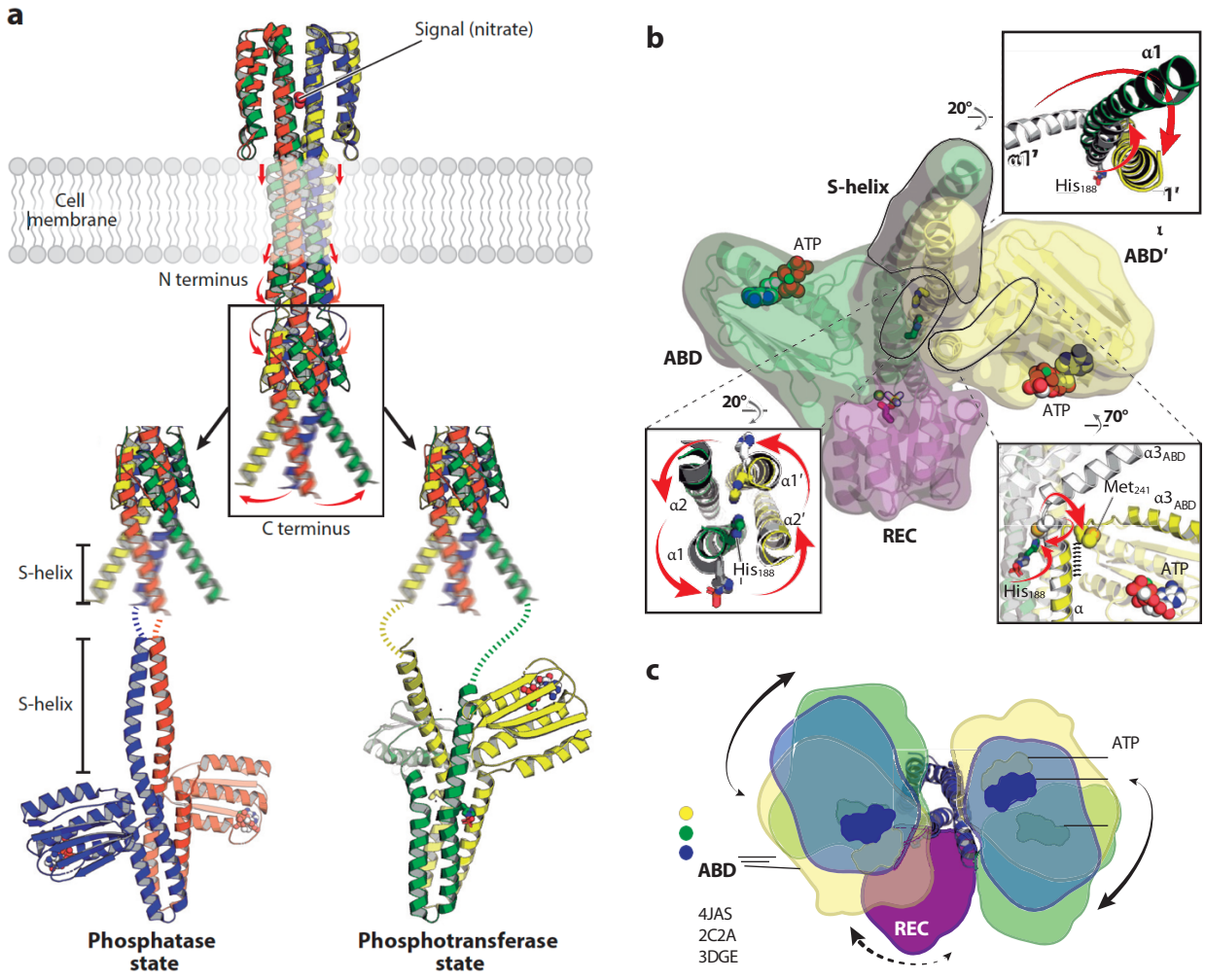

Figure 2. (*a*, *Top*) NarQ in the presence (*green* and *yellow*, PDB 5IJI) and absence (*orange* and *blue*, 5JEQ) of signal. The C termini were extended, illustrating the predicted structure of the S-helix (*blurred*). Red arrows point to signal-triggered movements. Disruption of the S-helix is expected when the kinase is on. (*Bottom*) Entire CAT of DesK (also a HisKA_3 HK) comparing the phosphatase (5IUN) and phosphotransferase states (5IUM). The S-helix is observed switching between coiled-coil stabilized (kinase off) and destabilized (kinase on). (*b*) Activation switch in HisKA_3 HKs. DesK:DesR complex in phosphatase state (5IUN); insets superpose it onto the active phosphotransferase (*white transparent*, 5IUM) illustrating the shifts. Single prime labels distinguish the second protomer within the dimer. Coiled-coil rearrangements at the S-helix (*top right inset*) trigger composite helical movements transmitted to the core of the DHp (*bottom insets*). Note the drastic rotational movement of the phosphorylatable histidine (*bottom left inset* His_{188}), buried in the phosphatase state. The interdomain linker (*bottom right inset*) modulates the ABD-DHp binding, strong in phosphatase and released in the active state. (*c*) Activation switch in HisKA HKs. Multiple structures of the HisKA HK HK853, alone and in complex with RR468, are superimposed, aligning the DHp segment involved in RR binding; only 3DGE is drawn for clarity. The ABDs are delineated precisely along the contours of molecular surfaces. The positions of ATP are depicted schematically as a reference of the binding pocket position and the ATP-lid on the surface beside it. Only one REC domain (*purple*) from the bound RR is shown; some shifting of its position is observed among different complexes (*dotted arrow*). Signal-triggered shifts at the S-helix seem to universally promote ABD-DHp association rearrangements in all HKs, ultimately modulating ABD positioning (*solid black arrows*). In HisKAs direct ABD:REC interactions through the ATP-lid also seem to be important in reorienting the REC domain into a productive position.

Abbreviations: ABD, ATP-binding domain; CAT, catalytic module; DHp, dimerization and histidine-phosphotransfer domain; HK, histidine kinase; REC, receiver domain.

