Hypothesis

Identification of communication networks in Spo0F: a model for phosphorylation-induced conformational change and implications for activation of multiple domain bacterial response regulators

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Received 20 January 1998

Abstract Fundamental to understanding the mechanism by which phosphorylation activates bacterial signal transduction response regulator proteins is the identification of regions and residues that are responsible for the phosphorylation-induced conformational change. Here we review results from structural and protein dynamics investigations, and combine them with mutagenesis studies on the response regulator protein Spo0F to suggest a model in which a network of buried and surface residues link surface regions required for protein:protein interactions to the site of phosphorylation. The network described for Spo0F may provide pathways through which information is transmitted from the site of phosphorylation, propagating a conformational change many angstroms away. The general applicability of the communication network model for all bacterial response regulator proteins is discussed.

Key words: Phosphorylation; Aspartyl phosphate; Protein dynamics; Response regulator; Spo0F

1. Introduction

Protein phosphorylation is a common mechanism for protein activation in signal transduction pathways of eukaryotic and prokaryotic cells [1]. In the case of eukaryotic signal transduction, the protein modification occurs at a tyrosine, serine or threonine residue. The details by which the modification causes protein activation has been determined through structural comparisons between the unphosphorylated and phosphorylated forms. Large long-range conformational changes have been observed in proteins phosphorylated at serine or threonine residues [2–4]. These proteins commonly make use of the formation of new hydrogen bonds between the phosphate oxygens and arginine side chains to propagate conformational changes. The rearrangement of the arginine residues creates longer range shifts in the secondary structure elements to which they are covalently linked or through hydrophobic interactions of neighboring side chains, thus providing a network that transmits the news of the phosphorylation event from the site of phosphorylation to regions of the protein that are many angstroms away.

To date, such details for the bacterial response regulator, a class of proteins phosphorylated at an aspartate residue, remain elusive. A short acyl-phosphate lifetime, generally on the order of seconds, precludes the facile elucidation of a phosphorylated response regulator structure. However, it is accepted that phosphorylation at the conserved aspartyl pocket of response regulators induces a conformational change that activates these proteins allowing the appropriate response to environmental stimuli [5]. Preliminary studies have illustrated that phosphorylation causes structural changes that propagate away from the site of phosphorylation ([6], Feher and Cavanagh, unpublished results). Despite the absence of detailed structural data on a phosphorylated response regulator protein, two structural studies of a unphosphorylated single domain response regulator, Spo0F, have allowed the identification of residues in the protein which (a) are sensitive to perturbations of the aspartyl pocket and (b) have a propensity for adopting multiple conformations. Mutagenesis results have identified residues essential for phosphorylation and subsequent activity in the sporulation signal transduction pathway. Together, these studies enable the identification of residues which may be responsible for interaction with the acyl-phosphate and propagating conformational changes to sites responsible for protein:protein interactions of the activated Spo0F$\sim$P.

2. The sporulation signal transduction system

Spo0F is a response regulator protein whose phosphorylation plays a central role in integration of cellular and extracellular signals responsible for initiating sporulation in *Bacillus subtilis* (Fig. 1). Spo0F is the target of two sensor histidine kinases, KinA and KinB, that transduce environmental signals by phosphorylating Spo0F at its aspartate binding pocket [7,8]. Phosphorylated Spo0F is the substrate for the Spo0B phosphotransferase that removes the phosphoryl group from Spo0F and transfers it to Spo0A. Phosphorylation of Spo0A causes a 20-fold increase in its DNA binding affinity, activating transcription of many sporulation-specific genes (sporulation sigma factors) and repressing the transition state regulator, abrB (reviewed in [9]). Phosphatase proteins interact with either Spo0F$\sim$P or Spo0A$\sim$P to inhibit the flow of the phosphoryl group through the pathway should cellular conditions become inappropriate for the onset of sporulation [10].

