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Structural transitions in the FixJ receiver domain

Patrice Gouet¹, Béatrice Fabry¹, Valérie Guillet¹, Catherine Birck¹,
Lionel Mourey¹, Daniel Kahn² and Jean-Pierre Samama^{1*}

Background: Two-component signal transduction pathways are sophisticated phosphorelay cascades widespread in prokaryotes and also found in fungi, molds and plants. FixL/FixJ is a prototypical system responsible for the regulation of nitrogen fixation in the symbiotic bacterium *Sinorhizobium meliloti*. In microaerobic conditions the membrane-bound kinase FixL uses ATP to transphosphorylate a histidine residue, and the response regulator FixJ transfers the phosphoryl group from the phosphohistidine to one of its own aspartate residues in a Mg²⁺-dependent mechanism.

Results: Seven X-ray structures of the unphosphorylated N-terminal receiver domain of FixJ (FixJN) have been solved from two crystal forms soaked in different conditions. Three conformations of the protein were found. In the first case, the protein fold impairs metal binding in the active site and the structure reveals a receiver domain that is self-inhibited for catalysis. In the second conformation, the canonical geometry of the active site is attained, and subsequent metal binding to the protein induces minimal conformational changes. The third conformation illustrates a non-catalytic form of the protein where unwinding of the N terminus of helix α 1 has occurred. Interconversion of the canonical and self-inhibited conformations requires a large conformational change of the β 3– α 3 loop region.

Conclusions: These unphosphorylated structures of FixJN stress the importance of flexible peptide segments that delineate the active site. Their movements may act as molecular switches that define the functional status of the protein. Such observations are in line with structural and biochemical results obtained on other response regulator proteins and may illustrate general features that account for the specificity of protein–protein interactions.

Introduction

A large variety of adaptative cellular responses to environmental stimuli, such as osmoregulation, sporulation, pathogenicity and chemotaxis [1] are mediated by two-component systems [2]. These signaling cascades are widespread in bacteria and are also found in lower eukaryotic organisms [3–5]. They involve two essential protein families, the sensor histidine kinases and the response regulator proteins. On detecting a stimulus, the sensor protein, which may be an input domain of the kinase itself or a receptor coupled to it, modulates the activity of the transmitter module of the kinase. Signal transduction is mediated by Mg²⁺-dependent phosphorelay reactions between the kinase and the phosphoaccepting receiver domain of the cognate response regulator protein.

The FixL/FixJ two-component system is responsible for the regulation of nitrogen fixation in the symbiotic bacterium *S. meliloti* [6,7]. The membrane-bound kinase FixL contains an oxygen-sensing domain and a transmitter

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module. In microaerobic conditions this module uses ATP to transphosphorylate a histidine residue conserved in the kinase family [8,9]. The phosphoryl group is then transferred to a conserved aspartate residue on the N-terminal regulatory, or receiver domain, of the FixJ response regulator, which displays a typical two-domain arrangement [10,11]. Phosphorylation of the receiver domain mediates dimerization of the protein [12,13] and relieves the inhibition of the C-terminal domain as transcriptional activator of the *nifA* and *fixK* genes [14–16].

Sequence and structure homologies between receiver domains suggested a common Mg²⁺-dependent mechanism of phosphorylation for all members of the response regulator superfamily [17–19]. The structures exhibit a common (β/α)₅ fold [18,20–26], and similar active sites where three invariant residues are located (Asp11, Asp54 and Lys104 in FixJ). Phosphorylation of Asp54 in FixJ induces a cascade of conformational changes; these were revealed by the X-ray structure of the phosphorylated receiver domain [13].