unwinding of the C-terminal end of DHp α2) but restraining its freedom. (b) They also force the His to rotate from an exposed position (a skip insertion in the heptad) to a buried one (core a position) (4, 131) (Figure 2b). This double effect allows the phosphatase state to keep the ATP substrates far from the reactive histidines, further burying their side chains, altogether hampering autophosphorylation and potential back-transfer from the P∼RR. Stabilized coiled-coil helices correlate with symmetric dimers in the off state, and vice versa, a link that appears to hold as well in HisKA HKs, where stabilization of an open state via extensive ABD-DHp interfaces favors the phosphatase reaction (34). The asymmetry/symmetry transitions in HKs, linked to on/off switching, appear as a general trend in different TCSs (4, 15, 85, 131). Strongly asymmetric structures correspond to active HKs, as a signature not only of the autokinase reaction but also of the phosphotransferase (131).

In HisKAs the His occupies a more external position on the helix (either a 'b' position right after a stutter at Ser/Ala or an 'f' position, depending on the precise assignment of heptads), and it is thus not anticipated to shift as dramatically as in HisKA_3 HKs. It has even been posited that it does not move whatsoever, based on NMR data (38). However, early reports had otherwise shown high dynamics at the His position (126), also consistent with crystallographic evidence: Comparison of protomers within dimers using several CpxA (PDBs 5LFK, 4BIU, 4BIV, 4BIW, 4BIX, 4BIY, 4CB0) and EnvZ (4CTI, 4KP4, 2LFR, 2LFS, 3ZCC, 3ZRV, 3ZRW, 3ZRX) crystal structures provides compelling indication of His movement. The CpxA structures suggest it will likely back off from the reaction center in preparation for phosphatase (82, 85, 86) (Supplemental Figure 1a), providing the required access for a hydrolytic water. The asymmetry of several of these configurations seems key for such differential His movements. The conclusion that asymmetry is relevant, and not derived from crystal packing restraints, stems from the fact that several of the asymmetric structures have been solved from different crystal packings yet display identical asymmetric features (4, 39, 85). Taken together, the above findings lead to the conclusion that signal sensing induces coiled-coil rearrangements in HisKA HKs, driving ABDs' mobility and relocation (Figure 2c). HK activation likely proceeds thereafter by means of a flip-flop mechanism, coupling autophosphorylation in one HK protomer, with phosphoryl-transfer to the RR occurring on the other (38). Such a mechanism explains a number of observations from different TCSs (see 86 and references therein) and is plausible for all HKs.