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PII S0014-5793(98)00182-3

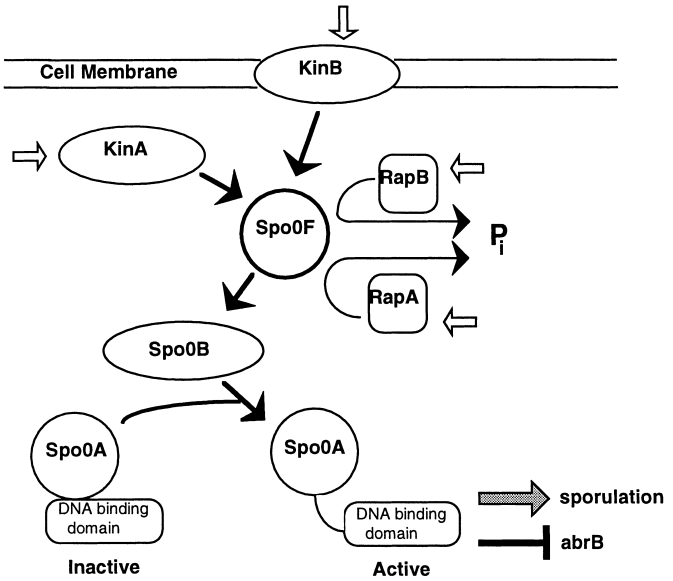

Fig. 1. Diagram of proteins involved in the phosphorelay required for initiating the sporulation signal transduction pathway in *B. subtilis* [7–10]. Open arrows indicate environmental and cellular signal inputs triggering the flow of the phosphoryl group (filled arrows) through the phosphorelay. Phosphorylated Spo0A activates sporulation gene transcription (shaded arrow) and represses the abrB gene (filled bar).

This phosphotransfer scheme involving phosphoryl group migration from histidine (kinase) to aspartate (Spo0F) to histidine (Spo0B), to aspartate (Spo0A) is termed a phosphorelay and although first described and understood in the *B. subtilis* sporulation system is now known to be utilized in eukaryotes [11] for signal transduction.

The wealth of biochemical and genetic data accumulated for Spo0F combined with its tractable size, make it an ideal candidate for structure-function investigations. In addition, the high level of homology and structural similarity manifested by response regulators, allows Spo0F studies to address both specific and general mechanistic questions involved in this family of proteins. The solution structure of Spo0F is illustrated in Fig. 2a. The aspartyl pocket is comprised of residues D10, D11 and D54; D54 forms the acyl-phosphate [12].

3. Structural changes at the aspartyl pocket are propagated to outlying protein regions

Magnesium is required for the phosphoryl-transfer reaction and a crystal structure has localized its coordination in Spo0F to residues of the aspartyl pocket [13]. NMR studies demonstrate that magnesium ion binding at the aspartyl pocket affects not only the structure of residues in the immediate vicinity of the coordination site but also regions far removed from that site (Fig. 2b). For example, the backbone amide proton of E110 is 15 Å from the magnesium coordination site, yet it has a significant chemical shift change between free and bound forms. The elements of secondary structure sensing the perturbation have been identified as: α1 helix, β3-α3 loop (so-called γ loop), β4-α4 loop, α4 helix, β5 strand and α5 helix. It is evident that for such remote regions to sense the perturbation within the aspartate pocket upon metal-ion binding (and hence upon phosphorylation), a network is in place which transmits information concerning the coordination condition of the pocket, particularly the state of D54.

4. Structural studies identify regions with propensity for multiple conformations

The results of Spo0F backbone dynamics investigations are shown in Fig. 2c. Highlighted on the ribbon structure are regions of Spo0F found to have a propensity for conformational flexibility. These regions were identified from measurements of the backbone amide nitrogen relaxation studies and other structural parameters such as dihedral and χ_1 angles [14]. Not surprisingly many of the regions identified comprise loops. However, several residues involved in this flexible family are buried and are involved in the interfaces of secondary structure (e.g. S93, H101). One notable region comprises residues involved in the α3 helix:α4 helix interface and β5 strand which all experience motions on a similar millisecond timescale, suggesting a conformationally concerted behavior. Structurally, α4 helix is an ideal candidate for conformational change; its sole hydrophobic contacts are made to residues of α3 helix (L66, K70) and β5 helix (H101) and not to the central hydrophobic core as is the case for the other helices (see Fig. 2a, legend). It is thought that those regions able to adopt multiple conformations are the ones most likely to be affected by phosphorylation and that the phosphorylation event would favor these regions to adopt a new conformation required for the function of the 'activated' protein.

