High energy exchange: proteins that make or break phosphoramidate bonds

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Several proteins that catalyze phosphoryl transfer reactions involving phosphohistidine residues have recently been structurally characterized. The architecture of two histidine kinases defines a new protein kinase fold. The diverse folds of several phosphotransfer proteins appear to be designed to foster protein–protein interactions between transfer partners.

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Introduction

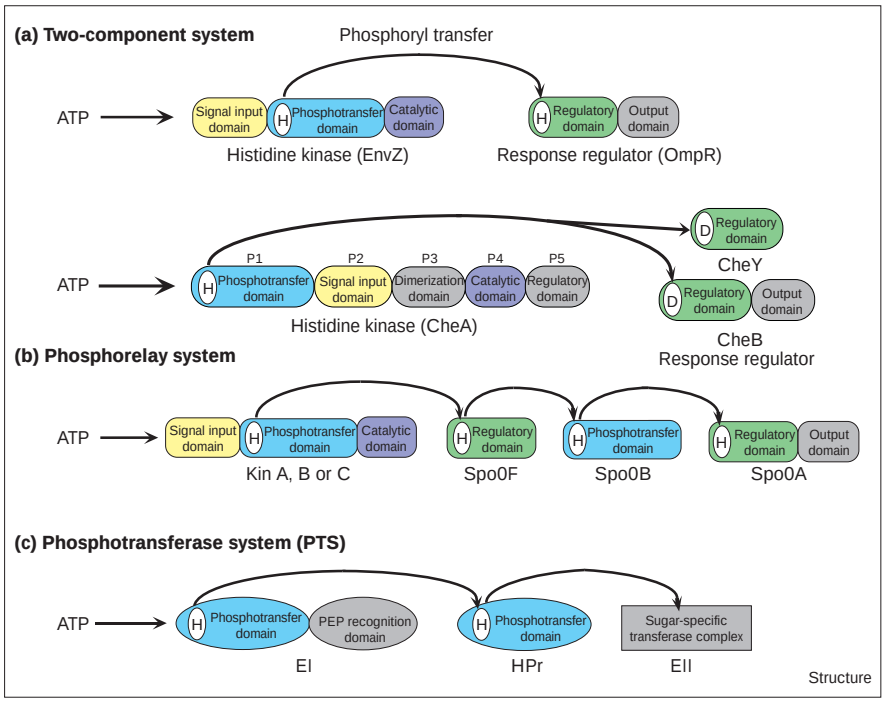
Protein phosphorylation is an important mechanism for the regulation of cellular responses in both prokaryotic and eukaryotic organisms. Proteins are prevalently phosphorylated on sidechains that contain a hydroxyl group, such as serine, threonine or tyrosine residues. In the past decade, however, an increasing number of examples of histidine phosphorylation have been identified. Whereas phosphorylation of serine, threonine or tyrosine results in the formation of a phosphoester linkage, phosphorylation of histidine occurs on nitrogen atoms, producing a high-energy phosphoramidate bond. Phosphohistidines have a large standard free energy of hydrolysis making them the most unstable of any known phosphoamino acid [1]. Thus, the formation of a phosphohistidine, unlike its phosphoserine or phosphothreonine counterparts, is a highly reversible process. Because of this inherent instability, phosphohistidines are not observed in cells as an irreversible post-translational modification, but instead have two main functions. They are utilized as enzyme intermediates, for example, in the catalytic mechanism of phosphoglucomutase or nucleoside diphosphate kinase. Alternatively, phosphohistidines function as high-energy phosphodonors in phosphoryl transfer systems, such as the bacterial two-component signaling systems or the sugar-specific phosphotransferase systems.

Two-component signal transduction pathways are used by organisms to respond to changes in a variety of environmental conditions, including the availability of nutrients and osmolarity [2]. Prevalent in bacteria, homologs of these sophisticated histidine–aspartate (His–Asp) phosphorelay pathways have also been identified in eukaryotic organisms, such as yeast, slime molds and plants [3]. The prototypical two-component system is composed of a histidine protein kinase and a response regulator protein (Figure 1a). The histidine protein kinase generally acts as a sensor, monitoring environmental stimuli through a signaling input domain. ATP-dependent autophosphorylation of the kinase occurs on a conserved histidine residue in the phosphoryl-transfer domain of the protein. This phosphoryl group is then transferred to a conserved aspartate residue in the regulatory domain of a response regulator protein. The regulatory domain interacts with an effector or output domain that mediates changes in gene expression or cell behavior in response to stimuli.