Further supporting evidence for a universal coiled-coil mechanism is provided by an elegant series of experiments with chimeric constructs (13, 14, 33, 36, 88, 89) showing that S-helix over-winding or stabilization switches HK activity from kinase to phosphatase (13). More recently, and now concerning a family of HKs differing from both HisKA and HisKA_3 (144), S-helix rearrangements have, once more, been found to be linked to the modulation of strong versus weak ABD-DHp interaction (correlated respectively with low versus high autokinase activity).

For space limitations we do not elaborate in detail on the mechanisms by which signal-independent RR activation is minimized (43). Suffice it to say that, other than by their cognate HKs, RRs could be phosphorylated by surrogate P∼HKs/P∼HPts, or yet by small-molecule phosphodonors (141). Should such alternative routes be triggered in a signal-independent way, the entire pathway's efficiency would be affected. Overall opposite to the HK activation scheme, RRs exhibit more flexible inactive structures (67, 104), becoming discrete once phosphorylated. Mg^{2+} coordination correlates with rigidification of loops surrounding the phosphorylation site (β1-α1, β3-α3, β4-α4) and helices α3 and α4 (43, 104, 129). A critical redisposition of the β4-β5

portion of the Rossmann β sheet follows, strikingly similar to the activation of distantly related enzymes (128), reshaping the domain's surfaces. The cognate HK is optimally designed, via coevolution of interaction surfaces, to select active/active-like conformations of the specific RR (25, 129, 131), facilitating phosphorylation and overall enforcing signal dependence.

3.2. Phosphates Lost in Transmission

Phosphates bind covalently and reversibly to HKs and RRs, acting as a tag that marks their active state in terms of 3D conformation and functional properties. Losing phosphoryl groups along the pathway is equivalent to losing electric current along a circuit (in the form of a leakage current or, when massive, a short circuit). Tracking the phosphoryl moiety from the γ position in ATP down to the P∼Asp in the RR, several steps represent potential leakage points: (a) HK-mediated ATP hydrolysis; (b) P∼His dephosphorylation with no transfer to the RR; or (c) premature P∼RR dephosphorylation, especially considering the capacity of most unphosphorylated HKs to catalyze the P∼RR phosphatase reaction. The regulation of such reactions influences the TCS pathways' efficiencies.

HK ABDs display intrinsic ATPase activity, yet typically with very low catalytic velocities (35, 130), a trait that, as described above, is common to all proteins bearing a Bergerat fold. The slow ATPase constants effectively maximize the odds for the γ-phosphoryl group to be transferred efficiently to the reactive histidine on the DHp domain, instead of being hydrolyzed via intrinsic ATPase catalysis. Selection pressure has probably resulted in the conservation of two glycines on helix α3—the G2-box motif—immediately C-terminal to the ATP-lid in ABDs. Such side chain–less residues provide with the required space to phosphotransfer to the DHp, simultaneously obviating sidechain assistance in positioning a water molecule in-line to attack ATP's γ-phosphate.

P∼HK dephosphorylation with no transfer to the RR can proceed in two ways, either by back-transfer to ADP, or by P∼His hydrolysis. The former reaction is minimized due to typical ∼10 fold ATP/ADP ratios in the cell (12), while affinities for both nucleotide forms tend to be similar (100, 118). ATP resynthesis by back-transfer in vitro is strongly inhibited just by adding constant ATP regeneration (92). As for the spontaneous hydrolysis of P∼HKs, the P-N bond stability in P∼His is pH dependent, and P∼His hydrolysis is favored as the pH of the aqueous environ- ment decreases (66). At physiologic pH, spontaneous dephosphorylation of HKs appears to be marginal. Nonetheless, enzyme-catalyzed P∼His dephosphorylation cannot be ruled out (8), the phosphatase SixA, for instance, dephosphorylates the HHK ArcB in vitro (94). The in vivo rel- evance of such activity on TCSs' P∼HK regulation (84) is, however, a matter of debate; SixA appears instead to be important in dephosphorylating other P∼His proteins (112). Additional P∼His phosphatases have not yet been clearly identified.

Autodephosphorylation of P∼RRs (140) is a well-described phenomenon that depends on a water molecule performing a nucleophilic attack on the Asp-bound phosphorus atom. This reaction is highly sensitive to Mg^{2+}, coordinated to the phosphoryl moiety and to key RR amino acids. The kinetics of such spontaneous dephosphorylation, spanning a 106-fold range (16), has been evolutionarily shaped by mutations in sequence-hypervariable RR loops surrounding the phosphorylation site (60), according to RR families (95).

