TWO-COMPONENT SIGNAL TRANSDUCTION

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Abstract Most prokaryotic signal-transduction systems and a few eukaryotic pathways use phosphotransfer schemes involving two conserved components, a histidine protein kinase and a response regulator protein. The histidine protein kinase, which is regulated by environmental stimuli, autophosphorylates at a histidine residue, creating a high-energy phosphoryl group that is subsequently transferred to an aspartate residue in the response regulator protein. Phosphorylation induces a conformational change in the regulatory domain that results in activation of an associated domain that effects the response. The basic scheme is highly adaptable, and numerous variations have provided optimization within specific signaling systems. The domains of two-component proteins are modular and can be integrated into proteins and pathways in a variety of ways, but the core structures and activities are maintained. Thus detailed analyses of a relatively small number of representative proteins provide a foundation for understanding this large family of signaling proteins.

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INTRODUCTION

Over a decade ago, the term “two-component” was coined to describe a new class of regulatory systems found in bacteria (1–3). To date, researchers have found hundreds of such systems in eubacteria, archaea, and a few eukaryotic organisms. Two-component systems serve as a basic stimulus-response coupling mechanism to allow organisms to sense and respond to changes in many different environmental conditions. These are sophisticated signaling systems marked by a highly modular design that has been adapted and integrated into a wide variety of cellular signaling circuits.

The prototypical system consists of a histidine protein kinase (HK), containing a conserved kinase core, and a response regulator protein (RR), containing a conserved regulatory domain. Extracellular stimuli are sensed by, and serve to modulate the activities of, the HK. The HK transfers a phosphoryl group to the RR, in a reaction catalyzed by the RR. Phosphotransfer to the RR results in activation of a downstream effector domain that elicits the specific response.

In this review, we summarize the current understanding of the basic biochemical and biophysical mechanisms of two-component signal transduction, with an emphasis on recent advances concerning structure/function aspects. Because of the enormous number of systems that have been identified to date and the pace at which new ones are currently being discovered, it is beyond the scope of this review to present a comprehensive cataloging of systems or components. A number of extensive reviews (4–6) and a monograph (7) have been published detailing the biology and chemistry of two-component systems. In addition to descriptions of individual systems in these sources, additional reviews have focused on well-characterized systems such as bacterial chemotaxis (8), aerobic/anaerobic
regulation in *Escherichia coli* (9, 10), the sporulation system of *Bacillus subtilis* (11, 12), and differentiation in *Caulobacter crescentus* (13–15) and *Myxococcus xanthus* (16, 17). The efforts of a large number of laboratories working with many different systems have gone into elucidation of the basic biology and chemistry of two-component signal transduction. The cumulative data have provided a basic understanding of how these systems transduce extracellular signals and elicit appropriate cellular responses.

**PHOSPHOTRANSFER CHEMISTRY**

**Reactions**

The chemistry of the basic two-component phosphoryl transfer signal transduction pathway involves three phosphotransfer reactions and two phosphoprotein intermediates (Figure 1a):

1. Autophosphorylation: HK-His + ATP ⇌ HK-His∼P + ADP
2. Phosphotransfer: HK-His∼P + RR-Asp ⇌ HK-His + RR-Asp∼P
3. Dephosphorylation: RR-Asp∼P + H₂O ⇌ RR-Asp + Pᵢ

The γ-phosphoryl group in ATP is first transferred to a conserved His side chain of the HK. The RR then catalyzes the transfer of this phosphoryl group from the phospho-His residue to a conserved Asp side chain within its own regulatory domain. Finally, the phosphoryl group is transferred from the phospho-Asp residue to water in a hydrolysis reaction. All three reactions require divalent metal ions, with Mg²⁺ presumably being the relevant cation in vivo.

**Phosphohistidine Chemistry**

The HKs are catalytically similar to Ser/Thr/Tyr protein kinases, but the chemistries differ. Ser/Thr/Tyr kinases create phosphoesters, whereas HKs create phosphoramidates. The hydrolysis of phosphoramidates has a significantly greater negative free energy than that of phosphoesters, and the use of these modifications in biological systems differs accordingly (18). The equilibrium for reaction 1 favors the unphosphorylated protein. Given the intracellular ATP/ADP ratio, only a small percentage of the HK population exists in a phosphorylated state. Thus, it is the flux of phosphoryl groups rather than stoichiometric phosphorylation that is relevant to the function of HKs.

The high-energy N∼P bond is ideally suited for phosphoryl transfer. In this capacity, phospho-His occurs as a phosphoenzyme intermediate in proteins such as succinyl-CoA synthetase (19), pyruvate phosphate dikinase (20), and nucleoside diphosphate kinase (21, 22). Phospho-His is also used by the EI and EII enzymes of the phosphoenolpyruvate:sugar phosphotransferase system as phosphotransfer moieties, paralleling their use in two-component pathways (23, 24).
Figure 1  Schematic diagram depicting the modular organization of representative two-component systems. Asp-containing domains are colored dark gray, His-containing domains are colored light gray, and variable auxiliary domains are colored white. (a) The prototypical two-component pathway exemplified by the E. coli osmoregulatory system uses a single phosphoryl transfer event between the orthodox histidine protein kinase (HK) EnvZ and its cognate response regulator protein (RR) OmpR. (b) The E. coli Arc system illustrates a phosphorelay involving the hybrid HK ArcB. Depending on aerobic conditions, ArcA is capable of receiving a phosphoryl group from either the catalytic core or the His-containing phosphotransfer (HPt) domain of ArcB. (c) The E. coli chemotaxis pathway involves an atypical soluble HK CheA that phosphorylates either of two RRs, the single domain RR CheY and the methylesterase CheB. (d) The B. subtilis sporulation control system is a multicomponent His-Asp-His-Asp phosphorelay system in which all of the signaling domains are independent proteins. Spo0F receives a phosphoryl moiety from either KinA or KinB and subsequently transfers it to the HPt Spo0B, which then phosphorylates the terminal RR Spo0A.
The characteristic acid lability and alkaline stability of phospho-His has provided an experimental basis for its identification in proteins (25–27). Nuclear magnetic resonance (NMR) studies have also been used to detect phospho-His within an HK (28). Phosphorylation of His can occur on either the N1 or N3 position of the imidazole ring, and both forms have been found in proteins. However, in all HKs characterized to date, N3-phospho-His has been observed, or it has been inferred based on hydrolysis rates (26–29).