The elegance of the His–Asp signal transduction system is its modularity. The basic components of the signaling cascade, the phosphotransfer and the regulatory domains, have been integrated in various ways and appropriately modified by both prokaryotes and eukaryotes to accommodate specific cellular needs. Such elaborate signaling cascades allow for multiple regulatory checkpoints as well as providing connections for communication between signaling pathways [4]. The archetype of the multistep phosphorelay is the pathway controlling the initiation of sporulation in *Bacillus subtilis* (Figure 1b) [5]. The sequence of the phosphoryl transfer steps in this four-step pathway parallels that of the two-component system. A histidine protein kinase (Kin A, B or C) is autophosphorylated using ATP and the phosphoryl group is subsequently transferred to an aspartate residue on Spo0F, a single-domain protein homologous to the N-terminal region of a prototypical two-component response regulator protein. The next protein in the signaling cascade, Spo0B, has the unique ability to transfer a phosphoryl group between two different response regulator proteins. Acting as a phosphotransferase, Spo0B dephosphorylates Spo0F~P, producing a high-energy phosphohistidine intermediate. The phosphoryl moiety of Spo0B~P is transferred to an aspartate residue in the regulatory domain of Spo0A, both an activator and repressor of downstream sporulation events. Similar His–Asp–His–Asp phosphorelay pathways have been identified in both yeast and *Bordetella pertussis* for the regulation of osmolarity and virulence, respectively [6,7].

The bacterial phosphoenolpyruvate (PEP): sugar phosphotransferase systems (PTSs) are in some ways analogous to the multicomponent phosphorelay systems [8]. Using a four-step process, the PTS simultaneously phosphorylates

Figure 1



Schematic diagram depicting the domain organization of various phosphorelay systems discussed in the text. **(a)** The prototypical two-component transfer system. **(b)** The multicomponent phosphorelay system. **(c)** The phosphoenolpyruvate (PEP) dependent sugar transport system of bacteria. Protein domains are colored according to their major functions: the signal input domains are colored yellow; phosphotransfer domains are colored blue; catalytic domains are colored purple; regulatory domains are colored green; and other associated domains are colored gray.

and transports hexose sugars across the cell membrane through several phosphohistidine intermediates (Figure 1c). Unlike the two-component and phosphorelay cascades, the phosphoryl moieties in the PTS are derived from PEP rather than ATP. Enzyme I (EI) is a protein common to all PTSs which autophosphorylates by PEP on a conserved histidine residue. EI subsequently donates a phosphoryl group to the histidine-containing phosphocarrier protein (HPr), a small 9 kDa protein also found in all PTSs. The resulting phosphohistidine intermediate interacts with a sugar-specific permease complex collectively known as enzyme II (EII). The EII complex contains three distinct domains termed EIIA, EIIB and EIIC. The soluble EIIA and EIIB domains serve as energy-couplers, while the integral membrane protein EIIC is responsible for the actual phosphorylation and transport of the sugar molecule. Although histidine protein kinases are not part of the PTS, phosphohistidine intermediates are used in three out of four steps as the phosphoryl transfer moiety.