Phosphate leakage can also occur if the HK phosphatase and phosphotransferase activities

are not properly regulated, resulting in futile cycles. Except if there is a physiologic advantage for such energy drain (70), inefficient energy loss appears to be minimized. The HK phosphatase activity is not the reverse of phosphotransferase (26, 58, 131) and was found to be strikingly analogous to that of dedicated P∼RR phosphatases such as CheZ, CheX, and Rap phosphatases (96, 97, 149), at both the functional and structural levels (56, 96, 97, 149). In HisKA_3 HKs, a glutamine (five residues C- terminal to the phosphorylatable His: His+5) is well located in the HK:P∼RR complex to position a water molecule in-line to attack the Asp-bonded phosphorus atom (131), structurally equivalent to the essential Asn/Gln of dedicated phosphatases. HisKA HKs locate a Thr/Asn (His+4) at this position (25, 86, 138). A potential HK-mediated phosphate drain is seemingly minimized by averting water to access the proper attacking position, through the strict coiled-coil-driven switching mechanism detailed above. Stabilization of distinct DHp kinking positions and angles (24, 85) not only relocates the ABDs (and through ABD:REC interactions affects REC positions in HisKAs) (Figure 2*c*) but also likely shifts the His position itself, hindering access to a nucleophilic water molecule (Supplemental Figure 1*a*). In HisKA_3 HKs, sequestration of Gln at His+5 by covariant RR residues, together with triggered movements of the RR β4α4 loop (Supplemental Figure 1*b*), seems also a determinant to avoid premature dephosphorylation of P∼RR when the pathway is turned on.

4. SPECIFICITY BETWEEN PARTNERS: HISTIDINE KINASES TALKING WITH RESPONSE REGULATORS

Each HK recognizes its cognate RR, minimizing nonspecific interactions or cross talk (116, 117). The specificity code is a mapping of sets of amino acids on each partner, according to physicochemical complementarity. Detection of evolutionary covariation by comparing sequences of HK/RR pairs (7, 41, 136) has led to pinpointing specificity determinants (22, 90, 101, 102, 116, 124, 136). Only a few residues convey most of the information, allowing for elegant rewiring of signals to surrogate responses by design (116). Natural sampling space for mutations in specificity-encoding positions is constrained (1, 20, 23, 103), for evolution to produce novel specificities without losing functionality along the way. Thus, the specificity code is also one that allows for evolvability.

Covariant residue pairs are located near, but not within, the phosphoryl-transfer reaction center. Specificity seems primarily encoded within helices α1 of both interacting domains (102), with residues also on DHp α2 and the REC β4α4 loop. In the special case of HHKs, the effective concentration of both domains is increased due to the covalent link (127), better outcompeting nonspecific partners. Specificity-determinant residues consistently show lower covariance compared to orthodox HK:RR pairs, resulting in higher promiscuity of HHK CAT modules with respect to several HHK REC domains (21).

The HK:RR interface tolerates high mutation rates on specificity-determining residues, while maintaining signal-dependent phosphoryl-transfer functionality (103), a feature that has been described as interfacial degeneracy. Considering the tight specificity that is observed in partner recognition, such extensive degeneracy might at first be unexpected. However, it was found to simultaneously be epistatic, in that individual substitutions are highly dependent on other residues (1, 103) providing a compensatory mechanism. We can now fully recognize the molecular bases of such observations by looking at 3D structures of HK:RR complexes (11, 25, 131, 137, 146,

148).

The HK:RR interfaces reveal loose interactions, consistent with the transient nature of their binding: limited areas (typically ~900A˚ 2), a very low number of polar interactions, and overall poor surface complementarities (Figure 3a). Such characteristics result in slippery interfaces, with large rigid-body shifts between the HK and the RR, but little rearrangements within each partner (25, 146, 148). The limited geometric complementarity between interacting surfaces supplies unoccupied volumes that allow for mutations to be explored without severely affecting binding capacity.

