Histidine kinases and two-component signal transduction systems

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The phosphorylation of histidine is the first step in many signal transduction cascades in bacteria, yeast and higher plants. The transfer of a very reactive phosphoryl group from phosphorylated histidine kinase to an acceptor is an essential step in many cellular signaling responses.

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Introduction

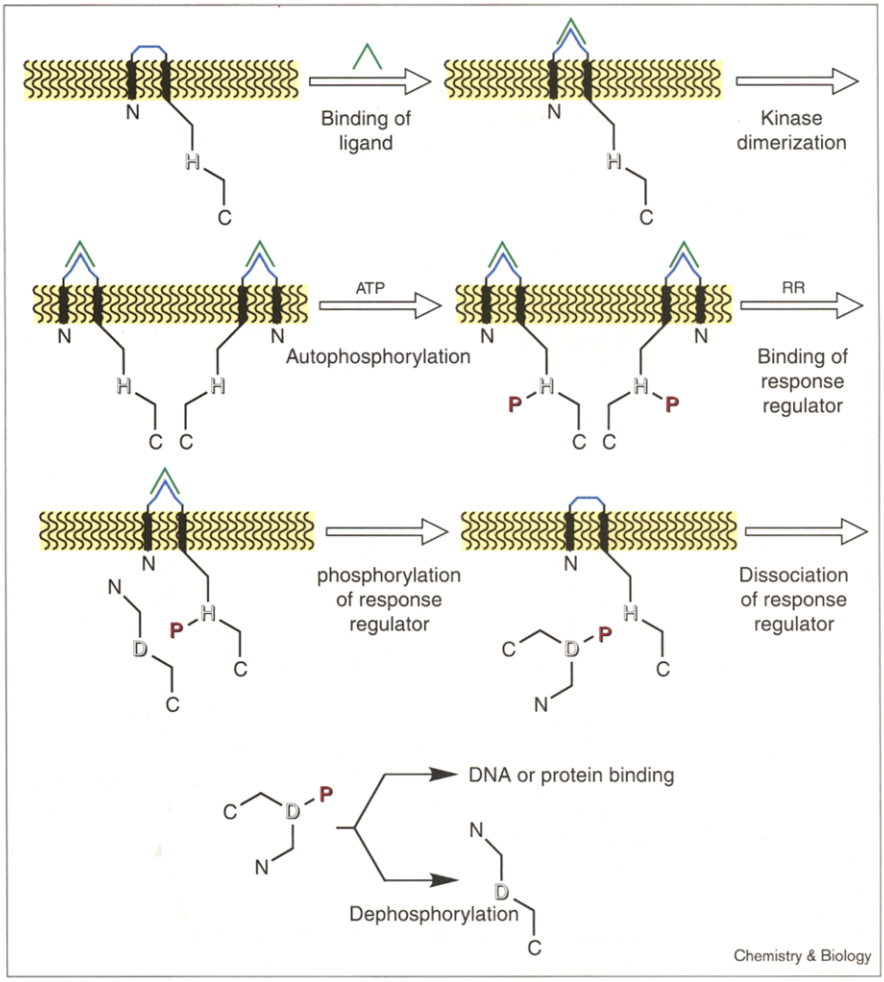
Two-component signaling systems are involved in a variety of responses to the extracellular environment, and have most often been found in prokaryotes [1–3]. These systems include a first protein, usually bearing a membrane-spanning region, containing a ~250 amino acid carboxy-terminal histidine kinase domain that is autophosphorylated on a histidine residue. An aspartate residue in a ~120 amino-acid amino-terminal domain in a second, cytosolic response regulator protein is phosphorylated by the phosphohistidine in the first protein. Some response regulators bear DNA-binding domains that can activate downstream responses, for example as transcription factors (Figure 1). Signal transduction in antimicrobial peptide production, osmoregulation, virulence factor production, sporulation, xenobiotic metabolism, cell-wall production, antibiotic resistance and chemotaxis is initiated by histidine kinases. Protein kinases and phosphatases (involving phosphorylated tyrosine, serine or threonine hydroxyl groups) have long been recognized as tremendously important elements of signal transduction. In contrast, phosphorylation of other heteroatoms in amino-acid sidechains is less known [4]. This review describes histidine kinases and phosphatases in general and their relation to the two-component signal transducing systems.