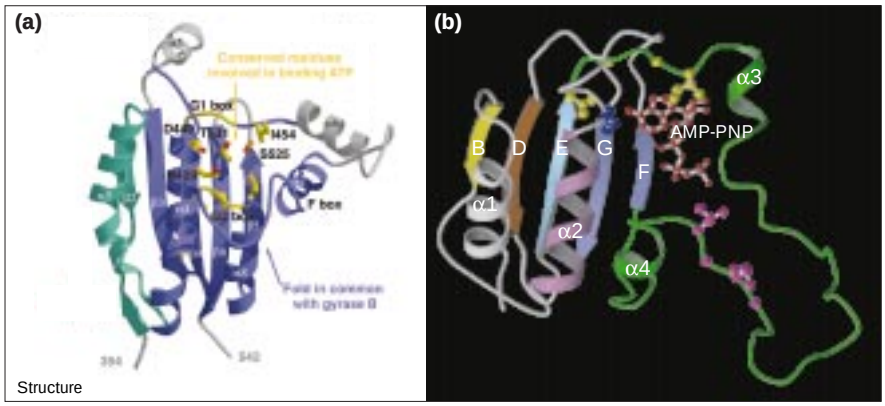
Structural information is available for most components of the PTS [9,10] and for a representative number of proteins that function in the two-component and multicomponent phosphorelay systems. This review discusses recently available structural data for three fundamental elements of the aforementioned pathways that function to create and/or break phosphoramidate linkages: the crystal structure of the C-terminal half of the histidine protein kinase CheA [11] and the nuclear magnetic resonance (NMR) structure of the catalytic domain of a histidine protein kinase, EnvZ [12]; the 2.6 Å crystal structure of Spo0B [13]; and the solution structure of the 40 kDa complex formed between the N-terminal domain of the EI protein and the phosphocarrier protein HPr [14].

The two-component histidine protein kinase

Both CheA and EnvZ are members of the large histidine protein kinase family (Figure 1a). CheA is a central component of the bacterial chemotaxis system responsible for the transmission of signals from transmembrane chemoreceptors into the cytoplasm. The protein is a dimer with each monomer being composed of five functionally and structurally distinct domains (P1–P5) connected by flexible linkers. Autophosphorylation of CheA occurs via a bimolecular reaction in which the catalytic kinase domain (P4) of one monomer phosphorylates His48 in the second CheA monomer. The high-energy phosphoryl group is subsequently transferred to either of the two response regulators in the chemotaxis pathway, CheY or CheB.

EnvZ is the transmembrane osmosensor protein of *Escherichia coli*. Together with its companion response regulator, OmpR, EnvZ regulates the expression of the OmpF and OmpC porins. The catalytic domain of EnvZ is located in the C-terminal region of the protein (residues 180–450). This cytosolic region of the histidine kinase can be subdivided into two stable functional domains [15]: subdomain A (residues 223–289) contains

Figure 2



Comparison of the catalytic domains of *T. maritima* CheA and *E. coli* EnvZ. (a) Ribbon diagram of the *T. maritima* CheA catalytic domain. (b) Ribbon diagram of the *E. coli* EnvZ catalytic domain. The residues from the N, F, G1 and G2 boxes that are implicated in nucleotide binding and the bound nucleotide analog in EnvZ are shown in ball-and-stick representation. (The figures were reproduced from [11] and [12] with permission.)

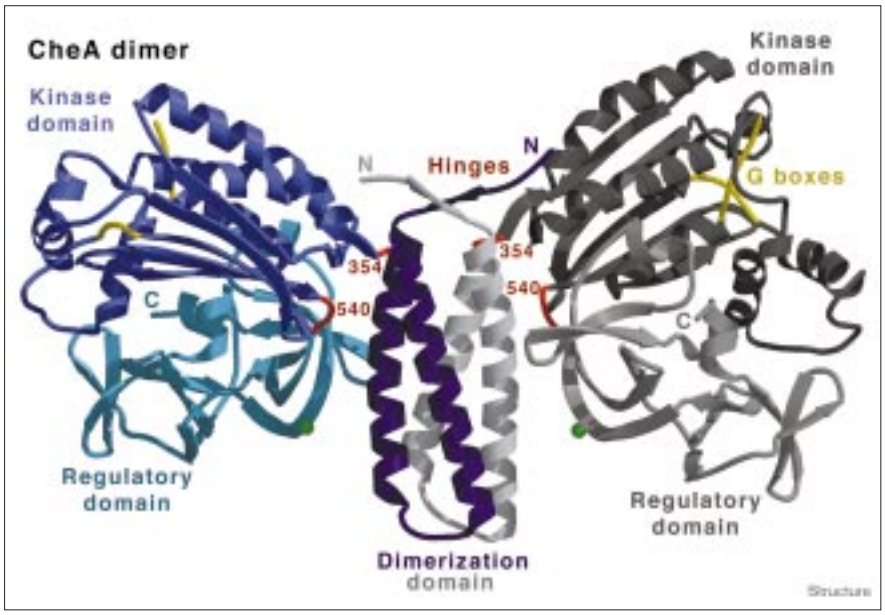
the autophosphorylation (His243) and OmpR interaction sites and subdomain B (residues 290–450) contains the catalytic core of the protein.