In vivo the production of the output response is highly regulated by the number of phosphorylated species. Their formation involves a selective recognition of the receiver domain by the cognate kinase and it was also proposed that the ability of this domain to bind the essential metal ion in the active site could be a means of modulating the functional status of the response regulator [24]. In this paper, we report seven X-ray structures of the unphosphorylated N-terminal receiver domain of FixJ (FixJN), with and without bound metal cations, at a maximum resolution of 1.6 Å. These structures, solved from crystals grown and soaked in different conditions, provide snapshots of the conformational states of FixJN. Leu55, the residue adjacent to the phosphorylatable aspartic acid, and loop $\beta 3$ – $\alpha 3$ play a central role in the observed conformational changes of the protein.

Results and discussion

Overview of the crystal structures

The topology of FixJN is described by a doubly wound five-stranded β/α fold where the parallel β sheet is sandwiched by helices $\alpha 1$ and $\alpha 5$ on one side and helices $\alpha 2$,

$\alpha 3$ and $\alpha 4$ on the other side (Figure 1a,b). The protein core is made of strands $\beta 1$, $\beta 3$ and $\beta 4$, which are lined by conserved hydrophobic residues [19]. The C-terminal ends of these strands and their connecting loops to the following helices ($\beta 1$ – $\alpha 1$, $\beta 3$ – $\alpha 3$ and $\beta 4$ – $\alpha 4$) define the active-site depression. The polypeptide stretch (residues 55–61) connecting strand $\beta 3$ to helix $\alpha 3$, known as the γ loop [19], displays two significantly different conformations (Figure 1a). One of them has never been observed in the structures of other receiver domains and its fold impairs metal binding in the active site. The two structures of FixJN where this conformation is found were called ‘self-inhibited’ and ‘atypical’. In the latter, unwinding of the N terminus of helix $\alpha 1$ up to Ser18 also occurs. The second conformation of the γ loop yields the ‘canonical’ structure of FixJN. The receiver domain displays a proper geometry of the active site for metal binding and, accordingly, subsequent binding of this ion induced minimal structural changes. Table 1 summarizes the occurrence of the three FixJN structures in the two crystal forms. It shows that two different species may be simultaneously present in one asymmetric unit, which indicates