Additional interfaces other than the DHp:REC should be considered, to uncover the full richness of the specificity code. A notable example concerns the ATP-lid on ABDs for its role in HK:RR binding (Figure 3b). HisKA HKs generally possess longer ATP-lids compared to HisKA_3 HKs. This loop participates in RR binding as observed in the T. maritima HK853:RR468 structure (25). This interaction could explain why phosphatase activities are nucleotide dependent in several HisKA HKs with long ATP-lids (34, 52, 147), as ATP binding is known to structure the ATP-lid possibly enabling proper association to the RR partner. Consistent with this scenario, phosphatase activity mediated by HisKA_3 kinases is not influenced by the presence of ATP

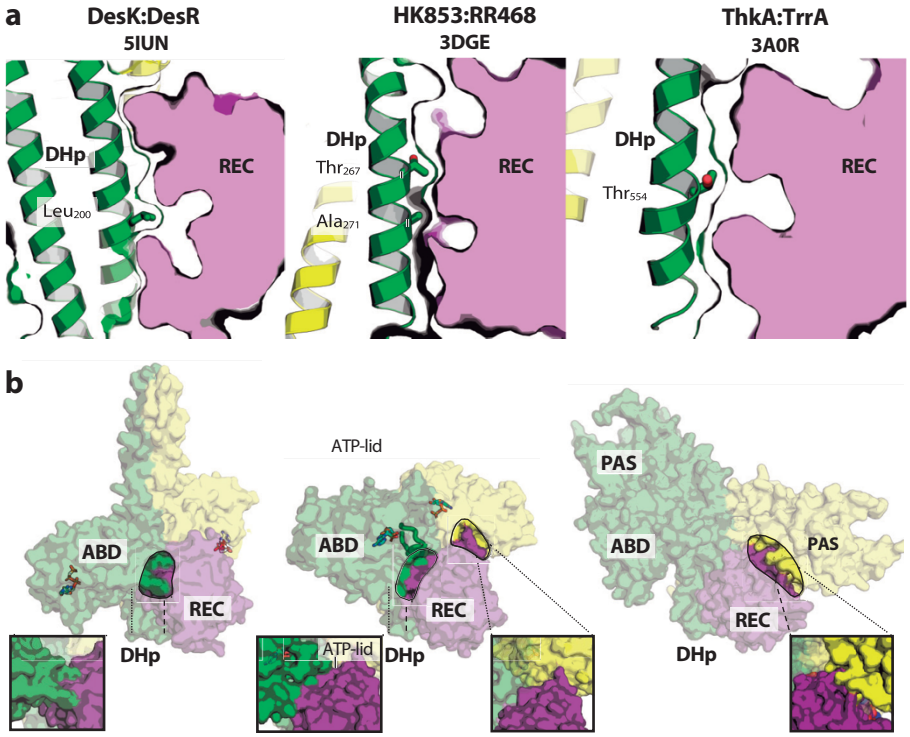

Figure 3. Molecular determinants of HK:RR specificity. (*a*) Three HK:RR complexes are shown with the projected volume of REC domains in solid magenta. HKs' DHp α helices are represented (distinct protomers colored) with superimposed transparent molecular surfaces. The interface is shown as a thin slab highlighting surface contours. Note the presence of unfilled cavities at the interfaces. Selected highly covariant residues in the HK are shown as sticks, observed to be facing such cavities, likely explaining high degeneracy. Similar traits are seen in other complexes of phosphorelays. (*b*) Transparent molecular surfaces of the same HK:RR complexes as in panel *a*, shown from a different view. ATP or ADP are shown in sticks when present. The ATP-lid of HK853 is depicted in cartoon representation. Bottom insets zoom in to the HK:RR interfaces. Note especially the ATP-lid portion of HK853 binding RR468 in the center, and ThkA PAS interacting extensively with TrrA to the right. Abbreviations: ABD, ATP-binding domain; DHp, dimerization and histidine-phosphotransfer domain; HK, histidine kinase; PAS, Per-Arnt-Sim domain; REC, receiver domain; RR, response regulator.

(3, 57), and these HKs possess a shorter ATP-lid. Indeed, the structure of the HisKA_3 DesK in complex with DesR reveals no HK:RR contacts via the ATP-lid loop (131).

Additional HK domains could also be important in RR binding. The structure of the T. maritima ThkA:TrrA complex (146) showcases a PAS domain, N-terminal to ThkA's CAT module, interacting extensively with TrrA. This explains why ThkA-mediated phosphatase activity on P∼TrrA depends on the presence of ThkA's PAS domain (146). Furthermore, in a different system, FixJ (RR) binding to FixL (HK) affects the O2-binding affinity of the kinase's sensory PAS domain (91). In sum, additional interactions beyond the DHp:REC interface appear to be also relevant in determining HK:RR specificity, especially since those additional regions (ABD, PAS, etc.) undergo large reorganizations during HK activation switching. Meaningful variations of the specificity code might thus be discovered, by considering the structures of TCS proteins in distinct functional states.