Comparisons of Figs. 2b and 2c indicate a remarkable correlation between those elements of secondary structure in Spo0F exhibiting conformational flexibility and those involved in a network sensitive to perturbations at the aspartyl pocket.

5. Structural and mutagenesis results identify potential residues for propagating conformational change

As noted above, a common mechanism employed for affecting conformational changes in proteins which become phosphorylated, is the formation of new hydrogen bonds between

the phosphate oxygens and amino acid sidechains which are able to offer the required donor protons. With this in mind, inspection of the Spo0F structure suggest the following candidates as residues able to interact with the acyl-phosphate and promote perturbations to far-reaching regions of the protein: R16, T82, and K104[1]. R16 and T82, when mutated to alanine, gives rise to proteins that interfere with formation of the acyl-phosphate and/or have decreased ability to interact with Spo0B [17]. K104 is an invariant residue in the response regulator family and is essential for activation [18].

The side chains of each of R16, T82 and K104 have been observed to adopt positions that would enable them to make direct (or indirect through solvent molecules) interactions with the acyl-phosphate (see Fig. 3a), and each of these residues has structural links to regions of the protein which (a) have been identified in Fig. 2 and (b) are important in protein:protein interactions. R16 resides in the N-terminal end of α1 helix. The δ-guanido group of this arginine is able to adopt a conformation to contact an acyl-phosphate at D54, and is observed in the wild type structure to populate this position as well as more solvent exposed conformations [14]. The position of R16 at the N-terminus of α1 helix provides a means for altering the region immediately surrounding it, namely the β1-α1 loop and the α1 helix:α5 helix interface, both identified as important for protein:protein interactions with phosphatases and Spo0B [17,19]. The structural evidence for T82 providing a link between the acyl-phosphate and a region with propensity for multiple conformations is most convincing of all the residues identified. Structural studies of the wild type protein show that T82 also can adopt multiple conformations, one with the γ-hydroxyl toward the aspartyl pocket [14]. Interaction of the γ-hydroxyl proton with the acyl-phosphate would have direct consequences on the H101 conformation resulting in the disruption of the T82:H101 hydrogen bond observed in the apo-form [14]. NMR data suggest that the position of H101 has a large structural impact on the region of the β4-α4 loop and α4 helix, a region identified to be important in protein:kinase interactions [17]. K104 resides on the β5-α5 loop. This surface bound residue reaches back from the β5-α5 loop to the active site and its potential role in response regulator activation has been described elsewhere [20–22]. Specifically, we suggest that residues R16, T82 and K104 interact with the acyl-phosphate and that these interactions propagate a conformational change through protein backbone regions to which they are covalently linked.

Another residue that potentially is involved in the communication network is M81. This residue when truncated to alanine alters Spo0F∼P stability without altering the kinetics of phosphoryl-transfer between KinA and Spo0B through Spo0F∼P [17]. This residue is buried and spans from the site of phosphorylation (ε-methyl of M81) to the β4 strand. Its proximity and hydrogen bonding interactions to residues previously mentioned residues (T82, K104) suggest it too may play a role in propagation of conformational change, either directly or indirectly.

In summary, Fig. 3a illustrates the key residues identified in the model for propagating conformational change from the aspartyl phosphate to the regions with propensity for conformational change. The concatenated structural rearrangements are mediated through shifts and alterations in hydrophobic packing (M81) and hydrogen bonding interactions (R16, T82, K104). In the case described here, α1 helix (R16), β4-α4 loop (T82, H101) and β5-α5 loop (K104) directly affect regions which have propensity for conformational change identified by the NMR dynamics studies: the β1-α1 loop, α1 helix:α5 helix interface, β4-α4 loop, α3 helix:α4 helix:β5 strand interface. Most importantly many of the regions sensitive to aspartyl pocket perturbations (Fig. 2b) and which have propensity for conformational change are involved in protein:protein interactions ([17,19], Tzeng and Hoch, unpublished results).

