Protein Phosphorylation in Chemotaxis and Two-component Regulatory Systems of Bacteria

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Transient protein phosphorylation is an important feature of many biological processes, including signal transduction, growth control, and metabolism. While attention has focused on protein phosphorylation in eukaryotic organisms, there is accumulating evidence of a wide variety of novel functional roles for phosphorylation in prokaryotes (reviewed in Ref. 1). Past studies of bacterial systems have demonstrated the involvement of protein phosphorylation in bacteriophage infection, in sugar transport, and in regulating the flow of metabolites (1). Recent evidence obtained from the study of the sensory transduction systems associated with chemotaxis and the regulation of genes involved in nitrogen assimilation and porin expression suggests that transient protein phosphorylation is central to the mechanism of many bacterial information processing systems as well. We review here recent work on this class of protein modification, with an emphasis on bacterial chemotaxis.

Two-component Regulatory Systems

A growing family of paired proteins, which are integral components of bacterial regulatory systems, has been defined on the basis of amino acid sequence similarities (2, 3) (Table I). In all cases, the protein pairs share the common function of allowing the cell to sense and respond to environmental stimuli. These two-component regulatory systems consist of "sensor" and "regulator" proteins and are found in very different functional contexts in many bacterial species. Members of the sensor class of proteins share sequence similarities spanning about 200 amino acid residues at their N-terminal ends (2, 3). Many members of this class appear to be transmembrane proteins (Table I) and are proposed to have an N-terminal "sensing" domain located in the periplasm as well as a C-terminal "signaling" domain in the cytoplasm (2, 3). Some sensor proteins (e.g. CheA and NtrB; Table I), however, are clearly located in the cytoplasm and are presumed to detect intracellular signals. For each sensor protein, there is a corresponding regulator protein. Proteins of the regulator class share sequence similarities over about 120 amino acids at their N-terminal ends. In the cases of the relatively small CheY and SpoOF, the conserved regulator sequence comprises the entire protein (2, 3). Most members of the regulator class act as positive regulators of transcription (Table I).

Phosphorylation had just recently been discovered in the nitrogen assimilation system of bacteria (15) when the existence of the two-component regulatory system family was recognized (2). The suggestion was made that protein phosphorylation might be a general feature of these systems and could be involved in the interaction between sensors and regulators (2, 3). The following summary of evidence from several two-component regulatory systems shows that protein phosphorylation is associated with these pairs of proteins and appears to be essential for their function.

Bacterial Chemotaxis

The bacteria Escherichia coli and Salmonella typhimurium mediate their swimming behavior in response to a wide variety of chemical and physical stimuli reviewed in Refs. 16-18, 66. transduce changes in chemoeffectors concentrations using a number of specific receptors. Changes in ligand concentration are transduced into intracellular signals that alter the direction of flagellar rotation. In the direction of flagellar rotation determine the cell's swimming behavior. The major components of the chemotaxis information processing network have been identified, and the general organization and roles of these gene products have been described (Fig. 1), but the detailed molecular mechanisms of signal transduction remain elusive.

E. coli has four well characterized species of transmembrane transducer proteins, each of which detects a different set of chemoeffectors. In most cases, the transmembrane protein acts as a receptor for a specific ligand or ligand binding protein in the periplasmic space. Information regarding this event is then transmitted by an unknown mechanism across the membrane into the cell. The next event appears to involve the participation of cytoplasmic chemotaxis proteins to process receptor information into a form effective for excitation and a form effective for adaptation. The excitation pathway involves CheA, CheW, CheY, and CheZ and creates a "second message" that is rapidly (-0.1 s) sent to the switch controlling flagellar rotation. A slower acting adaptation signal is also generated. It adjusts transducer sensitivity to the new environmental conditions. Adaptation correlates with the methylation and demethylation of the transmembrane transducer protein, which presumably alters its ability to generate an excitation signal. Two other cytoplasmic proteins are involved in adaptation; the CheB protein is a methytransferase, and the CheZ protein is a specific methylase.