4.1. Networking Connectivity: Room for Cross Talk?

In contrast to chemotactic TCSs implicating CheA-like class II HKs (105), there are no clear examples of class I HKs able to phosphorylate more than one cognate RR in vivo, without genetic manipulations of some sort. The strong encoded specificity leads to insulated HK:RR pathways (115). Phosphorelay TCSs do offer native examples of cross talk (63, 87, 137), often assembling branched networking connectivities (65). Fascinating specificity questions arise, considering that alternative RRs react with a single phosphotransfer partner while displaying substantial sequence variation among them. The inverse scenario also holds, one RR interacting specifically with two or more phosphotransfer proteins of varying sequence. How is this divergent specificity encoded? Such broadening of the code could enlighten the molecular bases of network signal integration (32), explaining striking examples of one intermediate RR receiving and transferring phosphoryl groups from/to multiple specific partners (78) and improving the performance of engineered rewiring approaches.

5. DIRECTIONALITY IN TCS SIGNALING: WHEN DO PHOSPHATES MOVE DOWN THE PATHWAY?

Eukaryotic-like protein kinases and phosphatases catalyze phosphoryl-transfer reactions that involve P-O phosphoester bonds to the side chains of Ser, Thr, and Tyr. Such esters are extremely stable (69, 122, 142), as compared to the P-N phosphoramidate bond in P∼His (66, 135) and the P-O phosphoanhydride in P∼Asp (99, 121). Differences are based on their electron structures and associated free Gibbs energies (5, 122, and references therein), although not necessarily in terms of kinetic rate constants. As an example of the latter, ATP itself is a high-energy phosphoanhydride, yet it is very stable in water, due to the action of the nonbridging O atoms of the γ-phosphate to hydrogen-bond incoming water molecules, diverting them from occupying a catalytically competent in-line attack position (29).

Most HK:RR systems catalyze irreversible P∼His→Asp phosphoryl-transfer, with negligible or undetectable back-transfer P∼Asp→His (3, 63, 106). In contrast, phosphorelay systems often catalyze reversible reactions in vitro and in vivo (18, 63, 73, 78). The law of mass action may explain a certain number of cases, especially modulating reversible velocities, but most irreversible reactions take place with significantly smaller in-cell HK concentrations compared to RRs' (19). Moreover, some HHKs are examples of irreversible transfer from the His1 to the Asp1, whereas that Asp1 transfers reversibly to/from His2, all within the same polypeptide (125), enforcing near identical relative concentrations.

A working hypothesis has been proposed correlating reversibility to coordinate reaction distance in TCS phosphoryl-transfer reactions (131). The suggestion is based on findings that 3D structures of HK:RR complexes engaged in irreversible reactions display significantly longer distances between the nucleophile and the phosphoryl-donor atoms in the reaction center (131). The opposite is true for reversible complexes, which consistently show short reaction coordinate distances (131). It has long been known that Mg^{2+} depletion favors P∼Asp→His back-transfer in otherwise irreversible systems (3, 79, 113). The cation position is indeed different in the reaction center of both types of complexes. During phosphoryl-transfer, the Mg^{2+} is fixed, coordinated to the RR, and its distances to the HK His and to the phosphoryl transition state are thus larger in irreversible than in reversible centers. The differing distance between entering and leaving groups anticipates a more dissociative character of the nucleophilic substitution (74) in irreversible