Figure 1

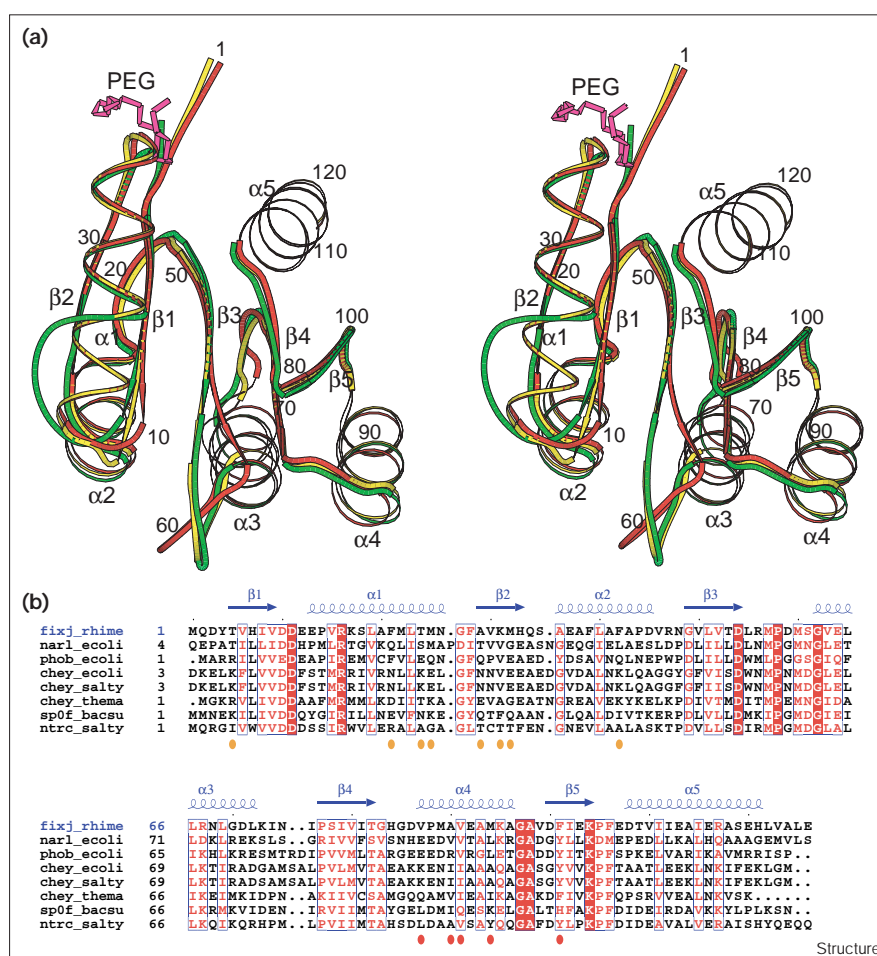


Table 1

Occurrence of the three FixJN conformations in the two crystal forms and their refinement statistics.

Crystal forms	Triclinic					Monoclinic	
	FixJN	FixJN–PtCl ₄ ²⁻	FixJN–Sm ³⁺	FixJN–Mn ²⁺	FixJN–Mg ²⁺	FixJN	FixJN–Mn ²⁺
Resolution (Å)	15–1.6	15–1.9	15–2.4	15–2.0	15–2.0	15–2.9	15–3.0
No. of copies in AU*	2(SI-C) [†]	2(SI-SI)	2(C-C)	2(C-C)	2(C-C)	2(SI-SI)	2(C-A)
No. of reflections	20,600	15,961	8,245	12,977	13,448	5,363	4,663
R factor (R _{free}) (%)	18.6 (22.2)	21.9 (26.8)	19.6 (26.5)	20.6 (25.3)	21.9 (27.4)	23.1 (29.2)	25.3 (33.6)
Rmsd bond lengths (Å)	0.012	0.012	0.011	0.012	0.012	0.010	0.010
Rmsd bond angles (°)	1.7	1.6	1.7	1.6	1.6	1.4	1.6
Nonhydrogen atoms	2,040	1,836	1,951	1,995	1,969	1,920	1,785
Heteroatoms	148 H ₂ O PEG [‡]	71 H ₂ O, 4 Pt ²⁺ , 4 Cl ⁻	75 H ₂ O, PEG [‡] , 4 Sm ³⁺	94 H ₂ O, PEG [‡] , 3 Mn ²⁺	91 H ₂ O, PEG [‡] , 2 Mg ²⁺	34 H ₂ O	12 H ₂ O 1 Mn ²⁺
Average B (Å ²)	22	25	22	26	27	14	57

*AU is asymmetric unit. [†]SI, self-inhibited; C, canonical; A, atypical. [‡]Ordered segment of 19 atoms of PEG 1500 has been modeled as -O-(CH₂CH₂O)₆-.

that the crystallization processes did not select one population. Soaking the crystals in solutions of metal ions (Sm³⁺, Mn²⁺ or Mg²⁺) induced the conformational change from the self-inhibited to the canonical structure, except in one case where it led to the atypical structure. In contrast, soaking with platinum chloride (PtCl₄²⁻) favored the self-inhibited structure over the canonical one (Table 1). The superposition of the self-inhibited and canonical structures of the asymmetric unit in the native triclinic crystals gave a root mean square deviation (rmsd) value of 0.7 Å for all C_α pairs when excluding residues 55–61 from the calculation. On the basis of this superposition, the γ loop regions differ by an rmsd value of 3.0 Å. Apart from the protein–protein contacts leading to the major interfaces described below, one crystal-packing interaction is observed in all triclinic crystals. Loops α2–β3 and α3–β4 from a symmetry-related self-inhibited molecule are in the vicinity of the His84 sidechain and of the α1–β5–α5 area of the canonical molecule, respectively. Both molecules undergo conformational changes (Table 1), suggesting that these packing contacts do not affect the structures. In the triclinic crystals, a continuous electron density was