**Phosphoaspartate Chemistry**

Phosphorylation of Asp produces a high-energy acyl phosphate. Phospho-Asp enzyme intermediates have been observed in acetate kinase (30) and postulated for the large haloacid dehalogenase super family of hydrolases (comprising P-type ATPases, phosphatases, epoxide hydrolases, and L-2-haloacid dehalogenases) (31). The best characterized phospho-Asp intermediate is that found in the P-type ion-translocating ATPases (32–34). The free energy of hydrolysis of the phospho-Asp residue in these proteins is significantly different than that measured in small-molecule acyl phosphates (35). This has led to the hypothesis that the energy within the acyl phosphate bond may be used to drive conformational changes in proteins (32, 33). In this proposed role, phospho-Asp differs from phospho-Ser/Thr, which alters protein activity by local electrostatic effects (36, 37). Presumably, it is the propensity of phospho-Asp to effect long-range conformational changes that is exploited in RRs.

Acyl phosphates are rapidly hydrolyzed in both acidic and alkaline conditions and have half-lives of several hours in neutral conditions (38, 39). Identification of phospho-Asp is technically difficult and has been achieved in only a small number of RRs (40, 41). Characteristic hydrolysis rates under different conditions have been more frequently used to infer the presence of phospho-aspartyl residues in RRs (42, 43).

The lifetime of phospho-Asp within RRs varies significantly. Typical half-lives range from seconds to hours (3, 43–46). Many RRs have autoprophosphatase activity that decreases the lifetime of the phosphoprotein (3). In a few cases, the RR stabilizes the phospho-Asp, increasing the half-life significantly beyond that of a typical acyl phosphate. For example, the phospho-Asp in yeast SSK1 has a half-life of ~2 days (47). Phospho-Asps in thermophilic proteins also appear to be stabilized, with half-lives at ambient temperature greater than their mesophilic counterparts (48, 49, 49a).

**GENOMIC DISTRIBUTION**

**His-Asp vs Ser/Thr/Tyr Phosphorylation Pathways**

Two-component systems are found in organisms of all domains: Eubacteria, Archaea, and Eukarya. However, their abundance in each domain differs substantially. His-Asp phosphotransfer systems account for the majority of signaling
pathways in eubacteria but are quite rare in eukaryotes, in which kinase cascades involving Ser/Thr and Tyr phosphorylation predominate. Although attempts have been made to rationalize the different distribution of signal transduction mechanisms, no persuasive explanation has been achieved. Clearly both phosphorylation schemes (His-Asp and Ser/Thr/Tyr) can function in both prokaryotes and eukaryotes. His-Asp phosphotransfer systems have been found in several eukaryotic organisms (for reviews, see 50–53), and Ser/Thr and Tyr kinases and phosphatases have been identified in bacteria (for a review, see 54). In fact, these modular signaling components appear to be intertwined within several signaling pathways. In eukaryotes, His-Asp phosphorelays are coupled to mitogen-activated protein (MAP) kinase cascades (55–57) and a cAMP-dependent protein kinase (58–60). In the cyanobacteria *Synechocystis* sp., the coupling of an RR domain to a Ser/Thr protein phosphatase domain and the clustering of genes encoding Ser/Thr kinases or phosphatases with those encoding two-component proteins have led to postulation that, within bacteria as well, multiple phosphorylation schemes may be combined in a single pathway (61, 62).

**Occurrence in Prokaryotes**

The availability of complete genome sequences has allowed definitive assessment of the prevalence of two-component proteins. In *E. coli* there are 30 HKs (5 of which are hybrid kinases) and 32 RRs (63). However, the number of two-component proteins differs greatly in different bacteria, ranging from 0 in *Mycoplasma genitalium* to 80 in *Synechocystis* sp., in which these proteins account for ~2.5% of the genome (61, 64). Preliminary analyses of other completed bacterial genomes have estimated the numbers of two-component proteins to be as follows: *B. subtilis*, 70 (65); *Haemophilus influenzae*, 9 (64); *Helicobacter pylori*, 11 (64); and *Thermotoga maritima*, 19 (65a). Similar analyses of two archaea have estimated the following: *Methanobacterium thermoautotrophicum*, 24 (66); and *Methanococcus jannaschii*, 0 (64).

**Occurrence in Eukaryotes**

In contrast to the hundreds of two-component proteins identified in prokaryotes, only a limited number have been found in eukaryotes. In the genome of the budding yeast *Saccharomyces cerevisiae*, there is only one phosphorelay system (SLN1-YPD1-SSK1, SKN7) involved in osmoregulation (56, 67–69). The fission yeast *Schizosaccharomyces pombe* contains an RR (MSC4) that regulates a stress-activated MAP kinase cascade (57). The pathogenic fungus *Candida albicans* contains at least two HKs involved in osmoregulation (CASLN1) (70) and hyphal development (COS1/CANI1K) (70, 71). Homologs of COS1/CANI1K have been identified in the filamentous fungi *Neurospora crassa* (NIK-1) (72) and *Aspergillus nidulans* (ANNI1K) (71). The slime mold *Dictyostelium discoideum* contains at least 11 HKs (DOKA, DHKA-D, and ESTs) involved in a range of activities that include osmotic response and development (73–77). Two-component proteins are
not limited to eukaryotic microorganisms; they have also been found in plants, such as *Arabidopsis thaliana* (ETR1, ETR2, ERS, and EIN4) (55, 78) and tomato (NR) (79, 80), in which they regulate ethylene-mediated fruit ripening. The only completed animal genome, that of the worm *Caenorhabditis elegans* (81), has revealed no two-component proteins.

### Differences Between Prokaryotic and Eukaryotic Components

Several features distinguish eukaryotic two-component systems from those of prokaryotes. Hybrid HKs that contain RR domains are rare in prokaryotes (5 of 30 in *E. coli*) (63), whereas eukaryotic HKs are almost exclusively hybrid kinases; the only known exception is *Arabidopsis* ERS (78). Specific sequences distinguish eukaryotic HKs and RR domains from those of prokaryotes (82), and phylogenetic analyses suggest that the eukaryotic HKs evolved from a single prokaryotic source represented by a cluster of bacterial hybrid HKs (BarA, RcsC, ArcB) (82). Prokaryotic RRs are predominantly transcription factors (at least 25 of 32 in *E. coli*), whereas there is only one known eukaryotic RR with a DNA-binding domain (*S. cerevisiae* SKN7) (83). In eukaryotes (as in prokaryotes), the ultimate response of two-component pathways is generally regulation of gene expression. However, in eukaryotes, other signaling components that are themselves regulated by two-component proteins effect the final response. This more-complex scheme provides a greater number of potential steps for regulation and may also facilitate transmission of signals from the cytoplasm to the nucleus, where transcription occurs. Despite the differences in configurations of eukaryotic and prokaryotic two-component signaling systems, the structures and activities of the modular domains of the proteins are conserved, and characterization of individual components provides a solid foundation for understanding other family members.