reactions, and vice versa (Supplemental Figure 2). The actual chemical role that a modulated Mg2+ distance could exert in phospho-His protein phosphoryl-transfer reactions has not yet been addressed (54), but a substantially distinct distribution of built negative charge is expected if the transition states are more or less dissociative (Supplemental Figure 2). It seems clear, though, that the entering group is required to be held firmly in place for nucleophilic attack, so that larger (looser) reaction centers are disadvantageous for catalysis (55); the cation being closer to the Asp could be critical in stabilizing the metaphosphate intermediate, with no equivalent group for back-transfer. Withdrawing the cation altogether reduces the catalytic efficiency in both directions but symmetrizes the reaction outcome, explaining reported results. Gathering direct evidence is challenging; mutagenesis or metal substitution approaches modify the reaction center itself, confounding straightforward interpretations. Yet, informative experiments can take advantage of TCS proteins known to be engaged in both reversible and irreversible reactions, according to the specific partner that they bind to. High-resolution structures of both complexes should reveal differential features, and particularly different His-Asp distances as anticipated. Indeed, two such examples provide supportive evidence, implicating HPt- and DHp-containing phosphotransfer proteins (Figure 4). The yeast HPt protein YPD1 transfers reversibly from/to the REC domain of SLN1, while it does so irreversibly to SSK1 (63). Corresponding crystal structures show a short distance (4.6–4.8 Å) in the YPD1:SLN1 complex (145, 150), whereas they move 6.4 Å apart in the YPD1:SSK1 complex (PDB 5KBX). A second example concerns the His-containing Spo0B

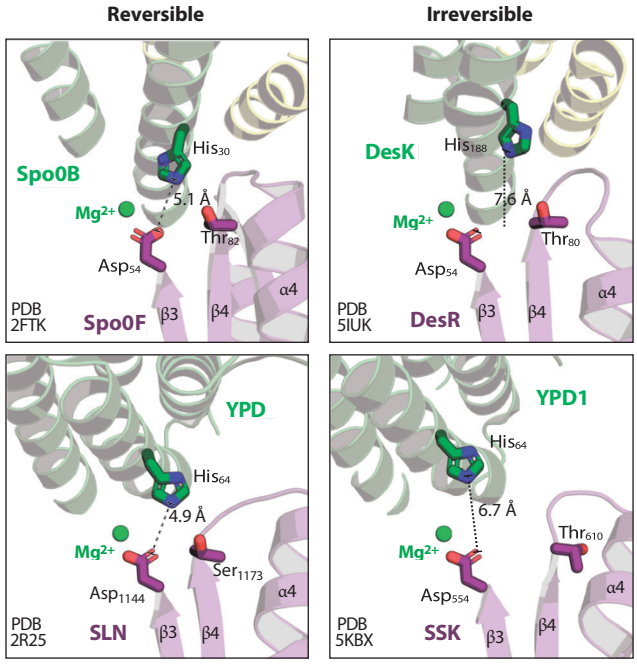

Figure 4. Phosphoryl-transfer directionality in TCS signaling. Examples of reversible (*left*) and irreversible (*right*) HK:RR complexes are illustrated, with HKs or HPts in green (and yellow), and RRs in purple. Note that reversible complexes display the His closer to the Asp residue; distances between reactive N and O atoms are marked. BeF_3^- moieties are not shown, for clarity. Anticipated intermediates correspond to more associative nucleophilic reactions in reversible cases, and dissociative in irreversible ones (see Supplemental Figure 2). For YPD1:SSK1, which crystallized with no Mg^{2+}-BeF_3^- (5KBX), the His/Asp rotamers and Mg^{2+} positions were obtained by extrapolating from superimposed YPD1:SLN1 complex (2R25). See Trajtenberg et al. (131) for details on the model construction of DesK:DesR.

Abbreviations: HK, histidine kinase; HPt, histidine phosphotransfer domain; RR, response regulator.

within the *B. subtilis* sporulation phosphorelay. Spo0B receives a phosphoryl group from Spo0F and donates it to Spo0A. Phosphoryl-transfer directionality incubating radioactive P∼Spo0B with the alternate partners shows reversibility with Spo0F, while the transfer is unidirectional to Spo0A (see figure 4 in Reference 18). Once again, the crystal structure of the Spo0F:Spo0B complex (132, 148) discloses a distance of 5.3 A˚ (averaged among the four independently refined complexes PDB 2FTK) between the reactive HisSpo0B and AspSpo0F, leading the authors to propose an associative nucleophilic substitution mechanism (Figure 4). The 3D structure of Spo0B in complex with Spo0A was recently solved (F. Trajtenberg, A. Buschiazzo, manuscript in preparation) and displays longer distances (spanning 6.5 to 8.5 A˚ on four independent complexes), separating the equivalent HisSpo0B and AspSpo0A atoms. These structures will guide mutagenesis strategies to graft directionality properties by protein engineering. Enzymatic catalysis is based upon lowering the energy of transition states. Kinetic control of otherwise reversible reactions (as driven by thermodynamic equilibrium) can lead to irreversibility, e.g., by withdrawing the P∼RR for oligomerization. With kinetic effects worth consideration, additional 3D structures and quantum chemistry studies of reversible versus irreversible complexes shall forward the mechanistic understanding of TCS directionality, with anticipated impact in synthetic biology.