assigned to 19 atoms of a polyethylene glycol (PEG) fragment bound in a pocket lined by residues from two adjacent molecules (Figure 2). Three oxygen atoms of the ether groups interact with the amino group of a lysine sidechain, and the conformation of this fragment is close to that of a crown polyether with a cationic group at its center [27]. The binding of a PEG molecule in crystal structures has only been reported for the peptide deformylase enzyme from *Escherichia coli* [28] and for the human peptidyl-prolyl *cis-trans* isomerase Pin1 [29].

The canonical structure

The active site of the receiver domain of FixJ comprises the conserved Asp10, Asp11 and Asp54 residues (Figure 3). Glu12 is oriented towards this acidic pocket, as is the invariant Lys104 from the β5–α5 loop. Lys104 makes no direct interaction with any of the acidic groups. A water molecule is found in the active site at hydrogen-bonding distance (2.5 Å) from the mainchain oxygen atom of residue 56 and the carboxylic oxygen atoms of Asp11 and Asp54. Leu55, Arg56 and Met57 from the γ loop are critical residues with respect to the different structures of

Figure 2

Stereoview of the PEG fragment (gray) in the final 2F_o–F_c electron-density map contoured at 1.0σ. Two adjacent FixJN molecules in the crystal (yellow and green) contribute to the binding of this fragment.

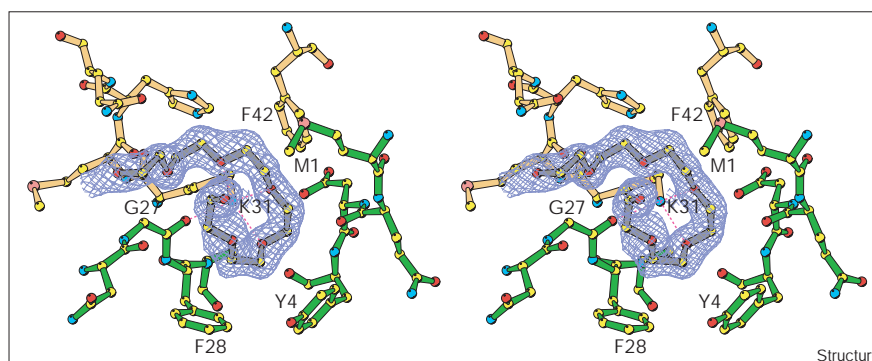
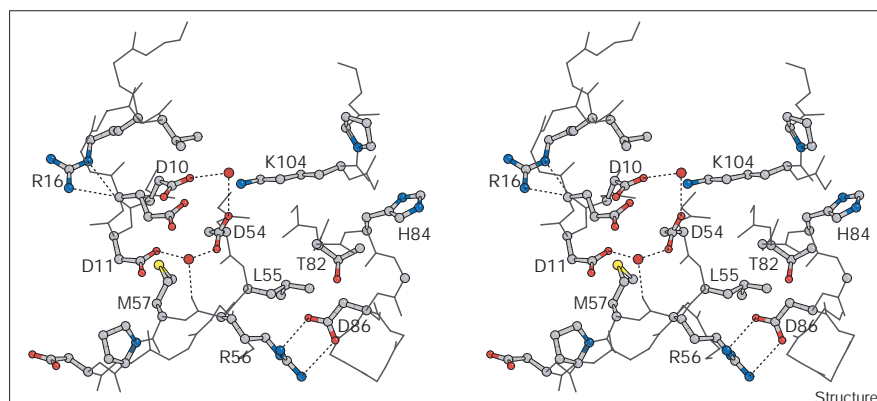


Figure 3



Stereoview of the active-site environment in the metal-free canonical structure. The water molecules are indicated by red spheres and the polar interactions by dotted lines. The color code for the atoms is oxygen in red, carbon in grey, nitrogen in blue and sulfur in yellow.