The 2.6 Å crystal structure of the C-terminal half of *Thermotoga maritima* CheA (residues 290–671) [11] and the multidimensional NMR solution structure of the catalytic domain of EnvZ (residues 290–450) [12] have provided the first picture of the topology of the conserved histidine kinase catalytic domain. The catalytic domain of a histidine protein kinase is an autonomously folding unit that functions to bind ATP and catalyze histidine phosphorylation. The catalytic domain contains several highly conserved motifs termed the N, F, G1 and G2 boxes that are characteristic of all prokaryotic and eukaryotic members of the histidine kinase family. It has been postulated that these elements are arranged in the tertiary structure of the protein to form a nucleotide-binding cleft within the active site [16]. The histidine substrate in these proteins is located in another conserved region of the kinase termed the H box. The positioning of the histidine-containing phosphotransfer domain relative to the catalytic domain varies in different histidine protein kinases (Figure 1a). For example, the phosphotransfer domain of EnvZ is adjacent to the conserved catalytic core of the enzyme, whereas in CheA the substrate histidine is located at the far N terminus of the protein in the P1 domain.

The fold of the histidine protein kinase catalytic domain is unlike that of any previously characterized serine, threonine or tyrosine kinase. It does, however, share structural homology with the ATP-binding regions of the heat shock protein Hsp90 and DNA gyrase B. As illustrated in Figure 2, the body of the kinase domain adopts an α/β-sandwich fold comprising five antiparallel β strands and three α helices. In both the EnvZ solution structure and the CheA crystal structure, the N box is located on the central α helix (α2 in EnvZ and α4 in CheA) and the G1 box is positioned on a loop emanating from the third β strand. This loop then forms a short α helix (α3 in EnvZ and α6 in CheA) extending outward from the body of the protein. In the CheA crystal structure, the F and G2 boxes are located on a flexible segment between the C terminus of helix α7 and the N terminus of helix α8. Analogously, the F and G2 boxes are part of a large disordered loop in the EnvZ solution structure. The EnvZ structure was determined in the presence of a nonhydrolyzable nucleotide analog, AMP-PNP. The triphosphate chain of the analog is solvent-exposed whereas the adenine ring of the analog lies adjacent to the disordered loop, in proximity to several highly conserved amino acid residues in the N, F, G1 and G2 boxes. The disorder observed in this region of the structure may be the result of instability in the truncated EnvZ fragment used for the structure determination. Alternatively, as pointed out by Bilwes *et al.*, [11] in analogy to DNA gyrase B, histidine protein kinases may undergo significant conformational changes upon binding ATP and thus the disorder observed in this region of the EnvZ and CheA catalytic domains may reflect inherent flexibility of the G2 loop itself. Unfortunately, the lack of bound nucleotide in the CheA crystal structure and the disorder surrounding the bound nucleotide in EnvZ, limits our understanding of interactions critical to the catalytic mechanism. Undoubtedly, future structural studies will resolve this situation.

The crystal structure of *T. maritima* CheA also provides insights into how CheA dimerizes and how it may bind CheW, a protein responsible for the coupling of CheA to the receptor. The dimerization domain of CheA (residues 290–354), also known as the P3 domain, is composed of two antiparallel α helices. As shown in Figure 3, two CheA monomers interact through a parallel association of the helices creating a central four-helix bundle. The extensive hydrophobic interface formed by this association is further stabilized by a six-residue β sheet at the N terminus of the monomer, which interacts with its noncrystallographic counterpart forming a cap over the end of the four-helix

Figure 3



Ribbon diagram of the CheA dimer formed by the association of two monomers of the C-terminal half of the CheA protein containing the dimerization (P3), kinase (P4) and regulatory (P5) domains. One CheA monomer is shown in blue and the other in gray. The dimerization domains form the central four-helix bundle. The kinase and regulatory domains of the monomers protrude out from this central four-helix bundle, such that the ATP-binding clefts are positioned on opposite sides of the dimer. (The figure was reproduced from [11] with permission.)

bundle. The kinase (P4) and regulatory (P5) domains of CheA protrude out from this central helical bundle like the wings of a butterfly (Figure 3). The overall spacing of these two domains presumably facilitates interactions between CheA and its molecular partners in the chemotaxis pathway.