A strong candidate for the unidentified chemotaxis excitation signal was recently provided by biochemical experiments with purified proteins, the results of which can be summarized as follows (see Fig. 1). CheA is an autophosphorylating protein kinase (19-21). In the presence of Mg^2+, the y-phosphoryl group of ATP is reversibly transferred to CheA residue histidine 48 (22), forming a phosphoenzyme intermediate. Phosphorylated CheA (sensor) can then transfer the phosphate group to either CheB (regulator) (20) or CheY (regulator) (20, 21), proteins of the adaptation and excitation pathways, respectively. The reactions of CheA and CheZ play a central role in the regulatory network. "Input" into CheA can be reconstituted by using membrane fractions containing the transmembrane transducer proteins. The presence of such membranes greatly stimulates the accumulation of CheY-phosphate in a reaction requiring CheA and CheW (20). Stimulation by the wild-type transmembrane transducer protein is regulated in response to the addition of an attractant, with a concentration dependence similar to that for attractant binding (23). Thus, the transmembrane transducers can modulate the phosphorylation reactions. Mutant transmembrane transducers that are "fixed" in a particular signaling mode have constitutive stimulatory effects, providing additional evidence that the in vitro reactions faithfully mimic the situation in vivo (23). "Output" from CheA involves the transfer of phosphate to CheB and CheY. CheB-phosphate (20) and CheY-phosphate (20, 21, 24) are both unstable and hydrolyze to yield the unmodified protein and inorganic phosphate. In addition, CheZ stimulates the dephosphorylation of CheY-phosphate (20). Rapid decay of the phosphorylated form of various chemotaxis proteins may be the basis for transient signaling by removing potential sources of "noise" from the system and allowing rapid transitions between two signaling states.

A model for chemotactic signal transduction which incorporates the observations of phosphoryl group transfer between proteins in vitro has been formulated (Fig. 1). Each of the various transducers is proposed to utilize CheW, perhaps in conjunction with CheY, to regulate CheA autophosphorylation. CheA would thus integrate the cell's responses to multiple stimuli. The level of CheA-phosphate controls the levels of CheB-phosphate and CheY-phosphate, leading to the formation of adaptation and excitation signals, respectively. Phosphorylation of CheB appears to increase its methylesterase activity (24). Phosphorylation of CheY is proposed to alter its ability to interact with the flagellar switch. Accelerated dephosphorylation of CheY by CheZ should make the excitation signal more sensitive to CheY-phosphate levels and thus the cell more responsive to environmental changes.

This model is also consistent with previous biochemical and genetic studies of chemotaxis (18-18). ATP is required for chemotaxis. CheB activity is regulated by CheA. Both CheY and CheZ are believed to act downstream of CheA and CheW in the signal processing pathway,
The well characterized examples described so far (Che and Ntr) differ from the majority of two-component regulatory systems in that
the sensors are located in the cytoplasm rather than on the membrane (Table I). In addition, bacterial chemotaxis apparently does not involve regulation of transcription. Demonstration of biochemical activities predicted from the study of Che and Ntr (e.g. sensor autophosphorylation, phosphoryl group transfer to regulator, altered activity of phosphorylated regulator) in other systems would greatly strengthen the case for a common mechanism of signal transduction in two-component regulatory systems of bacteria. The only evidence currently available comes from the EnvZ(sensor)/OmpR(regulator) system, which controls the relative expression of the outer membrane porins OmpC and OmpF in response to osmolarity changes. An envZ gene coding for a fusion protein missing the N-terminal 38 (of 448) amino acids of EnvZ retains the ability to complement an envZ::Tn10 mutation for porin synthesis, although this activity is apparently unregulated (30). The partial similarity of this EnvZ fusion protein is phosphorylated in the presence of [γ-32P]ATP to form an acid-stable base-stable bond (30), consistent with phosphorylation of a basic amino acid such as histidine. These results suggest that phosphorylation may also be central to the EnvZ/OmpR system.