FixJN. The leucine and methionine sidechains are located in two hydrophobic pockets that line up the active site and define its geometry. The guanidinium group of Arg56 and the carboxylate group of Asp86 form electrostatic interactions that stabilize the conformation of the adjacent $\beta 4$ - $\alpha 4$ loop (residues 83–86) (Figure 3). In all metal-bound FixJN structures, the cation (Sm^{3+} , Mn^{2+} or Mg^{2+}) replaces the water molecule in the active site, and is liganded to the same protein atoms. Three water molecules fill out the octahedral coordination of the Mn^{2+} or Mg^{2+} ions (Figure 4). The distance of these ions to all ligands is 2.4 Å. The N terminus of helix $\alpha 1$ is stabilized by the hydrogen-bonding interaction between the mainchain oxygen of Asp10 and the guanidinium group of Arg16 (2.7 Å) (Figure 3).

The self-inhibited structure

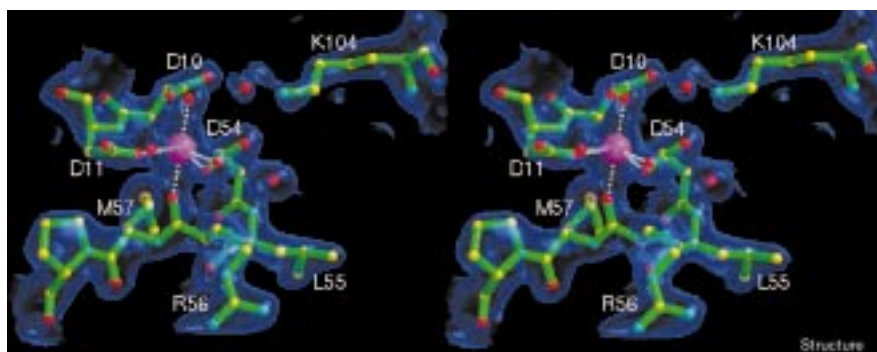
The fold of the γ loop in this structure significantly alters the location and the conformation of several residues. Compared with the canonical structure, the C_α atom of Leu55 moves by 4.5 Å and this residue shifts from the α to the β conformation, which results in an extension of strand $\beta 3$. The leucine sidechain is then located in the hydrophobic

pocket previously occupied by the sidechain of Met57, which points into the solvent. As a consequence, the sidechain of Arg56 moves by 9 Å, runs across the metal-ion-binding site and fills the active site (Figure 5). In the triclinic structure solved at 1.6 Å resolution (Table 1), the guanidinium group forms a salt bridge and a hydrogen-bonding interaction with Glu12 (2.8 Å) and Asp54 (3.2 Å), respectively, and the ammonium group of Lys104 forms a salt-bridge interaction with Asp54 (2.9 Å) (Figure 5). Loops $\beta 4$ - $\alpha 4$ and $\beta 5$ - $\alpha 5$ are shifted by 0.9 Å, and the N terminus of helix $\alpha 4$ moves away from the protein core by about 1 Å, allowing hydrogen-bond formation between the carboxylate of Asp86 and the mainchain nitrogen atom of residue 89. Hatched electron density was observed in some protomers for residues 13–17 at the N terminus of helix $\alpha 1$. The perturbation in this region probably arises from the close proximity of the guanidinium group of Arg56 and the Val15 sidechain (3.3 Å) (Figure 5).