6. Is such a network conserved throughout the response regulator family?

The model describing the propagation of conformational changes from the site of Spo0F phosphorylation to outlying regions of the protein includes many residues and dynamic characteristics observed for other response regulators. Structurally, the $(\alpha/\beta)_5$ global fold and many of the buried residues involved in defining the hydrophobic core of Spo0F (see text of Fig. 2a) are conserved throughout the family of response regulators [18] suggesting many of the hydrogen bonding and hydrophobic interactions identified as necessary for the network are also conserved. Further, the aspartyl pocket and residues homologous to Spo0Fs T82 and K104 are also highly conserved (invariant in the case of K104) and mutagenesis has demonstrated their important role for activation in other response regulators [18]. An arginine residue at positions equivalent to Spo0F R16 or residue 15 occurs in over 60% of response regulators [23]. Finally, the β4-α4 loop has been noted to have both dynamic properties and a putative role in protein:protein interactions in other single domain response regulators [22,24–26].

7. Extending the model to multiple domain response regulators

In general, response regulators are comprised of an N-terminal regulatory domain, homologous to Spo0F, coupled to one (or more) C-terminal output domains. Output domains vary in function; many can control gene expression by directly binding to regulatory regions of target genes or by interacting with other proteins involved in gene regulation. Generally, the unphosphorylated regulatory domain is thought to inhibit the output domain and a phosphorylation-induced conformational change in the regulatory domain relieves this inhibition [27–29]. Certainly, the existence of a communication network, such as that described above, is essential. In the case of single domain response regulators such as Spo0F, the conserved general network may be in place, but only a subset of it is required for its specific function.

The results from the dynamics experiments on Spo0F (Fig. 2c) show that there are regions with propensity for conformational change which extend beyond regions important for Spo0F interactions with the kinases, Spo0B and phosphatases

[1] Structural inspection also suggests K56 as a potential candidate for electrostatic interactions with the acyl-phosphate. Mutagenesis studies show this residue is involved in stabilizing the relatively long lifetime of phosphorylated Spo0F and interactions with KinA [16,17]. It is not included in the present discussion because its proximity to D54 and lack of hydrophobic interactions with other side chains suggests any structural affect upon phosphorylation would be localized to the γ-loop.