Chemistry of histidine phosphates

Any protein that participates in phosphoryl transfer could potentially use a phosphohistidine intermediate, generated by a nucleophilic catalysis mechanism. Enzymes that use a phosphohistidine intermediate include nucleoside diphosphate kinase [5–8], pyruvate phosphate dikinase [9], phospholipase D [10], succinyl-CoA synthetase [11], G-protein β subunits [12], mannitol-1-phosphatase [13] and fructose-2,6-bisphosphatase [14,15]. Sugar transport across the cell membrane is also associated with phosphohistidine intermediates (see below) [16]. The phosphate transfer potential (ΔG° of transfer) of phosphohistidine is estimated to be –12 to –14 kcal/mol [17].

Phosphohistidine was first isolated from mitochondria (which was later shown to reflect ATP synthesis in the citric acid cycle via succinyl-CoA synthetase) [18]. By treating histidine with phosphoramidate, Boyer and coworkers [19] prepared both isomers, 1-phosphohistidine and 3-phosphohistidine (Figure 2a). The latter is thermodynamically more stable. Although both isomers are fairly stable to base, they are quite unstable in the presence of acid. They also differ significantly in their hydrolysis rate — 1-phosphohistidine has a 5 minute half-life at pH 2.4 (46°C) and 3-phosphohistidine has a

Figure 1



A model for two-component signaling systems.

25 minute half-life. Many studies of phosphohistidines in proteins do not distinguish between these isomers. Because they show different hydrolytic reactivity *in vitro*, it is certainly expected they would show biochemically different rates of phosphoryl transfer to acceptor groups, which might have physiological consequences. The hydrolytic reactivity of phosphohistidine has been used to infer its presence in a protein, and even the particular isomer present. Hydrolysis is enhanced by hydroxylamine or pyridine, which has also been used as a diagnostic test to distinguish between phosphohistidine and other acid-labile protein phosphates.

Methods for preparing histidine-phosphorylated peptides have involved conventional synthesis followed by phosphoramidate phosphorylation [20]. As in the phosphorylation of histidine itself, these give initially a mixture of isomers that equilibrates to primarily 3-phosphohistidine. These peptides can be analyzed by mass spectrometry using a negative ion matrix-assisted laser desorption/ionization (MALDI) mode. The phosphorylated forms of histidine kinases can often be prepared *in vitro* by treatment with ATP, phosphoramidate or acetyl phosphate (Figure 2b). Clearly, the target histidine is especially reactive, as not all of the histidines in the protein become phosphorylated. Although nuclear magnetic resonance (NMR) properties of phosphohistidines might be used to distinguish between the isomers, the differences are usually smaller than other environmental influences on the resonances [7]. In CheA, 3-phosphohistidine has been identified by its more positive phosphorous chemical shift (by ~1 ppm) [21]. Separation methods for phosphohistidine, its peptides and proteins have been developed to deal with its acid lability [22–24].

Histidine kinases in two-component signaling systems in bacteria

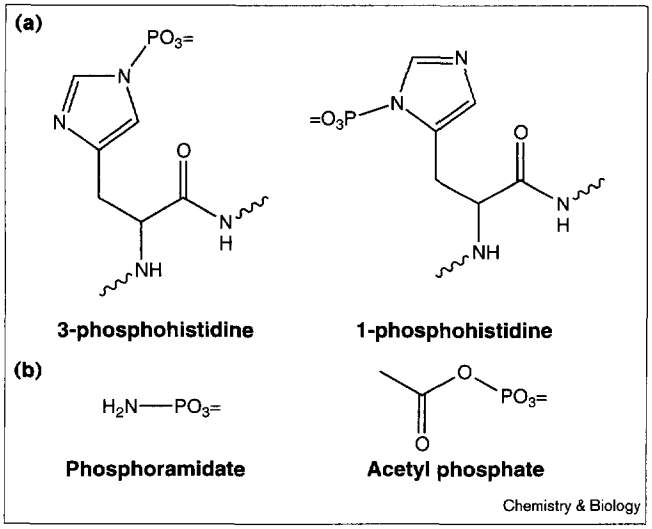
Two-component signaling systems are extremely common in bacteria (Table 1). In the genome of *Escherichia coli* alone, genes encoding 32 histidine kinases have been identified [25]. A number of histidine kinases have been subjected to biochemical study, and can be separated into

a few major classes (Figure 3). One group comprises the classical two-component signaling systems, in which the histidine kinase is at the carboxyl terminus of the protein. Another group comprises 'hybrid' proteins that also contain response regulator or receiver domains within their sequences, and in some cases even additional histidine phosphorylation sites [26]. For example, internal transfer of phosphate from histidine to an aspartate in a receiver domain in ArcB has been established [27], which is apparently required to enable further histidine phosphorylation, leading ultimately to the transfer of phosphate to the response regulator. A special subclass of the hybrid signaling systems is best exemplified by the well-characterized CheA (see below), which is not itself a membrane protein but is associated with a membrane receptor.