The atypical structure

The atypical structure of FixJN was found in one molecule of the asymmetric unit when monoclinic crystals were soaked with Mn^{2+} (Table 1). The fold of the γ loop

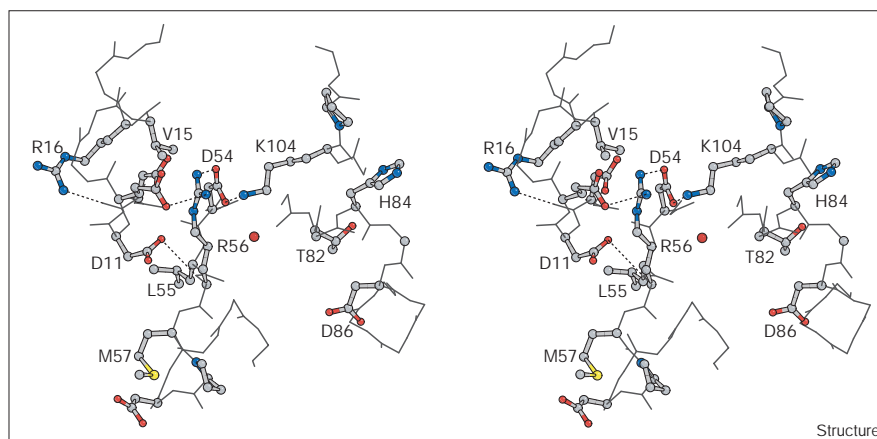
Figure 4



Stereoview of the active site in the Mn^{2+} -bound canonical structure of FixJN. The electron density is represented as a transparent solid, the water molecules as small red spheres and the Mn^{2+} as a large magenta sphere. Metal ion coordination bonds are illustrated by dotted lines.

Figure 5

Stereoview of the active-site environment in the self-inhibited FixJN structure. The water molecule is indicated by a red sphere and the polar interactions by dotted lines.



Structure

in the atypical structure is similar to the one of the self-inhibited structure with respect to the location of Leu55 and Met57 (Figure 6), but the conformation of the main-chain atoms of residues 56–61 differs by an rmsd value of 1.7 Å. The absence of electron density for Arg56 from the C_β atom suggests that the guanidinium group is no longer anchored to the active-site residues. In this structure, unwinding of the N terminus of helix α1 (residues 13–17) occurs and brings the C_α atom of the strongly conserved Arg16 8 Å away from its position in the self-inhibited and canonical structures (Figure 6). The invariant aspartic acids (Asp11 and Asp54) do not constitute an acidic pocket suitable for metal binding.

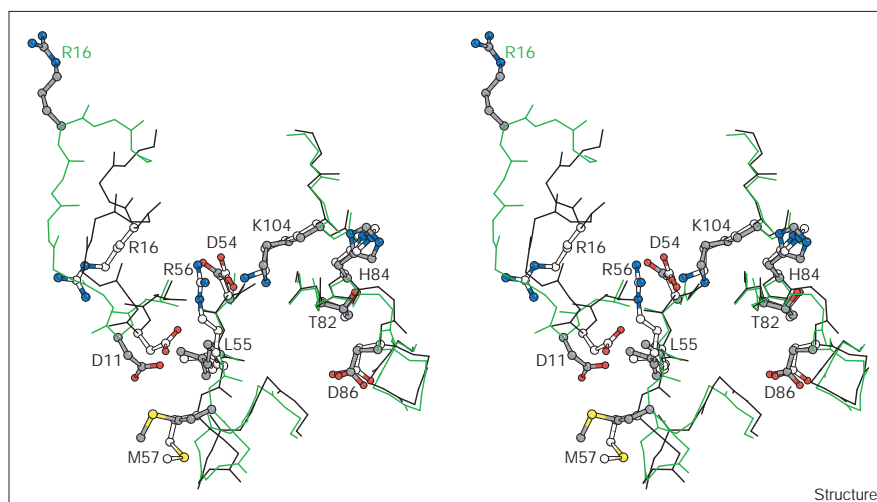
Molecular indicators of the conformational states of FixJN