### STRUCTURE/FUNCTION OF HISTIDINE PROTEIN KINASES

#### Activities and Architecture

In typical two-component systems, sensor HKs monitor external stimuli and transmit this information to the RR by a phosphorylation event. Both prokaryotic and eukaryotic HKs contain the same basic signaling components, namely a diverse sensing domain and a highly conserved kinase core that has a unique fold, distinct from that of the Ser/Thr/Tyr kinase superfamily. The overall activity of the kinase is modulated by input signals to the sensing domain. HKs undergo an ATP-dependent autophosphorylation at a conserved His residue in the kinase core. Autophosphorylation is a bimolecular reaction between homodimers, in which one HK monomer catalyzes the phosphorylation of the conserved His residue in the second monomer.
(28, 84–88). Unlike the typical protein kinase cascade in which one protein kinase phosphorylates multiple targets, in two-component systems, the RR stoichiometrically transfers the phosphoryl group from the phospho-HK to a conserved Asp residue in its regulatory domain. Therefore, control in two-component pathways is accomplished through the ability of the HK to regulate the phosphorylation state of the downstream RR. Besides directing the forward phosphorylation reaction, many HKs possess a phosphatase activity, enabling them to dephosphorylate their cognate RRs (89–91). These bifunctional HKs are commonly present in phosphotransfer pathways that need to be shut down quickly.

In the extremely diversified family of HKs, elegant signal transducers have been created from the simple combination of sensing, catalytic, and auxiliary domains. The modular nature of these proteins permits the structural architecture of individual HKs to be adapted to the specific needs of the signaling system. Members of the HK family range in size from <40 kDa to >200 kDa; the larger HKs consist of five or six structurally and functionally unique domains. Despite this diversity, HKs can be roughly divided into two classes: orthodox and hybrid kinases (6, 92; Figure 1).

Most orthodox HKs, exemplified by the *E. coli* EnvZ protein (Figure 1a), function as periplasmic membrane receptors. The osmosensor EnvZ has two transmembrane regions that separate the protein into a periplasmic N-terminal sensing domain and a cytoplasmic C-terminal catalytic region that is designated as the kinase core. Whereas EnvZ represents the most common membrane topology, other HKs contain multiple transmembrane segments. Examples include *Rhizobium meliloti* FixL, involved in controlling nitrogen fixation (93), and UhpB, part of the *E. coli* sugar transport system (94), which have four and eight transmembrane segments, respectively. Not all orthodox kinases are membrane bound. The chemotaxis kinase CheA (95) and the nitrogen regulatory kinase NtrB (96) are examples of soluble cytoplasmic HKs. Soluble HKs can be regulated by intracellular stimuli and/or interactions with cytoplasmic domains of other proteins.

The more elaborate hybrid kinases, found in some prokaryotic and most all eukaryotic systems, constitute the remainder of the HKs. These proteins contain multiple phosphodonor and phosphoacceptor sites. Instead of promoting a single phosphoryl transfer, hybrid kinases use multistep phosphorelay schemes. The overall complexity of the hybrid kinase structure allows different checks and inputs to be integrated into a signaling pathway. *E. coli* ArcB (Figure 1b), which functions in the anoxic redox control (Arc) system, has an architecture representative of most hybrid kinases (97). ArcB is composed of two N-terminal transmembrane regions followed by a kinase core, a domain similar to the regulatory domain of RRs, and finally a second His-containing region termed a His-containing phosphotransfer (HPt) domain. The great diversity of hybrid kinases is exemplified by TodS, a unique HK found in the *Pseudomonas putida* toluene degradation pathway (98). TodS is the only known HK containing two perfectly duplicated kinase cores, each containing all conserved HK motifs. TodS also has an N-terminal leucine zipper motif, which is uncommon in prokaryotes. Finally, TodS is a dual-sensing kinase, possessing not only an N-terminal toluene-sensing domain, but also a
putative oxygen-sensing (PAS) domain, identified based on sequence homology to the conventional \textit{R. meliloti} HK FixL (98).

**Kinase Catalytic Core**

The unifying structural feature of the HK family is the characteristic kinase core composed of a dimerization domain and an ATP/ADP-binding phosphotransfer or catalytic domain (99). This concept began to evolve when deletion analysis of EnvZ indicated that the cytoplasmic domain of the protein contains at least two functionally and structurally separable domains (100). However, this novel kinase architecture was not defined until the overall topology of the HK core was determined from the crystal structure of the C-terminal half of the \textit{T. maritima} CheA protein (101) and the NMR solution structures of both the catalytic (102) and dimerization (103) domains of the \textit{E. coli} EnvZ protein.

The kinase core is \(\sim\)350 amino acids in length and is responsible for binding ATP and directing kinase transphosphorylation. There are five conserved amino acid motifs present in both eukaryotic and prokaryotic HKs (4, 6, 92). The conserved His substrate is the central feature in the H box, whereas the N, G1, F, and G2 boxes define the nucleotide binding cleft (Figure 2). In most HKs, the H box is part of the dimerization domain. However, for some proteins, like CheA, the conserved His is located at the far N terminus of the protein in a separate HPt domain (Figure 1c). The N, G1, F, and G2 boxes are usually contiguous, but the spacing between these motifs is somewhat varied.

The fold of the HK catalytic domain is unlike any known Ser/Thr/Tyr kinase (reviewed in 99, 104), suggesting that the mechanism of action of this kinase may be different than that of other previously characterized prokaryotic and eukaryotic kinases. The main body of the domain is an \(\alpha/\beta\)-sandwich fold consisting of five antiparallel \(\beta\) strands and three \(\alpha\) helices (Figure 2a). This domain is structurally homologous to the ATPase domains of DNA gyrase B, MutL, and Hsp90 (101, 102). The ATP-binding site of the HKs consists of conserved residues from the N, G1, F, and G2 boxes. In both the CheA and EnvZ structures, this binding pocket is a highly flexible region of the protein. This flexibility may reflect conformational changes that accompany ATP binding. Crystallographic studies of MutL revealed structural shifts in the loops surrounding the nucleotide binding site upon ATP binding and hydrolysis. These changes modulate protein-protein interactions between MutL and other proteins involved in repair (105). Structural similarity has led to the proposal that HKs may undergo similar ATP-coupled conformational rearrangements (99).

Is the novel fold of the HK class of protein kinases indicative of specific catalytic requirements for phosphorylation of His rather than Ser/Thr/Tyr? The answer appears to be no. HK homologs are known in which the phosphorylated His is substituted by Ser (plant, cyanobacteria, and cryptophyte phytochromes) (106–109) or Tyr (\textit{Caulobacter crescentus} DivL) (110). It seems more likely that the HK fold reflects a distinct evolutionary origin and, perhaps, constraints other than those associated with catalysis. The substrate residues of HKs lie within helical
structures, unlike the substrate residues of Ser/Thr/Tyr kinases, which are found in more extended secondary structure conformations (111–113). Different active site architecture may be advantageous in sterically facilitating interactions with structurally distinct substrates.