Histidine kinases include a sensor domain, usually in an extracellular loop, a substrate domain, containing the phosphorylated histidine, and a catalytic region, which binds ATP. Some histidine kinases, like ArcB, also contain HPt (histidine-containing phosphotransfer) domains that are homologous to the HPr (histidine-containing protein) in sugar transport. Recent research on histidine kinases has also identified a conserved coiled-coil domain in the linker between the second transmembrane helix and the kinase domain [28]. In an earlier study, a fragment of VanS predicted to include this coiled-coil domain was able to inhibit the phosphorylation of a response regulator by VanS *in vivo* [29], which suggests that the coiled-coil domain might be responsible for receptor dimerization. The cytoplasmic signaling domain of EnvZ has been subdivided into a short dimerization domain in the linker region, which also contains the phosphorylated histidine, and the catalytic domain [30] (Figure 4).

It is important to keep in mind, when considering the actions of two-component signaling systems, that phosphorylation of the response regulator does not necessarily lead to the downstream response, as might easily be assumed.

Figure 2



(a) Phosphohistidines in proteins and **(b)** reagents used for chemical phosphorylation.

The role of the histidine kinase might, alternatively, be the maintenance of the response regulator in its phosphorylated form, which prevents it from triggering cellular responses. The dephosphorylated response regulator might, in fact, be the species that promotes subsequent events. Given the natural hydrolytic instability of an aspartyl phosphate, this might be the case more often than not, although a sufficient number of systems have not been studied to establish this trend.

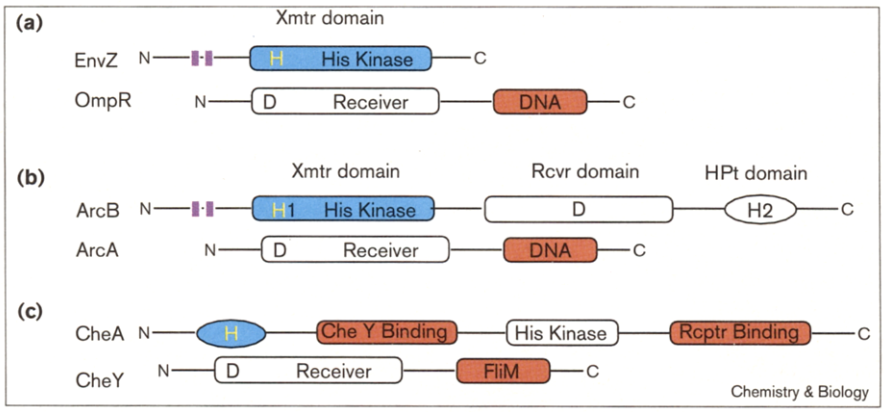
CheA, the histidine kinase involved in bacterial chemotaxis, has been extensively studied, providing information for evaluation of more recently identified proteins. Several accessory proteins are also associated with CheA, complicating the analysis of this system. Autophosphorylation occurs between two juxtaposed subunits of a kinase

Table 1

Some bacterial two-component signaling system histidine kinases.

Histidine kinase	Control function	Source	Receiver
CheA	Chemotaxis, flagellar motor	*E. coli*	CheY
EnvZ	Osmosensing, outer membrane proteins	*E. coli*	OmpR
VanS	Cell-wall biosynthesis	*Enterococcus faecium*	VanR
KinA	Osmosensing	*E. coli*	Spo0F
PhoR	Phosphate metabolism	*E. coli*	PhoB
FrzE	Fruiting body formation	*Myxococcus xanthus*	FrzE (internal receiver)
RscC	Cell capsule synthesis	*E. coli*	RscC (internal receiver)
VirA	Host recognition, transformation	*Agrobacter tumefaciens*	VirA (internal receiver)
ArcB	Anaerobiosis	*E. coli*	ArcB (internal receiver) and ArcA
BvgS	Virulence	*Bordetella pertussis*	BvgS (internal receiver)

Figure 3



Classes of histidine kinases and representative examples of two-component signaling systems. **(a)** Classical, **(b)** hybrid and **(c)** cytoplasmic. Blue, transmitter domains; red, protein- or DNA-binding regions; purple, transmembrane helices; yellow, transferring phosphohistidine.