The P5, or regulatory, domain of CheA comprises residues 540–671. Its topology is similar to that of the eukaryotic SH3 domain — a small β-barrel domain that frequently mediates protein–protein interactions in eukaryotic cellular signaling cascades [17]. Likewise, in CheA the P5 domain is also involved with protein–rotein interactions and represents the first example of an SH3 domain in bacteria.

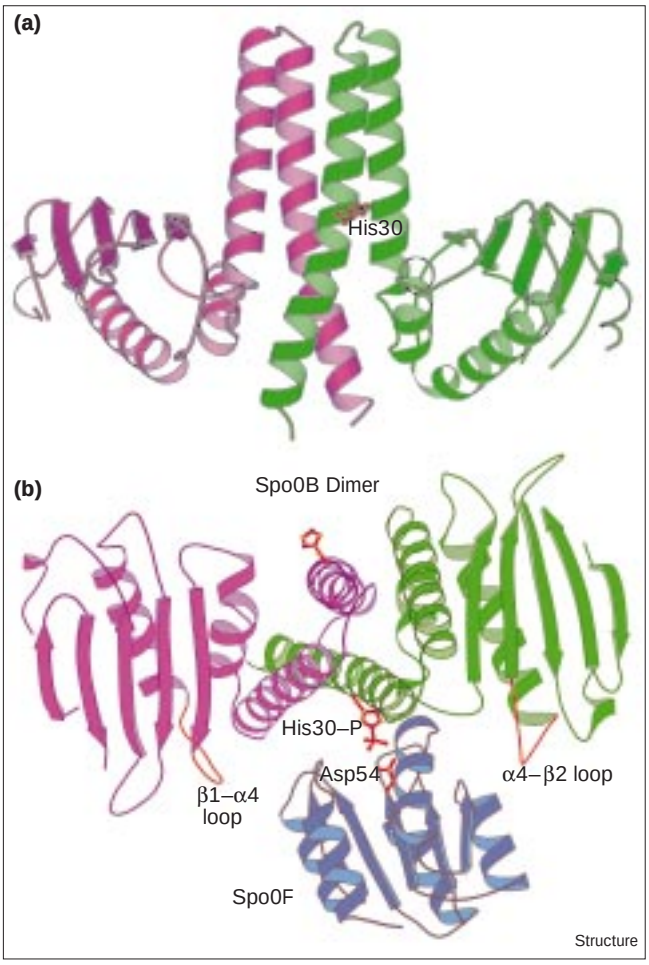
Phosphorelay phosphotransferase

One step of the multicomponent phosphorelay system that controls *B. subtilis* sporulation is now better understood due to the recent determination of the 2.6 Å crystal structure of Spo0B [13]. Spo0B is functionally similar to the phosphotransfer domain (P1) of the two-component histidine kinase CheA and the histidine-containing phosphotransfer (HPt) domain of the ArcB sensor kinase. But, whereas the P1 [18] and HPt domains [19] have similar structures, displaying the same overall four-helix-bundle topology and the same arrangement of active-site residues, Spo0B is structurally distinct. The N-terminal domain of Spo0B comprises a helical hairpin assembled from two long antiparallel α helices (α1 and α2) connected by a tight turn. His30, the phosphorylation site, is located in the middle of helix α1. The C-terminal domain of Spo0B consists of a five-stranded mixed β-sheet structure (β1 to β5) with two α helices (α3 and α4) that lie parallel to the first and fifth β strands, respectively.

In contrast to the monomeric P1 or HPt domains, Spo0B exists as a dimer in solution. Dimer formation results from the parallel alignment of the N-terminal helical regions of the protein, such that helix α1 of one monomer interacts with helix α2 of the other monomer (Figure 4a). The result is a symmetric four-helix bundle stabilized by hydrophobic interactions across the dimer interface with the C-terminal α/β domain protruding out from the center of the bundle. Unlike the P1 domain of CheA, both monomers contribute to the formation of the active site. The solvent-exposed His30 is poised at the top of a cleft created by residues from the C-terminal domains of opposing monomers. Moreover, because of the parallel packing of the monomers, the Spo0B dimer has two active-site histidines located on opposite faces of the molecule. Therefore, although the overall architecture of the CheA and Spo0B dimers are similar, the individual domains of the proteins serve quite different functions.