The dimerization domains of both EnvZ (103) and CheA (101) form antiparallel four-helix bundles, similar to the structure of the HPt domains discussed below.
The dimerization domain of EnvZ, like those of the majority of HKs, houses the conserved His, which is positioned midway along the exposed face of helix 1 (Figure 2b).

There are unanswered questions concerning the relative three-dimensional arrangement of the catalytic and dimerization domains. The only structural information for an intact kinase core is derived from the atypical kinase CheA, which lacks a contiguous H box (Figure 1c). In the crystal structure of the CheA dimer, catalytic domains (P4) protrude out from opposite sides of the central helical structure formed by the dimerization domains (P3) (Figure 2c). The P3 and P4 domains are connected through a short, flexible linker extending from the base of the four-helix bundle, with the active site of the catalytic domain facing away from the central core. This orientation may be well suited for transphosphorylation of the CheA HPt domain (P1) that is located at the N-terminus of the protein. However, such an arrangement, if maintained in conventional HKs, would place the H box of the dimerization domain far from the kinase active site. For typical kinases, it is necessary to postulate either a different domain arrangement than that found in CheA or a substantial conformational change that would bring the His and ATP into sufficiently close proximity for phosphotransfer.

Histidine-Containing Phosphotransfer Domain

A small number of two-component systems contain HPt domains. In prokaryotes, HPts are almost exclusively components of hybrid kinases, whereas in eukaryotes, they are found as separate proteins. HPt domains are ∼120 amino acids in length and contain a His residue capable of participating in phosphoryl transfer reactions. The HPt domains do not exhibit kinase or phosphatase activity (114) and so are ideally suited to serve as specific cross-communication modules between different proteins. In recent years, the structures of four HPt domains/proteins have been solved. These are the P1 domain of *E. coli* CheA (115), the HPt domain of *E. coli* ArcB (116), the *B. subtilis* Spo0B protein (117), and the *S. cerevisiae* YPD1 protein (118, 118a).

It is interesting that all of the HPts share a common four-helix bundle motif (Figure 3) despite their overall lack of sequence similarity. Moreover, the structural architecture of the HPt domain is homologous to the dimerization/His-containing domain of EnvZ, and as in the dimerization domain, the active site His in the HPt is located on a solvent-exposed helical face. Sequence similarity of specific residues surrounding the His residue has prompted postulation of structural and functional roles for these residues (118). Despite the overall fold conservation, specific differences in helix length and orientation in each HPt domain may provide structural features needed for individual functions. These structural variations likely result in modifications of surface properties designed to promote proper intermolecular contacts. The functional forms of YPD1, the ArcB HPt domain, and the CheA P1 domain are monomeric. They have somewhat irregular four-helix bundles, which may make them amenable to interactions with nonhelical partners. In contrast,
Figure 3  His-containing phosphotransfer (HPt) domains. The HPt domains represented by (a) the C-terminal domain of *E. coli* ArcB, (b) the P1 domain of *E. coli* CheA, (c) *S. cerevisiae* YPD1, and (d) *B. subtilis* Spo0B contain four-helix bundles within monomeric or dimeric structures. The conserved His (blue) in each structure protrudes from the four-helix bundle (gold). The asymmetry of these bundles coupled with additional structural elements (green) produces protein surfaces specific for cognate partners. The overall architecture of the Spo0B protein is similar to the CheA kinase core shown in Figure 1c.

Spo0B exists as a dimer and has a classic bundle that more closely resembles the EnvZ and CheA dimerization domains.

**Sensing Domain**

Environmental stimuli are detected either directly or indirectly by the N-terminal sensing domain of the HK. These diverse sensing domains share little primary sequence similarity, thus supporting the idea that they have been designed for
specific ligand/stimulus interactions. In numerous cases, the specific stimuli and mechanism of sensing remain undefined. For others, structural information, such as that obtained recently for the sensing domain of PhoQ (119), may help to define extracellular receptor/ligand interactions. However, the mode of transmission of the signal to the intracellular kinase core remains elusive.

Cytosolic sensing modules have also been integrated into HKs. Examples include the versatile PAS domains. These adaptable domains monitor changes in light, redox potential, oxygen, and small ligands, depending on their associated cofactor (120). PAS domains are small (∼100 amino acids) and are typically located adjacent to the last transmembrane region of the sensing domain and N terminal to the kinase domain. The PAS domain of the HK FixL houses a heme group, the coordination state of which regulates kinase activity (121, 122). The crystal structure of this sensing domain (123) shows it to consist of a five-stranded antiparallel β barrel leading to a glutamine-rich helical region called the Q-linker, a motif that has been observed in other HKs (124). KinA, a soluble HK in the B. subtilis sporulation control pathway, is unusual in containing three PAS domains (120). Numerous other examples exist (120), highlighting the importance of PAS domains in two-component systems.

Linker Domain

In transmembrane HKs, the sensing domain is connected to the cytoplasmic kinase core through a transmembrane helix and a cytoplasmic linker. Although the least understood segment of any HK, multiple studies indicate that these linker regions are critical for proper signal transduction (125–129). The linker regions are of variable lengths, ranging from 40 to >180 amino acids (65). Computational analyses of sequence similarities of these regions revealed a 50-residue α-helical, coiled coil-like (CC) motif that, in most cases, directly precedes the H box of the kinase domain (130, 131). Similar repeats have also been identified in other multidomain proteins such as adenylyl cyclase, PP2C phosphatase, and diguanylate cyclase/phosphodiesterase (131). These repeats may promote intramolecular associations or, as suggested by a mutational analysis of the EnvZ linker region (127), correct structural alignment of monomers within the HK dimer. Alternatively, because of the dynamic nature of helices, these CCs might be used as a structural relay, sensing conformational changes in the periplasmic region of the kinase and communicating this signal to the kinase core.

STRUCTURE/FUNCTION OF RESPONSE REGULATORS

Activities and Architecture

In most prokaryotic systems, RRs are the terminal component of the pathway, functioning as phosphorylation-activated switches to effect the adaptive response. The RR catalyzes phosphoryl transfer from the phospho-His of the HK to a conserved
Asp in its own regulatory domain. Small molecules such as acetyl phosphate, carbamoyl phosphate, imidazole phosphate, and phosphoramidate can serve as phosphodonor to RRs (132), demonstrating that the RR can catalyze phosphoryl transfer independently of assistance from an HK. As discussed previously, most RRs also catalyze autodephosphorylation, limiting the lifetime of the activated state. Recent data support the view that phosphorylation promotes a conformational alteration affecting a large surface of the RR. This altered molecular surface facilitates a distinct set of inter- or intramolecular interactions through which the response is achieved. This basic scheme allows for a great variety of regulatory mechanisms optimized for the diverse effector functions of different systems.