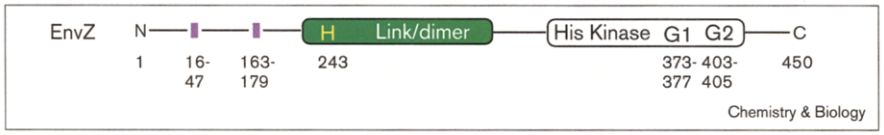
dimer, which has a K_D value ~0.2 μM [21]. The autophosphorylation activity of CheA is reduced when an attractant occupies the sensor site. CheW can influence the activity of CheA, and proteins in another branch of the chemotaxis-response system, like CheB, can also be influenced by CheA by their state of phosphorylation. A complex forms between phosphorylated CheA (P-CheA) and CheY (K_D ~30 nM) that triggers phosphate transfer, which in turn prompts dissociation of P-CheY. The functional domains of CheA include the phosphorylation site at the amino terminus and a middle region involved in dimerization. Interestingly, it appears that the active site that catalyzes phosphorylation is composed from residues in both subunits of the dimer. Studies of heterodimers show modified kinetics when mutations are introduced into the subunit undergoing phosphorylation [31].

An interesting feature of two-component signaling systems is that transfer from the phosphohistidine is in some cases not limited to the cognate response regulator. The structural similarities of the transmitter domain of CheA and the HPt domain of ArcB (Figure 3b) have provoked investigation of the ability of the latter to phosphorylate the CheY response regulator [32]. It was demonstrated both *in vitro* and *in vivo* that the HPt domain of ArcB can phosphorylate the CheY response regulator. In another example, the cytoplasmic domain of VanS can, albeit at a 10^4-fold slower rate, phosphorylate the *E. coli* response regulator PhoB both *in vitro* and *in vivo* [29,33]. Several other response regulators (ArcA, OmpR, and CreB) with comparable levels of sequence homology to VanR were not phosphorylated by VanS, however, suggesting that other factors determine catalytic specificity.

One of the most compelling reasons to study bacterial two-component signaling is its involvement in resistance to the antibiotic vancomycin and related glycopeptides. Vancomycin is used as a last-resort defense against hospital-acquired infections. Researchers had supposed that it would be difficult for bacteria to become resistant to vancomycin on the basis of its mode of action — binding to the D-Ala–D-Ala intermediate in the cross-linking reaction that forms bacterial cell-wall peptidoglycans (which are required for cell viability). Naturally, Darwin taught better, and three classes of vancomycin-resistant enterococci have been identified. High level and inducible glycopeptide resistance is classified as either VanA or VanB and results from the bacteria synthesizing modified peptidoglycan precursors that terminate in D-Ala–D-Lac, for which vancomycin has a thousand-fold lower affinity. The distinction between the A and B strains is related to the ability of the VanS to respond to only vancomycin (B) or to both vancomycin and its relative teicoplanin (A). VanC resistance is constitutive and results from the substitution of D-serine for D-lactate in the peptidoglycan termini.

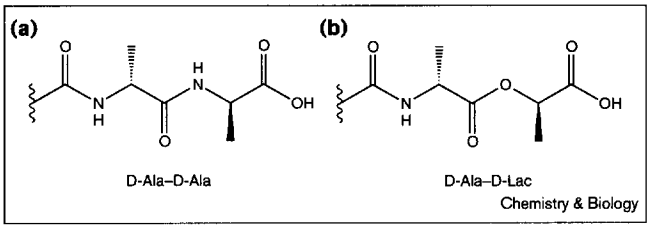
The pathway by which resistance is expressed involves genes that can be transmitted through transposon Tn*1546*. The VanS protein is a histidine kinase that responds to the presence of the glycopeptide, phosphorylating the response regulator VanR. Phosphorylation of VanR enhances its binding capacity for the *vanH* and

Figure 4



Subdomain organization of a representative classical two-component signaling system histidine kinase, EnvZ.