6. CONCLUDING REMARKS AND PERSPECTIVES

A vast volume of information has been generated over the last decades, studying a tantalizing num- ber of different TCSs that govern a diverse range of biological processes. Remarkably, all HKs are orthologous, as well as the set of RRs, meaning that from unique ancestors, the current plethora of systems and cross-connected networks have evolved. A core of mechanistic principles must be at work, shared by all systems regardless of individual variations and family classifications. From a molecular perspective, a converging picture is indeed emerging, with long α helices acting as machines, transporting information efficiently through central spines in HKs. Another general feature is the low shape complementarity of HK:RR protein:protein interfaces. Ideal complementarity would increase specificity, but likely at the price of slower evolution rates toward novel TCSs. As an example, antigen:antibody interfaces are typically more complementary (reaching very high affinities and specificity), but then adaptability has been solved by a stochastic genetic mechanism of variability generation, which is absent in TCS-encoding genes. Selective pressure on TCSs, in the form of rapidly varying environments, seems to maintain nonideal HK:RR interaction surfaces, enabling rapid evolution of new pathways via gene duplication. Going deeper into the atomic level, yet another universal trait is uncovered in that TCS reaction centers are built from two proteins meeting, so that catalysis is intertwined and amenable to modulations that can influence the pathways' forward and backward directions. More 3D structures of HK:RR complexes are needed, particularly from mesophile organisms, to uncover a clearer picture of molecular interactions, dynamic features and enzymatic mechanisms. Cryo–electron microscopy will likely be a powerful approach to image full-length multi-domain TCS complexes, better dis- criminating different functional states. DHp mutants should be further exploited in structural and biochemical studies, aimed at trapping functional switching states. Future work is expected to ad- dress the association between structural symmetry and functional states in HKs, which is still a matter of debate.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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TERMS AND DEFINITIONS

Allosterically regulated: when structural rearrangements at the protein's orthosteric site (key for function/activity) are modulated by a molecule binding at a distant, allosteric site

Structural rearrangements: mechanical shifts of protein regions, or order-disorder modulation of their conformational dynamic behaviors

Posttranslational modifications: in signaling proteins often rely on phosphorylation and dephosphorylation of specific residues

Histidine kinases (HKs): have the enzymatic capacity to phosphorylate a side chain nitrogen of a histidine using ATP as the phosphodonor

Response regulators (RRs): switch between on and off states according to the phosphorylation status of a specific aspartate residue

ATP-binding domain (ABD): also known as catalytic and ATP-binding (CA), although autophosphorylation catalysis always implicates both DHp and ABD

REC (receiver) domains: prototypical of response regulators; receive the phosphoryl group from HKs

Histidine phosphor transfer (HPt) domains: can be part of HHKs, or standalone monomers; structurally different from DHp domains; they share an all-helical architecture

SIDEBAR

COILED COILS AND SIGNALING IN BIOLOGY

α helices span approximately seven residues (named a through g) every two turns. If positions a and d possess hydrophobic side chains, they will both stick from the same side of the helix, poised to interact with neighbor helices comprising similar heptad-repeating hydrophobic patterns (80). This side-by-side interaction results in a left-handed superhelix or coiled-coils structure, with a knobs-into-holes arrangement of packing side chains (31). Simple movements at the end of a coiled coil are instrumental in biology to transmit information. Such movements translate into substantial positional shifts of distantly located residues, exploiting the stiffness of α helices and sequence-encoded heptad-repeat deviations (111). Strict heptad-repeating patterns show deviations that optimize the hydrophobic core packing to accommodate different

coiled-coil lengths and architectures, also dealing with the opposing periodicity tendencies of 3.6 versus 3.5 residues/turn in free versus coiled-coiled α helices. Frequent deviations as seen in HKs consist of insertions of one, three, or four amino acids (respectively called skips, stammers, and stutters), observed at any given point. Operating as local hydrophobic core adjustments, insertions also generate shifts in the rotary phase and backbone traces of the individual helices (17), an effective machine to transmit information in signaling proteins.