Most RRs consist of two domains: a conserved N-terminal regulatory domain and a variable C-terminal effector domain (Figure 4). The majority of RRs are transcription factors with DNA-binding effector domains (25 of 32 in E. coli) (63). These DNA-binding domains can be subdivided into three major families, represented by OmpR, NarL, and NtrC (14, 7, and 4 members, respectively, in E. coli) (4, 63, 133). Not all RRs have DNA-binding domains. A few have C-terminal domains that function as enzymes, such as the chemotaxis methylase CheB (134) or the Dictyostelium cAMP phosphodiesterase RegA (58–60). Some RRs lack C-terminal effector domains altogether. The chemotaxis protein CheY associates in an intermolecular fashion with its effector protein FliM, a component of the flagellar motor (135). Another isolated regulatory domain is found in the B. subtilis sporulation factor Spo0F, which shuttles phosphoryl groups between the HK KinA and the HPt protein Spo0B (136). Thus, Spo0F functions similarly to the RR domains that are incorporated into hybrid kinases (137).

Regulatory Domain

The single-domain chemotaxis protein CheY has served as a representative model for RR regulatory domains (18, 138; Figure 4a). This 128-residue protein is a doubly wound α/β protein with a central five-stranded parallel β sheet surrounded by five α helices (139, 140). The site of phosphorylation in CheY is Asp57 (40), located in the solvent-exposed loop between β3 and αC. This residue lies adjacent to other acidic residues, Asp12 and Asp13 in CheY. Two other highly conserved residues, Thr87 and Lys109, complete the cluster of conserved residues surrounding the active site of the regulatory domain.

The carboxylate side chains of the acidic cluster are involved in coordination of the Mg$^{2+}$ that is required for phosphoryl transfer and dephosphorylation (141–143). Structures of Mg$^{2+}$-bound CheY indicate an octahedral coordination involving Asp57, Asp12, the backbone oxygen of Asn59, and three water molecules (144, 145). The coordination suggests a mechanism for phosphoryl transfer proceeding through a bipyramidal pentavalent phosphorus transition state (144, 146), an intermediate that is likely to be involved in the autodephosphorylation mechanism as well (141). Thr87 and Lys109, which are not absolutely required for
Figure 4  Response regulator protein (RR) family members. RR proteins commonly contain two domains, a conserved regulatory domain (cyan) and a variable effector domain (green). The conserved regulatory domain, represented by (a) S. typhimurium CheY, contains a cluster of conserved Asp residues (side chain and Cα atoms shown as red spheres) that bind Mg^{2+} (dark blue) and form the active site for phosphoryl transfer. The variable architectures of effector domains are illustrated by (b) the C-terminal domain of E. coli OmpR, (c) E. coli NarL, and (d) S. typhimurium methylesterase CheB. The transcription factors NarL and OmpR each contain a recognition helix (magenta), whereas CheB contains a catalytic triad (magenta spheres). In the intact response regulators NarL and CheB, juxtapositioning of the regulatory domains and the functional regions of the effector domains suggests a structural basis for inhibition.
phosphorylation/dephosphorylation, have been implicated as being involved in the phosphorylation-induced conformational change (142, 147, 148).

In addition to numerous structures of CheY proteins (139, 140, 144, 145, 147, 149–153), X-ray and/or NMR structures are available for the regulatory domains found in Spo0F (154, 155), NtrC (156), PhoB (157), CheB (159), Spo0A (49a), and FixJ (159a). The overall features of these proteins are similar to those of CheY. The most notable structural differences occur in differing lengths and conformations of surface loops (159) and in the orientations of helices relative to each other as they pack against the central β sheet (155).

Effector Domain

A comprehensive description of effector domains will not be attempted because of their great diversity. The majority of effector domains have DNA-binding activity and function to activate and/or repress transcription of specific genes. However, the specific DNA sequences that are recognized, the arrangement of binding sites, and the specific mechanism of transcriptional regulation differ for each RR, even within the same subfamily. Detailed analyses of individual RRs have revealed a great deal of complexity in the functioning of these transcription factors, as illustrated by representative members of the three major subfamilies.

OmpR, a well-characterized member of the largest subfamily of RRs, functions as both an activator and repressor to regulate differentially the expression of the ompC and ompF genes that encode outer membrane porin proteins. OmpR-DNA interactions have been extensively studied and have established a phosphorylation-regulated hierarchical binding of tandemly arranged OmpR dimers to the F and C boxes preceding the porin genes (160–166). Crystal structures of the DNA-binding domain of OmpR (167, 168) define a novel subclass of winged-helix transcription factors (Figure 4b). The fold, conserved in all members of the subfamily (169, 170), contains a recognition helix that interacts with the major groove of DNA and flanking loops or “wings” that are proposed to contact the minor groove. Despite structural similarity, subfamily members have different modes of action. For instance, transcriptional activation by OmpR involves interaction with the α subunit of RNA polymerase (171, 172), whereas PhoB interacts with σ^70 (173, 174).

Another subfamily of RRs is represented by NarL, a transcription factor that both activates and represses genes involved in nitrate and nitrite metabolism (175, 176). NarL-regulated operons are also regulated by the transcription factor Fnr and contain Fnr-binding sites as well as multiple, diversely arranged, “NarL heptamer”-binding sites (177, 178). The crystal structure of NarL has defined a four-helix fold for the 62-residue DNA-binding domain (158; Figure 4c). The fold contains a typical helix-turn-helix motif that has allowed postulation of specific interactions between residues of the recognition helix and bases in the NarL heptamer (158).

The most structurally and perhaps functionally complex RR subfamily is that represented by the nitrogen regulatory protein NtrC, a transcriptional enhancer that
activates the $\sigma^{54}$-holoenzyme form of RNA polymerase (179, 180). The effector region of this subfamily consists of two domains: an ATPase domain and a helix-turn-helix DNA-binding domain (181–183). NtrC dimers, capable of binding to DNA (184), oligomerize into octamers upon phosphorylation (185). Oligomerization stimulates ATP hydrolysis (186, 187), which provides energy for open complex formation and activation of transcription (188).

**Activation by Phosphorylation**

The great diversity of effector domains raises the question of how a conserved regulatory domain can function to regulate so many different effector domain activities. From a large accumulation of data, an answer is beginning to emerge. The regulatory domains of RRs are thought to exist in equilibrium between two conformational states, inactive and active. Phosphorylation of the regulatory domain shifts the equilibrium toward the active form. The different molecular surfaces displayed in the two forms can facilitate specific protein-protein (or possibly protein-DNA) interactions. Any type of regulation that can be achieved through inter- or intramolecular interactions can potentially be exploited by this versatile family of proteins.