Figure 5



Muramyl dipeptide cell-wall peptidoglycan termini in strains of **(a)** wild-type and **(b)** vancomycin-resistant enterococci.

vanR promoter regions, so phosphorylation induces transcription of these and other Van genes involved in the synthesis and incorporation of D-lactate into peptidoglycan. The distinction between the VanA and VanB phenotypes is related to the ability of the VanS kinase to respond to only one or both of the glycopeptides [34] (see below).

Regulation of the VanS/VanR system has been studied in the Wanner and Walsh laboratories [35–37]. Model systems constructed in *E. coli* show that P-VanR activates transcription of *vanH* and itself in VanA-type strains, but only *vanH* in VanB-type strains. VanS is a phosphatase that acts on the phosphorylated form of VanR through a pathway that does not involve the active-site His164 (VanS mutants lacking His164 still have phosphatase activity). Given their involvement in closely analogous signal transduction pathways, it might be expected that cross-talk between the two response regulators in activating the others' promoters would occur, but to date no such activity has been observed.

Using a reporter-gene assay in a VanA-type enteroccoci strain, over 6000 compounds were screened for their ability to induce the expression of the vancomycin-resistance genes [38]. Interestingly, beyond the glycopeptides to which this system obviously responds, other cell-wall biosynthesis inhibitors, cyclic peptide antibiotics, as well as a macrocyclic lactone antibiotic induced expression of these genes (Figure 6). The only obvious common denominator between these compounds is hydrophobicity — determining why these compounds stimulate this system might provide insight into the mechanism of resistance itself.

The relevance of two-component signaling systems to antibacterials is not limited to vancomycin-resistant *Enterococci*, as the resistance of *Streptococcus pneumoniae* to penicillin is also based on a histidine kinase [39].

Figure 6

Compounds that induced expression of vancomycin-resistance genes in a VanA-type enterococci strain. Bacitracin, which inhibits cell-wall biosynthesis, acts on the undecaprenyl phosphate carrier required for transport of peptidoglycan precursor disaccharide pentapeptides across the plasma membrane. Moenomycin, which also inhibits cell-wall biosynthesis, inhibits transglycosylation of the undecaprenyl-pyrophosphate-disaccharide pentapeptide substrate. The mechanism of AO341β is unknown.

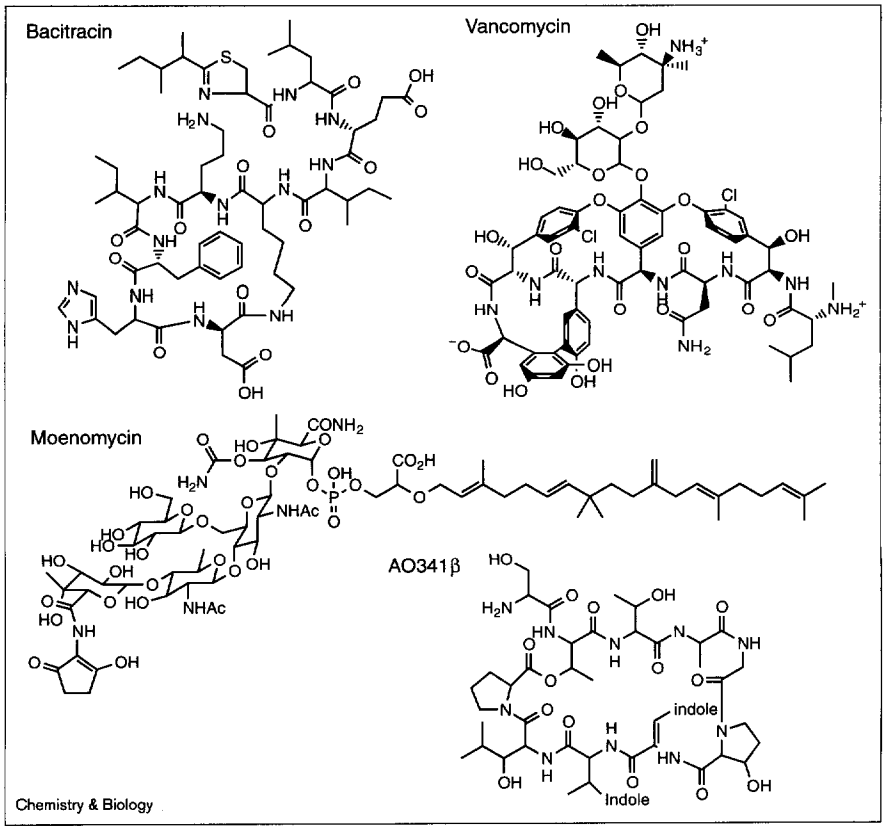
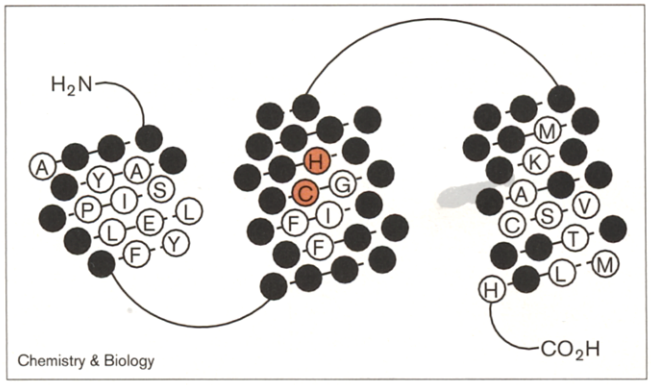