**Cross-talk**

A single bacterial cell (e.g. E. coli) has multiple two-component regulatory systems which respond to many different types of environmental stimuli. Interference between the various systems might occur if signals were transmitted via a common small “second messenger” molecule (31). Instead, phosphoryl group transfer between proteins provides the evolutionary economy of a common mechanism while permitting the necessary specificity for each system. There are apparently enough differences between the various sensor and regulator proteins (Table I) to keep the systems operating separately under normal circumstances and yet enough similarities that they can be forced to interact with one another if cognate components are missing. Observation of apparent “cross-talk” under artificial circumstances provides further evidence for a common mechanism of signal transduction. For example, several cases of inefficient in vitro phosphorylation involving mismatched sensor and regulator proteins have been reported. CheA can phosphorylate HtrC correctly to activate it for translocation (32); EnvZ can apparently activate NtrC as well (A. Ninfas, M. Icenogle, and T. Silhavy, cited in Ref 26). NtrC can phosphorylate both CheY (32) and SpoOA (27). In addition, cross-talk may occur in vivo. There are several cases in which the phenotype of a cell bearing a sensor mutation can be interpreted as resulting from a low level of cross-talk between improper sensor/regulator pairs (2, 3, 32, 33).

**Sensor/Regulator Sequence Similarities**

A model for how the sensor and regulator proteins might function was suggested based on the distribution of conserved and nonconserved sequences within these proteins (2, 3). The unique N-terminal domain of the sensor protein would detect the environmental stimuli particular to a given system and communicate intramolecularly with the conserved C-terminal domain. Phosphorylation of the sensor (e.g. sensor autophosphorylation, phosphoryl group transfer to regulator, altered activity of phosphorylated regulator) in other systems would greatly strengthen the case for a common mechanism of signal transduction in two-component regulatory systems of bacteria. The only evidence currently available comes from the EnvZ(sensor)/OmpR(regulator) system, which controls the relative expression of the outer membrane porins OmpC and OmpF in response to osmolarity changes. An envZ gene coding for a fusion protein missing the N-terminal 38 (of 448) amino acids of EnvZ retains the ability to complement an envZ::Tn10 mutation for porin synthesis, although this activity is apparently unregulated (30). The partial similarity of this EnvZ fusion protein is phosphorylated in the presence of [γ-32P]ATP to form an acid-stable base-stable bond (30), consistent with phosphorylation of a basic amino acid such as histidine. These results suggest that phosphorylation may also be central to the EnvZ/OmpR system.

**Minireview: Protein Phosphorylation in Bacterial Chemotaxis**

Protein phosphorylation in bacterial chemotaxis occurs predominantly in the cytoplasm, with the exception of EnvZ. The principal sites of phosphorylation in the EnvZ protein are the conserved N-terminal sequences (Ref. 27; true for CheY by definition). However, in the one case (CheA) where the site of sensor phosphorylation is known, the unique (N-terminal) portion of the molecule contains all the determinants necessary for phosphoryl group transfer from the sensor to the regulator (22, 25). In contrast, to the regulator phosphorylation requires the conserved C-terminal portion of CheA in addition to the N-terminal domain (22, 25). Thus, the conserved sensor sequences may contain the autokinase activity but not the site of phosphorylation. An alternative hypothesis which accommodates this data would be that protein-protein interaction between the conserved domains is responsible for communicating excitation of the sensor to the regulator. Phosphorylation of the regulator would then be responsible for stabilizing the alternate protein structure that results from this interaction, allowing the regulator to function in the next step of the pathway. Phosphorylation of the regulator is the consequence of sensor/regulator communication in this scheme rather than the means of such communication.