Accordingly, there are a number of different mechanisms for RR activation. Each mechanism is based on a distinct regulatory interaction(s) specific to the unphosphorylated and/or phosphorylated regulatory domain. In some cases, activation involves a relief of inhibition, as observed in RRs that can be activated by removing the N-terminal regulatory domain (134, 189–191). In other cases, the phosphorylated regulatory domain plays an active role. Phosphorylation can promote dimerization (192, 193), higher-order oligomerization (184, 185, 194), or interactions with other proteins (135, 195) or DNA (45, 160). Some proteins use a combination of these mechanisms (164, 196). Phosphorylation need not necessarily correspond to activation. In yeast osmoregulation, phosphorylated SSK1 is considered the “off” state (197).

Crystal structures of two intact RRs have provided a structural basis for inhibition of effector domain activity by the unphosphorylated regulatory domain. The regulatory domain of the transcription factor NarL blocks access of DNA to the recognition helix (158), and the regulatory domain of chemotaxis methylesterase CheB blocks access of the chemoreceptor substrate to the esterase active site (159; Figure 4c, d). In both proteins, it appears that phosphorylation-induced activation must involve a repositioning of the N- and C-terminal domains. Despite analogous mechanisms of regulation, different surfaces of the regulatory domains of NarL and CheB are found at the domain interfaces (159).

Structural analysis of a phosphorylated RR has been hindered by the short lifetime of the phosphorylated state. Data from a number of studies are consistent with a long-range conformational change (198–201), but direct evidence has, until recently, been elusive. NMR analyses of unphosphorylated and phosphorylated CheY have shown substantial chemical shift perturbations that map over a large
The concept of an equilibrium between active and inactive states, supported by NMR dynamics studies of NtrC (200), provides an attractive explanation for these seemingly contradictory observations. If two conformations are accessible to both the unphosphorylated and phosphorylated proteins, then the solution conditions, such as those encountered in crystallization, could potentially influence the conformational equilibrium. The equilibrium hypothesis also aids in understanding the observation that phosphorylation-independent activating mutations found for one RR are not generalizable to the family (202–205). A propagated conformational change involves interactions of numerous residues. Even if a residue functions in an analogous manner in different proteins, alteration of any single residue in a given protein may or may not be sufficient to shift the equilibrium markedly between inactive and active states.

Recently determined structures of the phosphorylated regulatory domains of NtrC (206) and FixJ (159a) and of a stably modified phosphoprotein analog, phosphono-CheY (207; CJ Halkides, MM McEvoy, P Matsumura, K Volz, FW Dahlquist, manuscript in preparation) confirm that phosphorylation causes long-range structural perturbations, altering the molecular surface of the regulatory domain (Figure 5). The crystal structure of the CheY analog shows structural changes in \( \alpha_D, \beta_5, \) and \( \alpha_E \). The NMR structure of NtrC shows a displacement of \( \beta_4, \beta_5, \alpha_C, \) and \( \alpha_D \) away from the active site, thereby exposing a hydrophobic surface that is proposed to allow transmission of the activation signal to the effector domain (206). Phosphorylation does not alter the overall fold or result in any substantial changes in secondary structure. Rather, the secondary structure elements are slightly repositioned, causing backbone deviations of only a few angstroms. These changes, however, dramatically affect the molecular surface, altering both topological and electrostatic features.

Not surprisingly, the broad surface that is altered by phosphorylation overlaps regions that have previously been identified in many RRs as sites involved in phosphorylation-regulated protein-protein interactions (Figure 5). These surfaces include the interdomain interfaces of NarL (158) and CheB (159), as well as the interaction surfaces of CheY with HK CheA, phosphatase CheZ, and flagellar switch protein FlhM (209–216); of Spo0F with HK KinA and HPt Spo0B (217); and of PhoB with HK PhoR (218). Additionally, in Spo0F, this surface is characterized by motions in the microsecond to millisecond time range, implying a conformationally dynamic region (219).

The phosphorylation-induced conformational change affects a large face of the regulatory domain, providing ample molecular surface to be exploited by multiple protein-protein interactions. Indeed, many RRs are involved in phosphorylation-modulated interactions with several different macromolecular targets. These include HKs, auxiliary phosphatases, effector domains, other regulatory domains within dimers, and possibly other components of the transcriptional machinery.
Figure 5 Correlation of phosphorylation-induced conformational changes with protein-protein interaction surfaces in the regulatory domains of response regulator proteins (RR). (a) A ribbon diagram of CheY with active-site Asp residues shown in red, establishes the orientation of the molecule depicted in subsequent space-filling representations. (b) Surfaces involved in phosphorylation-induced conformational changes are depicted in two views related by an 180° rotation about the indicated axis. Residues that differ in the nuclear magnetic resonance (NMR) structures of unphosphorylated and phosphorylated NtrC (206) are mapped in green onto the corresponding surface of CheY; residues that show significant NMR chemical shift perturbations in phosphorylated CheY are mapped in blue (199); and overlapping residues appear as cyan. (c) Protein-protein interaction surfaces in different RRs are mapped onto the surface of CheY. Residues involved in interactions of CheY with FliM (209, 213, 216), CheA (214, 215), and CheZ (212, 213, 216) are shown in magenta; interactions of Spo0F with KinA and Spo0B (217) in cyan; interactions of PhoB with PhoR (218) and a proposed dimerization surface of PhoB (157) in gold; the domain interface of NarL (158) in red; and the domain interface of CheB (159) in blue.
The emerging picture of RRs has significant parallels with the small G proteins of the Ras family. These switch proteins exist in two conformational states determined by the diphosphate or triphosphate state of the bound guanine nucleotide. Large surface regions differ in the “on” and “off” conformations of the G proteins. Subsets of these regions are used for specific interactions with many different signaling partners (220). Thus RRs, which have fundamental structural and catalytic similarity to small-G proteins (221–223), appear to have functional similarity as well.

SYSTEM ARCHITECTURE

The elegance of the two-component system is embodied in its modularity. This modularity extends into the individual systems themselves, in which the basic coupling of HKs and RRs has been adapted to accommodate the specific needs of the signaling pathway. The most commonly occurring phosphotransfer system is composed of a single HK and a single RR. However, there are other systems in which the combination of HK, RR, and HPt domains has been used to create a more complex signaling circuit known as a phosphorelay system. Whereas the classical phosphotransfer pathways are predominant in bacteria, the phosphorelay pathways are more prevalent in eukaryotes. The additional complexity of the phosphorelay system provides for multiple regulatory checkpoints as well as a means of communication between individual signaling pathways.