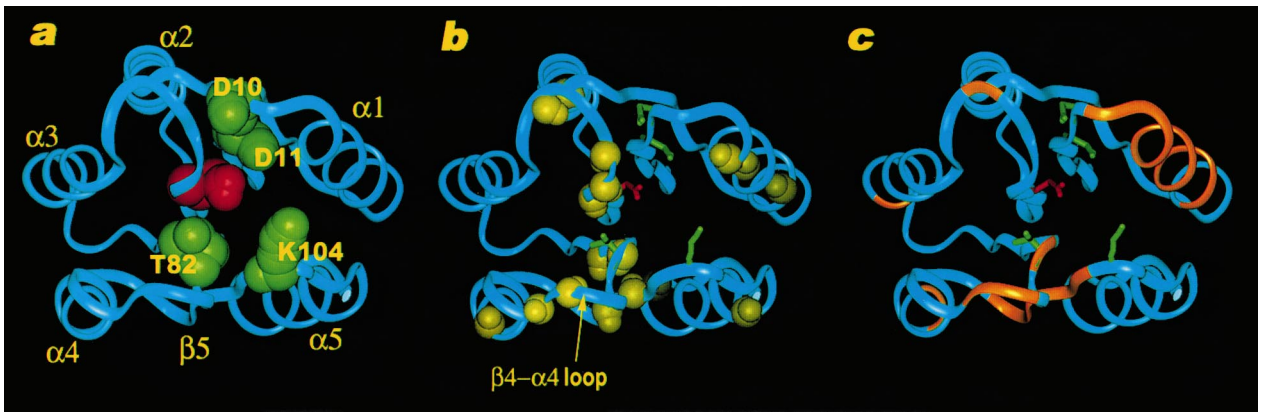

Fig. 2. a: The solution structure of Spo0F has an $(\alpha/\beta)_5$ fold [14]. The α helices span residues I15–E26, L37–K45, I63–I72, I90–G97, and I108–L118, the β strands span residues K5–V9, Q29–A33, L50–L53, R77–M81, and T100–F102. The three central β strands, β1, β3, and β4, constitute the hydrophobic core of the protein. Nine hydrophobic residues define the packing of helices to this central core; L19 and F23 (α1 helix), A39 and V43 (α2 helix), I65, L66, and M69 (α3 helix) and I111 and V115 (α5 helix). Residue D54 (red) is the site of phosphorylation. Other residues important in phosphorylation and activation of response regulators are D10, D11, T82, and K104 are illustrated (green). The $^{\gamma}$OH of T82 points towards the aspartyl pocket and is close enough to participate in phosphorylation. b: Ribbon diagram of apo-Spo0F solution structure with amides highlighted (yellow) that exhibited >50 Hz chemical shift change when Mg^{2+} binding to the aspartyl pocket was followed by NMR spectroscopy [15]. From the set of well resolved resonances the following had notable chemical shift changes: K56, I57, M60 (all three in the immediate vicinity of the magnesium coordination site), I15, N20, V22, G36, M55, T82, E86, L87, E92, H101, F102, E110 and L119. Residues directly involved in Mg^{2+} coordination are D54 and D11 carboxylate oxygens, D11 carboxylate through a solvent molecule, and the carbonyl oxygen of K56 [13]. c: Ribbon diagram of apo-Spo0F solution structure with protein backbone highlighted (orange) which exhibit multiple conformations on the millisecond to microsecond timescale. Residues Q12, Y13, G14, Gly59, Lys70, Thr82, Gly85, Lys94, Thr100 and Phe102 have backbone fluctuations on this timescale (judged by requirement for R_{ex} terms in spectral density fits), H101 and F102 side chains have millisecond timescale motion (judged by slow exchange between two conformers on the NMR timescale), dihedral angle averaging for Thr82, Glu86, Leu99, His101 and Ala103 and χ_1 angle averaging for Thr82 and Ser93 support the notion that these residues have conformational averaging of their backbone and/or sidechain positions [14].

(β1-α1 loop, β3-α3 loop, α1 helix:α5 helix interface, and β4-α4 loop ([17,19], Tzeng and Hoch, unpublished results). It is not immediately evident why the entire α3 helix:α4 helix:β5 strand interface of Spo0F should exhibit conformational flexibility. We suggest that the network illustrated in Fig. 3a constitutes a general mechanism for propagating conformational change for the response regulator family. The potential for conformational change to propagate through the α3 helix:α4 helix interface to the C-terminal regions of these helices via changes to T82:H101 and possibly through hydrophobic interactions of buried residues L53:L66:S93 (see Fig. 3a,b) suggests how two domain response regulators may become sensitive to the phosphorylation state of the regulatory domain. In support of these ideas, inspection of a recently published structure of a two domain response regulator, NarL, suggests the C-terminal regions of the α3 and α4 helices provide critical contacts between the regulatory and output domains and we propose that it is through these interactions