Figure 7



A helical network model for the ethylene-binding domain of ETR1 developed by comparing with a number of plant-derived hybrid histidine kinases. The essential cysteine and histidine residues are shown in red. Black circles indicate nonconserved residues; conserved residues are designated in single-letter amino-acid code.

Phosphohistidine in two-component signaling systems in plants

The fascinating discovery that the putative receptor for the plant hormone ethylene is a two-component signaling system was made in 1993 [40]. Dominant negative mutations in the *ETR1* gene of *Arabidopsis* cause insensitivity to ethylene. ETR1 contains both histidine-kinase and response-regulator regions like the hybrid bacterial histidine kinases, suggesting that there may be further histidine or aspartate phosphorelays involved in transduction of the ethylene signal. Secondary structure prediction on ETR1 has identified several subdomains, including the putative transmembrane region (Figure 7). The cytoplasmic domain of ETR1 has recently been shown to have histidine kinase activity [41]. Structure–function studies have pointed to a putative ethylene-binding site in the transmembrane region composed from a copper ion ligated by essential cysteine and histidine residues [42].

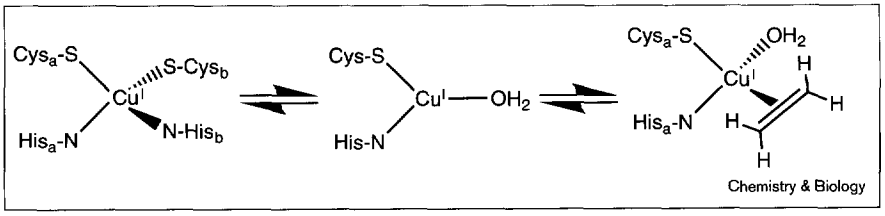
This structural information provides a challenge to explain how ethylene triggers signaling. A copper involved in olefin binding would be expected to exist in the +1 oxidation state, giving it 10 d-electrons. If the metal-binding site is composed from a histidine kinase dimer, each could contribute two 2-electron ligands to copper, making it coordinately saturated and unable to accept an olefin ligand. If the dimer were to 'breathe' to nonmetalated and metalated monomers (perhaps with the addition of a weak ligand such as water, to maintain copper in a 16-electron state), however, ethylene binding could occur, preventing re-association of the dimer (Figure 8). Ethylene would thereby stop signaling. The genetic evidence (a dominant mutation) indicates that ETR1 actively signals in the unbound state (keeps response pathways off) and that ethylene binding in the wild-type turns signaling off. The dominant mutations do not bind ethylene (or even copper) but apparently continue to signal an 'off' state to the response pathways. Although this model cannot account for the agonist/antagonist properties of a number of different olefins, it provides a provocative starting point for study.

NMR structures of phosphohistidine proteins

The structures of many phosphohistidine-containing proteins have been determined using X-ray crystallography (see above). No structure of a histidine kinase from a two-component system has been solved, however, although histidine phosphotransfer domains have been solved using NMR [43].