Intriguingly, the fold and subunit organization of Spo0B are remarkably similar to those of the dimerization and catalytic domains (P3 and P4) of the histidine kinase CheA. However, the dimerization domain of CheA lacks the consensus residues of the histidine phosphorylation site (H box) found in Spo0B, and the α/β domain of Spo0B lacks the consensus nucleotide-binding motifs (N, F, G1 and G2 boxes) found in the histidine kinases. It is likely that these bacterial signaling proteins are evolutionarily

Figure 4



The Spo0B dimer and its proposed interaction with Spo0F. (a) Ribbon diagram of the Spo0B dimer with each monomer shown in a different color. Helices α1, β1, α1′ and β2′ (where a prime designates a structural element from the second monomer) form the parallel four-helix bundle. His30, the active-site histidine residue (shown in red), is located in the middle of helix α1 facing outwards into the solvent. (b) Ribbon diagram depicting a model of the Spo0F–Spo0B complex. The active-site residues, His30 of Spo0B and Asp54 of Spo0F, are depicted in red. The Spo0B dimer is colored as in (a) and Spo0F is in blue. (Part (b) was reproduced from [13] with permission.)

related, and, as such, represent a clear example of how similar folds can be adapted to different functions.

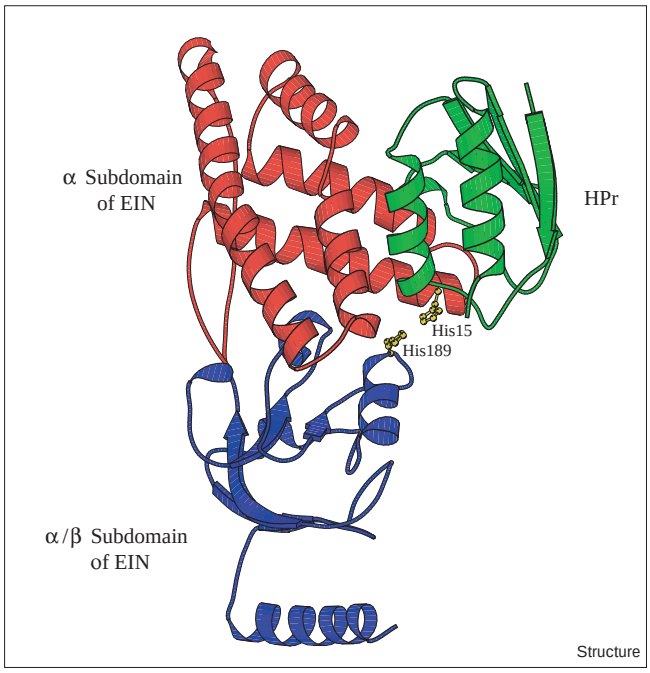
Using the crystal structure of unphosphorylated Spo0F, Varughese and colleagues [20] proposed a model for the interaction of Spo0B and one of its two cognate response regulators. As shown in Figure 4b, the active-site cleft of the Spo0B molecule readily accommodates the loop containing the conserved aspartate residue of Spo0F without any major structural changes to either protein. The complementary shape of these proteins allows them to interact in two ways, with the restriction that the plane of the Spo0F β sheet must lie perpendicular to the helical axis of the four-helix bundle of Spo0B. Both Spo0B monomers contact the Spo0F protein. Moreover, because Spo0A is homologous to Spo0F, it is believed that similar interactions will be seen for the Spo0B–Spo0A complex.