Phosphotransfer Systems

The majority of two-component systems have a very simplistic design. A transmembrane sensor HK uses a single phosphoryl transfer event to activate a cytoplasmic RR protein that elicits an appropriate adaptive response. The archetype of this basic His-Asp system is the E. coli EnvZ-OmpR osmosensing pathway responsible for modulating the expression of the outer membrane porin proteins OmpF and OmpC (224; Figure 1a). There are, of course, variations on this simple two-step scheme, in which multiple HKs phosphorylate the same RR or a single HK controls several RRs. For example, in the chemotaxis system, a single HK, CheA, competitively phosphorylates two RRs, CheB and CheY (225; Figure 1c). An even more complex scheme occurs in the system that controls nitrate/nitrate-responsive gene expression, in which two HKs, NarX and NarQ, regulate two RRs, NarL and NarP (225).

Phosphorelay Systems

More elaborate versions of the two-component system are the multiple phosphotransfer pathways known as phosphorelays (137, 227, 228). The architecture of the phosphorelay cascade extends the fundamental His-Asp sequence into a four-step reaction. The basic design of the phosphorelay incorporates five phosphoryl
transfer reactions and four phosphoprotein intermediates mechanistically linked by phosphoryl transfer events:

1. Autophosphorylation: HK-His\(_1\) + ATP ⇔ HK-His\(_1\)∼P + ADP
2. Phosphotransfer I: HK-His\(_1\)∼P + RR-Asp\(_1\) ⇔ HK-His\(_1\) + RR-Asp\(_1\)∼P
3. Phosphotransfer II: RR-Asp\(_1\)∼P + HPt-His\(_2\) ⇔ RR-Asp\(_1\) + HPt-His\(_2\)∼P
4. Phosphotransfer III: HPt-His\(_2\)∼P + RR-Asp\(_2\) ⇔ HPt-His\(_2\) + RR-Asp\(_2\)∼P
5. Dephosphorylation: RR-Asp\(_2\)∼P + H\(_2\)O ⇔ RR-Asp\(_2\) + P\(_i\)

His- and Asp-containing domains are used as phosphotransfer elements. They can exist as isolated domains or can be covalently coupled, as for hybrid kinases discussed previously.

The *B. subtilis* sporulation control system is an example of a His-Asp-His-Asp phosphorelay (136; Figure 1d). In this relay, multiple HKs function as phosphoryl donors to Spo0F. The phosphoryl group is subsequently transferred to the HPt protein Spo0B and finally to Spo0A, a transcription factor. Other well-characterized examples of phosphorelays include the *Bordetella pertussis* virulence control system BvgS/BvgA (229) and the *S. cerevisiae* osmoregulation system SLN1/YPD1/SSK1 (68). The multiple domains in phosphorelay systems provide potential for alternate pathways of phosphoryl transfer. In the hybrid kinase ArcB, either His-containing domain (the dimerization domain or the HPt) can be phosphorylated from ATP and can donate phosphoryl groups to the RR ArcA. Data suggest that different pathways are used in aerobic and anaerobic conditions (114).

Integration of Systems

As described above, basic signaling elements can be assembled into either simple phosphotransfer or more elaborate phosphorelay pathways. On yet another level, these distinct signaling pathways can be integrated into cellular networks. In *B. subtilis*, each characterized two-component system appears to interface with at least one other phosphotransfer pathway (230). One example of this integration is between the pathways controlling phosphate utilization (PhoR/PhoP), aerobic and anaerobic respiration (ResE/ResD), and sporulation (KinA-B/Spo0A). Respiration and phosphate utilization are coregulated; phospho-PhoP is required for expression of ResD and vice versa (231, 232). Furthermore, once the cell commits to sporulation, respiration and phosphate utilization are down-regulated. Phospho-Spo0A is a negative regulator of both phospho-ResD and phospho-PhoP and therefore mutually exclusive with both of these responses (231, 233).

In eukaryotes, two-component systems are commonly found as parts of larger signal transduction cascades. *S. cerevisiae* has a single phosphorelay system consisting of a hybrid HK (SLN1), an HPt domain (YPD1), and two RRs (SSK1 and SKN7) (68, 69, 234). Under normal physiological conditions, SSK1 is continually phosphorylated. However, in high osmolarity, SLN1 is less active, and SSK1 becomes dephosphorylated and subsequently activates the downstream
HOG1-dependent MAP kinase cascade (56, 197). Another example is the Dic-
tyostelium osmoregulation system (58–60, 75). Phosphorylation of the RR RegA by the hybrid kinase DhkA causes inhibition of the cAMP-dependent phosphodiesterase activity of RegA, increasing cAMP levels, which, in turn, regulate the activity of protein kinase A.

Structural and functional conservation of two-component proteins suggests the potential for phosphotransfer between noncognate pairs. Although such transfer is commonly observed in vitro (27, 235), it appears to be rare in vivo. There are a few systems designed for phosphotransfer between noncognate pairs (176, 236). However, the issue of cross-talk outside such systems remains an open question (237). In at least one case, RR activation, initially attributed to cross-talk, was found to involve the phosphodonor acetyl phosphate (238, 239).

**REGULATORY MECHANISMS**

The sole purpose of two-component signal transduction systems is to allow for regulation; the signaling pathway merely provides steps at which the flow of information can be modulated. A great diversity of regulatory mechanisms has been overlaid on the central phosphotransfer/phosphorelay pathways, allowing optimization of signal transmission for the specific needs of each system. Some systems output a graded response, such as the EnvZ-OmpR system that mediates the differential expression of porin genes *ompF* and *ompC* (224). Others, such as the *B. subtilis* Spo system that controls commitment to sporulation, output an all-or-nothing response (240). Regardless of the output, both types of systems can involve a significant amount of regulation and often involve a number of auxiliary protein components. The primary targets for regulation are the activities of the HK and dephosphorylation of the RR.

**Regulation of Histidine Kinase Activities**

HKs can have two activities that determine the level of RR phosphorylation: autophosphorylation activity and RR phosphatase activity. Not all HKs possess phosphatase activity; for some HKs, regulation occurs exclusively at the level of autophosphorylation (241). In many other systems, it is RR phosphatase activity rather than autophosphorylation that is regulated (2, 90, 91, 242, 243). Either of these activities can be regulated directly or indirectly by stimuli.

In typical transmembrane HKs, sensing domains directly bind ligands or detect other physical stimuli. More complex schemes involve indirect detection of signals through interaction with other protein components. For instance, the autophosphorylation activity of the chemotaxis HK CheA, which forms a complex with chemoreceptors and an adapter protein CheW (244, 245), is either inhibited or stimulated by signals transmitted from the chemoreceptors (246, 247). The RR phosphatase activity of the soluble HK NtrB is regulated by an auxiliary protein.
P_{II}, whose ability to interact with NtrB depends on its uridylylation state, a modification catalyzed by a uridylyltransferase enzyme, which is itself regulated by intracellular 2-ketoglutarate and glutamine (248). The ability of HK PhoR to mediate repression of the Pho regulon when phosphate is in excess, requires an intact phosphate uptake complex (PstA-C and PstS), together with an accessory protein, PhoU (249). These proteins are proposed to form a membrane-associated repression complex that regulates PhoR activity (250). Regulation of HK activities by intrinsic domains has also been found. The turgor sensor KdpD contains an auxiliary ATP-binding domain, and ATP binding, but not hydrolysis, is required for phosphatase activity (251).