In several areas may help determine the functional roles of the conserved sensor/regulator regions and of phosphorylation. The site of CheB phosphorylation is unknown; however, the N-terminal portion of CheB (sensor) is required for CheB-mediated regulation of CheB activity in response to some, but not all, chemotactic stimuli (34). It would be interesting to know the relationship between CheB phosphorylation and the regulatory requirement for the N terminus of CheB. Information regarding the nature of the phosphorylated residues in additional sensor and regulator proteins would also be useful. Another approach involves the characterization and identification of mutations that affect sensor/regulator interaction and function. Finally, the recent determination of the crystal structure of CheY (35) may allow specific correlations between structure and function for at least one regulator protein.

**Chemical Properties of Sensor/Regulator Phosphorylation**

In general, a protein can be phosphorylated as an intermediate in an enzyme-catalyzed reaction, or phosphorylation can act to regulate protein function. Sensor phosphorylation appears to be in the former class, since in the two best characterized examples (CheA and NtrB) phosphorylated sensor molecules are intermediates in a protein kinase reaction (15, 19–21, 27, 28). Regulator phosphorylation appears to belong to the latter class, based primarily on the observation that activation of NtrC function correlates with phosphorylation (15). Furthermore, CheB-phosphate may have increased methylesterase activity (24). Direct demonstration of additional cases where phosphorylated regulators have altered function is required.

Phosphorylation of proteins can occur on hydroxy (Ser, Thr, Tyr), basic (Arg, His, Lys), or acidic (Asp, Glu) amino acids. The first class is most widely observed and perhaps not coincidentally is also the most stable to routine laboratory protocols. It is possible that the relatively infrequent reports of phosphorylation of acidic or basic amino acids reflects their instability rather than true biological abundance. The sensors CheA (22) and NtrB (28) are both phosphorylated on histidine residues. The regulator NtrC is phosphorylated on aspartate (28), while the regulator CheY may also be phosphorylated on an acidic residue (A. Stock and J. Stock, cited in Ref 18). Two-component regulatory systems in general may utilize unstable phosphoamino acids for transferring phosphoryl groups, for example, by switching between or stabilizing alternate configurations of a protein.

Phosphohistidine has been observed in more than 20 different proteins.
proteins from a wide variety of organisms (36–53), primarily as an enzyme intermediate. We could discern no obvious features common to the sequences surrounding known histidine phosphorylation sites (data not shown), beyond those previously noted for a limited subset of enzymes (59). One provocative finding is that mutation of a conserved histidine in the p72 protein of Moloney murine sarcoma virus or in the CDC28 protein of S. cerevisiae caused ablation of protein kinase activity (44, 55). At least 33 additional eukaryotic serine/threonine kinases contain the conserved histidine (54, 56).

Although histidine phosphorylation has not been demonstrated in any of these cases, perhaps it is being masked by a more stable regulatory phosphorylation of the kinase.

Phosphopaste has been observed primarily in E. coli, cation-translocating ATPases from bacteria, fungi, plants, and animals (57–63) and rarely in other proteins (64, 65). The instability of acyl phosphates is apparently exploited in the two-component regulatory systems to provide a transient signal. One expectation of a common signaling mechanism is that the regulator proteins might all utilize phosphopaste, since NtrC does. However, the relative stability of the phosphorylated residue in the regulator proteins will depend on the microenvironment in each particular protein. The relative in vivo half-lives of CheY-phosphate (~5–15 s; Refs. 20 and 24) and NtrC-phosphate (~250 s; Refs. 57 and 58) in the presence of Mg2+ correlate well with the different time scales necessary to carry out their respective biological functions of chemotaxis and the regulation of transcription.

Summary/Conclusion

Two-component regulatory systems appear to be widespread in bacteria. Phosphorylation has been demonstrated in three of the known systems and correlated with in vivo function in two cases (Che and Ntr). Although phosphorylation of sensor and regulator proteins has so far been observed exclusively in vitro, transient protein phosphorylation could provide a basis for the mechanism of signal transduction in these bacterial systems. There is currently insufficient evidence, however, to establish the precise functional relationship(s) between the conserved sensor and regulator sequences, phosphorylation, and the detailed mechanism involved in signal transduction via the sensor and regulator proteins.

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