Sugar-specific phosphotransferase system

A direct view of a protein–protein phosphoryl transfer complex has been provided by the NMR solution structure of the *E. coli* PTS complex EIN–HPr (Figure 5) [14]. As stated previously, EI serves as the phosphodonor to HPr in the second step of the PTS. EI is a large protein (64 kDa) composed of an N-terminal phosphotransfer domain (EIN) attached by a short linker to a C-terminal PEP-recognition domain (EIC). The crystal structure of EIN [21] showed that it is composed of two distinct structural subdomains (Figure 5). The α subdomain contains two long helix hairpins oriented at approximately 60° with respect to one another. The α/β subdomain is composed of a four-stranded parallel β sheet, a three-stranded antiparallel β sheet, and three α helices which, taken together, form a β sandwich. The phosphorylation site, His189, is located at the N terminus of helix H6 in the interface between the two domains. The structure of HPr had also been solved previously [22,23]. HPr, the smallest protein in the PTS, consists of three α helices and a four-stranded antiparallel β sheet. The phosphorylation site of HPr, His15, is located near the N terminus of helix H1. The solution structure of the EIN–HPr complex indicates that no significant conformational changes occur upon formation of the bimolecular complex; the structures of the free proteins can be superimposed with those of the complex with average root mean square (rms) deviations of ~1 Å. A large interface is created between the two proteins (~950 $Å^2$) formed exclusively by helices H1 and H2 of HPr and helices H2′, H2, H3 and H4 of EIN. The majority of the protein–protein interface contacts are hydrophobic. In addition, there are about a dozen electrostatic interactions including six salt bridges.

The phosphorylated complex is too short-lived to be studied by NMR, so the transition state was modeled by the authors. Previous studies suggest that the transition state of the phosphoryl transfer reaction involves the formation of a pentacoordinated phosphorus intermediate. Therefore, the final model includes a trigonal bipyramidal phosphoryl group, where the axial positions are occupied by the Nε2 and Nδ1 atoms of His189 and His15, respectively. The rms deviation between the modeled transition-state complex and the original EIN–HPr complex was 0.38 Å. The most significant change was a 120° rotation of the sidechain torsion angles of His189. The phosphorus itself was positioned in a cleft composed of the N-terminal end of helix H2 and H4 of EIN and the N-terminal end of helix H1 in HPr. In addition, a number of electrostatic interactions stabilize the transition state in favor of phosphorylated HPr over EIN.

Figure 5



Ribbon diagram of the EIN–HPr complex. The α subunit of EIN is colored in red, the α/β subunit of EIN is colored blue and HPr is in green. Ball-and-stick representations of His15 of HPr and His189 of EIN, located at the interface between the two proteins, are shown in gold.

Conclusions

The proteins discussed in this review, the catalytic domain of EnvZ, the C-terminal region of CheA, the bi-functional Spo0B protein of the sporulation pathway and the EIN–HPr protein complex of the sugar-specific PTS, are all responsible for creating and/or breaking phosphoramidate bonds. Nevertheless, this group of functionally similar proteins exhibits a significant diversity of folds. Perhaps this is not surprising. A recent study by Thornton and colleagues indicated that protein fold classes tend to be more correlated with ligand type than with enzyme classification [24]. The histidine protein kinase catalytic domains of EnvZ and CheA, although unrelated to the superfamily of serine/threonine and tyrosine protein kinases, have folds similar to those of Hsp90 and DNA gyrase B, other enzymes that utilize ATP. The phosphotransfer domains represented by CheA P1, ArcB HPt, Spo0B, EIN and Hpr, the ligands of which are actually other proteins, appear to be structurally designed to promote optimal protein–protein interactions with cognate partners.

Protein complex formation in these phosphotransfer systems, exemplified by the Spo0B–Spo0F model and the bi-molecular EIN–HPr structure, occurs with very little change in the overall architecture of the individual protein components. Minimal conformational changes appear to be limited to residues in or surrounding the active site in order to prime the complex for catalysis. The active sites themselves show little structural conservation. The phosphorylated histidine residues are all found on the solvent-exposed surfaces of helices, but their positions along the helix vary. For example, in the Spo0B protein His30 is positioned in the middle of a long helix, whereas in the PTS proteins HPr and EIN the histidine residues are located at the N termini of relatively short helices. Residues flanking these regions also vary, perhaps reflecting differences in the mechanisms of phosphoryl transfer. The phosphorelay systems require divalent metal ions for transfer, whereas PTSs do not. Given the very high energy of the N–P bond, activating the phosphoryl group for transfer is not a difficult task. The challenge, for which these active sites appear to be designed, is to ensure the correct specificity and directionality of phosphoryl transfer.

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