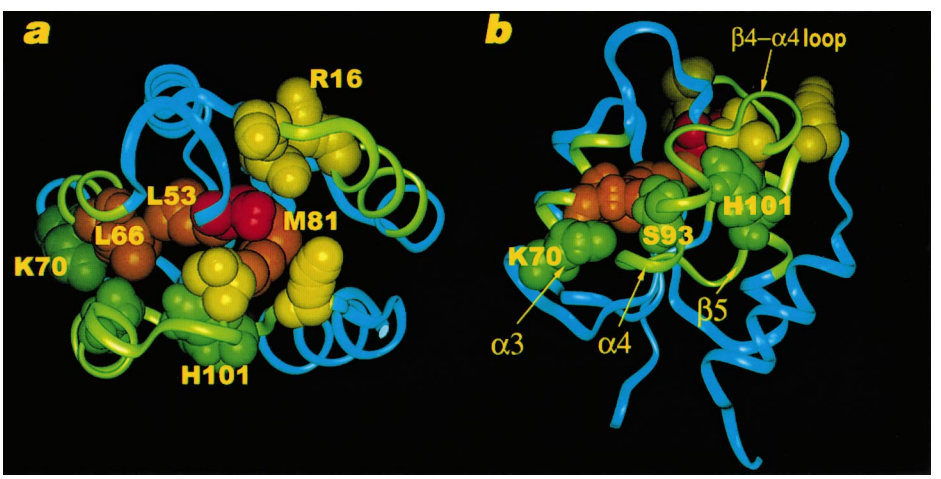

Fig. 3. The network. Residues R16, T82 and K104 (yellow) are candidates for direct interaction with the acyl-phosphate. Residues M81, L53 and L66 (orange) are buried residues which could also play a role in propagating conformational change from the acyl-phosphate to remote surfaces of the protein. Residues and backbone regions in green are involved in the network putatively perturbed by changes in R16, γ loop, M81, K104 that are immediately surrounding D54. Two views of the protein are given (a) is the same orientation as in Fig. 2, looking down onto D54 (red) and (b) is looking toward the C-terminal ends of α3 and α4 helices.

that the regulatory domain relays the signal that phosphorylation has occurred.

NarL, a protein involved in the nitrate-dependent regulation of many anaerobic electron transport and fermentative genes in *Escherichia coli*, represents the only published structure of a two domain response regulator to date [30]. The N-terminal regulatory domain of NarL displays the expected conserved $(\alpha/\beta)_5$ fold, and the aspartyl pocket containing the site of phosphorylation, D59. The C-terminal output DNA binding domain is a compact bundle of four α-helices with the middle two forming a helix-turn-helix DNA binding motif. In the published unphosphorylated form, the α3-β4 loop and C-terminus of α4 helix of the regulatory domain form contacts with the output domain, presumably accounting for the inhibition of DNA binding in this form. Changes in this interface can assist in either rotation or displacement of the output domain so that it becomes accessible for DNA binding. How does phosphorylation at the aspartyl pocket activate the protein to cause a conformational change at the surface at the opposite end of the regulatory domain?

It is evident from the structure of NarL that the bottom of the regulatory domain represents the interface between the regulatory and output domains of this protein. In particular the C-terminal end of α3 helix appears to be an integral part of this interface. Moreover, though many interactions are likely to contribute to stabilizing the structure of NarL, an important one appears to be the potential for a hydrogen bond between one of the δ-guanido protons of Arg^{75} and the carbonyl backbone oxygen of Lys^{201}, at a distance of 3.2 Å. This interaction may well provide a necessary restraint for keeping the C-terminal output domain to remain in position relative to the N-terminal receiver domain prior to phosphorylation. Across the response regulator family, there is high level of amino acid conservation at this position, with ~70% of response regulators having a lysine or arginine [23]. Indeed, the equivalent residue in Spo0F is a lysine at position 70, a residue involved in dynamic motion (Fig. 2c).

8. Concluding remarks

Although some aspects of the model proposed here have been suggested before, this expanded model extends our understanding of how Spo0F and potentially the family of response regulators function. Previously, residues homologous to Spo0Fs T82, H101 and K104 have been identified as important in post-phosphorylation events for the single domain response regulator CheY. Recent studies on CheY mutants at positions T82 and Y106 correlate the interaction of these two residues and the position of the Y106 ring to the signaling state of CheY [31,32]. Serrano et al. [22] have proposed that K109 is important in transmitting information of the acyl phosphate to β4-α4 loop in CheY. Nohaile and coworkers have also observed perturbations in the a3-a4-a5 face and phosphorylation site are active site in NtrC and have suggested the α4-β4 and β5-α5 loops are important in communicating structural changes to the surface [33]. Our model extends these ideas by identifying the dynamic character of the interfaces of the secondary elements α3 helix:α4 helix:β5 strand (highlighted orange in Fig. 2c) and including additional residues involved in the transmitting formation from the acyl-phosphate event to this area (L53, L66, M81 and T82). This revised model predicts perturbation of α3 helix:α4 helix:β5 strand interface as important for single domain response regulators on the 'top' surface and perturbations at the C-terminal end of this dynamically concerted region is important for many two domain response regulators. In addition, a new network is proposed that includes α1 helix (through R16), a region that appears to be important in response regulator regulation by phosphatases (Tzeng and Hoch, unpublished data; [34]). This model is testable in two domain response regulators and predicts that mutations in the C-terminal regions of α3 helix and α4 helix, especially residues homologous to Spo0F's K70, will disrupt regulation of the output domain by the acyl-phosphate containing regulatory domain.

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