### Regulation of Response Regulator Dephosphorylation

As discussed previously, many RRs have autophosphatase activity, with dephosphorylation rates tuned to the time-scale requirements of the specific system. RR dephosphorylation is also influenced by the phosphatase activities of HKs that are regulated by a number of different mechanisms, as discussed above. In most instances, the phosphatase mechanism appears to be distinct from a reversal of phosphotransfer and does not require the H box His of the HK (176, 252–254). In some HKs (89, 251), but not others (46, 91), phosphatase activity is stimulated by ATP.

RR dephosphorylation can also be effected by auxiliary proteins. The *B. subtilis* sporulation system involves a set of highly regulated phosphatases (RapA, RapB, and RapE) that dephosphorylate Spo0F (255), and an unrelated phosphatase (Spo0E) that dephosphorylates Spo0A (256). In bacterial chemotaxis, an auxiliary protein, CheZ, oligomerizes with phospho-CheY and accelerates its dephosphorylation (195, 257, 258). The phosphoAsp phosphatases appear to have great specificity for their substrate proteins, raising the question of whether they directly participate in catalyzing hydrolysis or rather function by stimulating the autophosphatase activity of the RR.

### Other Modes of Regulation

A small number of additional regulatory mechanisms are involved in specific systems. Phosphotransfer itself can be regulated. Within the *Agrobacterium tumefaciens* hybrid HK VirA, the C-terminal Asp-containing domain modulates the phosphotransfer ability of the kinase core by physically interacting with the autophosphorylation site (259). Additional regulatory potential exists in systems with an HK that can phosphorylate more than one RR. In these systems, competition for phosphoryl groups can influence activation of different branches of the signaling pathway (176, 225).

A novel regulatory mechanism involving multiple RRs has been postulated for the chemotaxis system of *R. meliloti* (260). This system contains two CheY proteins, CheY1 and CheY2, and lacks a phosphatase CheZ. Phosphorylated CheY2 is

---
responsible for the motor response, whereas CheY1 regulates the phosphorylation state of CheY2. In the absence of forward phosphotransfer, CheY1 serves as a “phosphatase” for phospho-CheY2, acting as a sink for phosphoryl groups that flow backwards in the pathway through CheA to CheY1.

All of the previously discussed regulatory mechanisms alter the level of phosphorylation of the RR. An alternative strategy is regulation of the level of the RR itself through control of gene expression. Many of the two-component systems that regulate transcription are subject to autoregulation. In these systems, the phosphorylated RR functions as an activator or repressor of the operon encoding the two-component proteins themselves (261–264).

POTENTIAL TARGETS FOR ANTIMICROBIAL THERAPY

The search for structurally unique antibiotics that inhibit new molecular targets has led researchers to prokaryotic two-component systems. Two-component systems are attractive for several reasons. First of all, they are widespread in bacteria and, so far, absent in mammals. Therefore, general HK or RR inhibitors could potentially be broad-spectrum antibiotics. Alternatively, by targeting specific HKs or RRs, selective inhibition may be achieved. The problem is that most two-component systems are nonessential. However, there is an interdependence not only among the proteins in these systems, but among the systems themselves. Cessation or slowing down of these intracellular networks may be a way to effect a cellular shutdown. Perhaps the most attractive reason for targeting two-component systems is that they are used by pathogenic bacteria to control the expression of virulence factors required for infectivity. Several well-characterized virulence systems are the *A. tumefaciens* VirA/VirG system, the *B. pertussis* BvgA/BvgS system, and the *Salmonella typhimurium* PhoP/PhoQ system. Interestingly, some bacteria have developed two-component systems that regulate resistance to certain chemotherapeutics. These include the vancomycin resistance systems in *Enterococcus faecalis* (VanR/VanS) (265) and *Streptococcus pneumoniae* (VncS/VncR) (266), as well as the system associated with tetracycline resistance in *Bacteroides fragilis* (RprX/RprY) (267).

A small number of investigations of two-component inhibitors have appeared in the literature. There are few reported natural inhibitors of two-component systems. Initially, derivatized unsaturated fatty acids were shown to be noncompetitive inhibitors of the autophosphorylation activity of KinA (268), as well as of CheA and NtrB (269). Limited success with these compounds spurred researchers to develop synthetic inhibitors (270). One class is the diphenol-methane compounds, originally identified because of their ability to inhibit NtrB in gram-positive bacteria (271). A number of compounds currently under investigation include hydrophobic tyramines (272), salicylanilides (273), triphenylalkyl derivatives, cyclohexenes, and benzoxazines (270). These compounds not only inhibit two-component
functions but also affect bacterial growth rates. The exact mechanism of action for most of these inhibitors has not been firmly established. Initial observations suggested that the target of these antibacterial compounds was the HK. However, recent studies (274, 275) indicate that inhibition is occurring on multiple metabolic and biosynthetic levels. It may be also be possible to extend the use of these two-component inhibitors into the unicellular eukaryotic realm. Specifically, an HK inhibitor was shown to hinder proliferation of the human pathogens *Trypanosoma brucei rhodesiense* and *Leishmania donovani* by interfering with succinyl CoA phosphorylation (276).

**SUMMARY AND PERSPECTIVES**

In slightly over a decade since two-component systems were first described, numerous studies involving many different systems have provided a fundamental understanding of the essential features of phosphotransfer signal transduction pathways. The biochemical activities of the conserved protein components are known, and three-dimensional structures are available for all of the conserved modular domains. However, there are still several central questions regarding the functioning of HKs and RRs. In many systems, the stimuli sensed by the HKs are not well defined, and the molecular mechanism of signal transmission across the membrane from the sensing domain to the kinase core has not been determined. In RRs, the detailed mechanisms that couple phosphorylation-induced conformational changes in regulatory domains to activation of effector domains remain to be elucidated. In addition to these issues concerning the conserved components, there are many questions regarding regulation within individual pathways. As the number of characterized two-component systems grows, so does the inventory of variations on the basic scheme. Although, from one perspective, these system-specific regulatory mechanisms might be considered mere details, they are essential to the appropriate coupling of stimuli to adaptive responses within each system. With the basic framework in hand, researchers are well poised to advance the understanding of the extremely elegant and sophisticated regulation involved in two-component signaling systems.

**ACKNOWLEDGMENTS**

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