Two proteins from the phosphoenolpyruvate (PEP)–sugar phosphotransferase system, which does not involve a histidine kinase *per se*, have been studied [44]. This system operates as shown in Figure 9, with a first protein Enzyme I undergoing phosphorylation on His189 by PEP and transferring its phosphate to His15 of HPr, which then phosphorylates enzyme IIA, and so on down a cascade that eventually leads to sugar transport. Some of the phosphohistidine intermediates in this phosphotransferase system are the 1-isomers, whereas others are the 3-isomers. Enzyme I is too large for its structure to be solved directly, but the structure of its amino-terminal domain, containing the phosphorylation site, has been reported [45]. It consists of an α-helical domain (residues 33–143) flanked by an α/β domain (residues 1–20 and 148–230), with the active site His189 located at the interface between the two domains. An NMR structure is also available for HPr [46]. Its phosphoprotein form is fairly unstable towards hydrolysis, with a half life of only a few minutes, but it can be maintained in a phosphorylated form by an *in situ* regeneration system via PEP and

Figure 8



A postulated mechanism for ethylene-triggered histidine-kinase signaling.

enzyme I. HPr experiences only local structural changes upon histidine phosphorylation, which primarily relate to hydrogen bonding of the phosphate to contiguous amide protons at the amino terminus of a helix. Finally, the structure of the complex between the amino terminus of enzyme I and Hpr has recently been solved, supporting the expected structural complementarity between the two protein surfaces [47].

The two-component osmosensor EnvZ from *E. coli* has two functional domains in its cytoplasmic carboxyl terminus: domain A contains the site of phosphorylation, whereas domain B contains the catalytic residues, including several regions highly conserved among histidine kinases (Figure 4). The structure of domain B of EnvZ has been solved [48]; it adopts an α/β sandwich fold. ATP is bound through two glycine-rich regions with its γ-phosphate exposed on the protein surface, ready for transfer to the histidine in domain A. Another of the ATP-binding residues, Asn347, binds to the β phosphate and a Mg^{+2}. When Asn347 is mutated to aspartate, ATP binding and ATP-dependent autophosphorylation activity are lost. This Asn347→Asp mutant retains the ability to phosphorylate a response regulator, however. The overall structure is distinct from the serine/threonine/tyrosine protein kinases, and has surprising similarity to heat-shock protein 90 and DNA gyrase B.

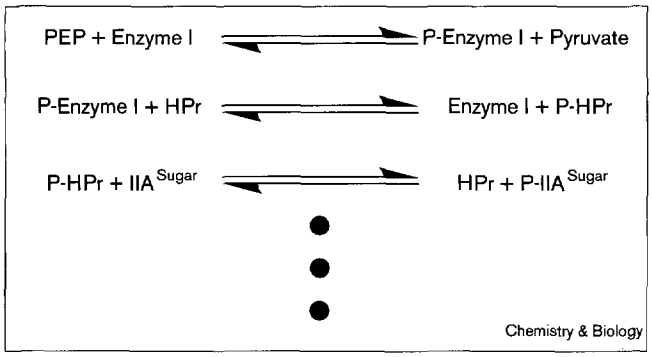
Low-resolution structures of two subdomains of the CheA protein have been determined [49,50]. The amino-terminal domain containing the phosphorylation site is mainly a four-helix bundle with the target histidine at a helix interface. Similar to earlier observations [46], only small perturbations to the overall protein structure occur upon phosphorylation. The carboxy-terminal domain responsible for interactions with the receiver CheY is structurally composed of a β-sandwich motif [51].

A useful compilation of structural information and analysis of histidine active sites determined using NMR is reported by McEvoy and Dahlquist [1]. One theme in the phosphosugar-transport system is the location of the phosphorylated histidine at the amino terminus of a helix, where the helix dipole can contribute to stabilization of the charge of the phosphate group. No such trend is seen in the two-component regulators, however.

Phosphatases for phosphohistidine proteins

Natural pathways for dephosphorylation of the histidine of two-component histidine kinases include transfer to the response regulator and autolysis. In some cases, a specific phosphatase may exist. One unambiguous demonstration of two-component phosphohistidine phosphatase activity is in the SixA protein [52]. SixA dephosphorylates the histidine kinase of ArcB in the anaerobiosis system in *E. coli*. SixA has an Arg–His–Gly (RHG) sequence at its amino terminus that is a putative nucleophilic phosphoacceptor. This sequence is essential for activity, and is homologous to sequences in other phosphatases, such as fructose-2,6-bisphosphatase. Natural eukaryotic histidine phosphatases are also known, including PP1, PP2A and PP2C [53,54]. These are generally thought of as protein serine- or threonine-phosphatases, but in fact have even greater activity on phosphohistidine.

Figure 9



Phosphosugar transferase system.

Two-component signaling systems in other eukaryotes

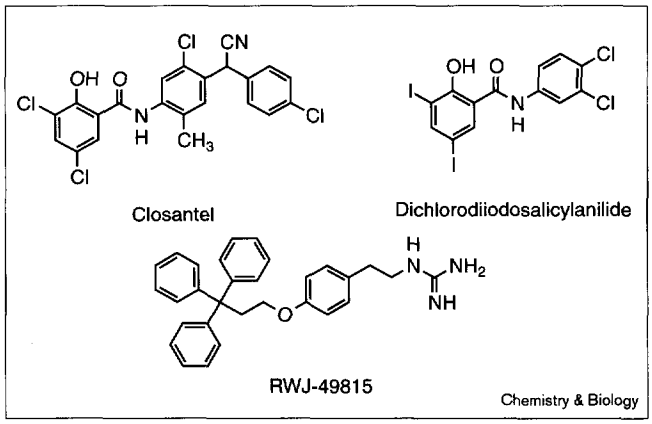
Only recently has a histidine kinase been identified in a eukaryote other than plants. Yeast *Sln1* is a hybrid kinase–response regulator involved in osmosensing [55]. By study of suppression of its otherwise lethal deletion, it has been possible to identify its connection to mitogen-activated protein kinases (MAPKs) [56].

P-Selectin has been shown to undergo histidine phosphorylation in its cytoplasmic tail upon platelet activation [57]. The half-life of this histidine phosphate is extremely short (< 15 s). It has not been shown that a response regulator is associated with this histidine kinase, however, and it is unclear, given the very short cytoplasmic tail of P-selectin, whether autophosphorylation would be possible.

Inhibitors of histidine kinases

The novelty of histidine kinases has left little opportunity for accidental or directed discovery of compounds that inhibit them. Workers at R.W. Johnson Pharmaceutical Research Institute have discovered two classes of histidine-kinase inhibitors [58–60]. Using a radioactive ATP incorporation assay and KinA (involved in osmosensing in *E. coli*) as the target they identified closantel, a veterinary anti-helminthic, and tetrachlorosalicylanilide, a topical antibacterial (Figure 10), as kinase inhibitors. Some optimization of these structures led to dichlorodiiodosalicylanilide, which is about twice as potent (IC_{50} = 21 μM) as either of the 'lead' compounds. Dichlorodiiodosalicylanilide has a minimal

Figure 10



Inhibitors of histidine kinases.

inhibitory concentration (MIC) value of ~0.5 μg/ml against methycillin-resistant *S. aureus* and vancomycin-resistant *E. faecium*. The hydrophobic tyramines, exemplified by RWJ-49815, represent another class of histidine kinase inhibitor. RWJ-49815 competes with ATP for binding (IC_{50} = 1.6 μM) and has an MIC value of 1 μg/ml against vancomycin-resistant enterococci. It is interesting that these compounds show activity directly, rather than simply acting to reverse antibiotic resistance and act as a synergist, which could suggest they target multiple two-component signaling systems *in vivo*.

Future prospects

The biology of two-component signaling systems is fairly well understood, but advances clearly must be made in the chemistry, which may also contribute to the elucidation of biological questions. For example, antibodies against phosphohistidine proteins are not available, as contrasted with O-linked phosphoproteins, because of the instability of phosphohistidine. The availability of anti-phosphohistidine antibodies would allow a variety of valuable immunochemical experiments, making analogues of phosphohistidine that are stable enough to be presented as an antigen to the mammalian immune system important. Methods to stabilize phosphohistidine reversibly would also be valuable. These could enable phosphohistidine proteins to be purified and unambiguously identified, yet placed back into their biological context to observe their behavior. Caging methods for the rapid photochemical release of stabilized and inactive phosphohistidine proteins would enable kinetic studies of fast signaling processes. More reliable and more easily applied methods for identifying the position of histidine phosphorylation are also needed. These would permit the phosphohistidine isomer reactivities to be broadly determined in many sequence contexts. Finally, it is expected that much more potent drug candidates targeting histidine kinases will be discovered through screening, rational design and combinatorial chemistry efforts. The resulting reagents could provide further mechanistic insight into the operation of these fascinating signal transduction systems, as well as provide an avenue to antibacterials with a novel mode of action.

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