The N terminus of helix α1, and the loops β1–α1, β3–α3 and β4–α4 define the active-site area of the receiver

domain. The atypical structure, where the N-terminal part of helix α1 unwinds up to Ser18, has never been observed in the crystal structures of isolated receiver domains, but motions of this region have been described in Spo0F [30,31], and unfolding of the first turn of helix α1 occurred in CheY in complex with the phosphotransfer domain of ArcB [32]. This structure of FixJN was found when the crystals were soaked with Mn²⁺, a cation present at high concentrations in the nodules on the plant roots where *S. meliloti* resides [7]. The atypical structure may illustrate a folding intermediate from the self-inhibited to the canonical structures, but the possibility that functional aspects may be related to this structure cannot be ruled out. Indeed, the residues that most severely influence the protein–protein interactions for phosphotransfer between Spo0F and Spo0B are located in the region of helix α1 that unwinds in FixJN [33].

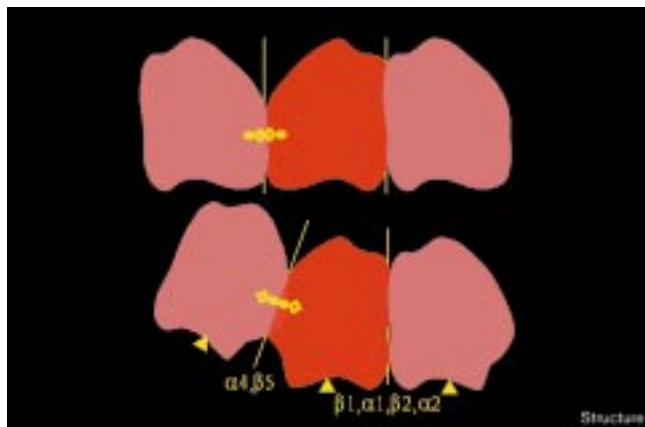
Figure 6

Stereoview of the superposed atypical structure (mainchain atoms in green; filled sidechain bonds) and self-inhibited structure (mainchain atoms in black; open sidechain bonds).



Structure

Figure 7



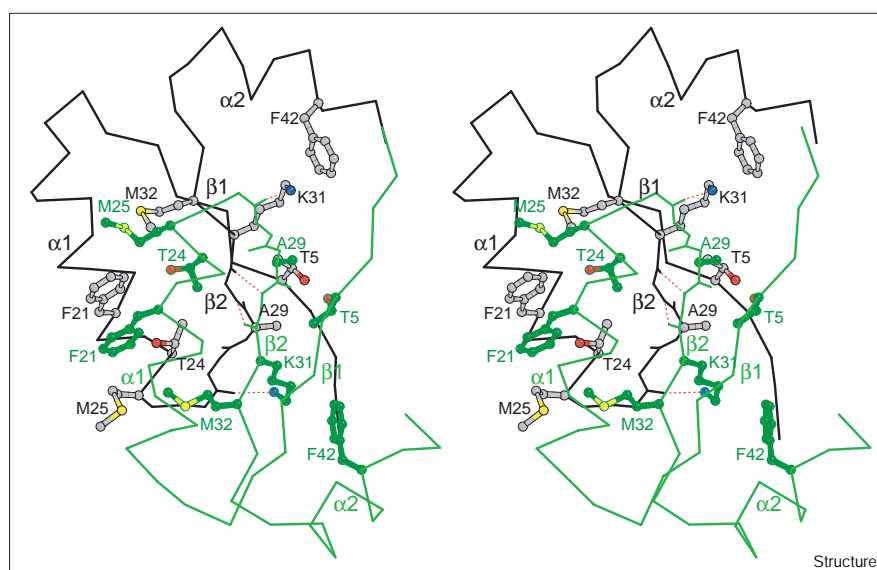
Cartoon representing the protein-protein interfaces mediated by twofold axes (yellow vertical lines) in the crystals for unphosphorylated FixJN (top) and phosphorylated FixJN (bottom). The $\beta 1$ - $\alpha 1$ - $\beta 2$ - $\alpha 2$ interface is not affected by the phosphorylation state of the protein. The $\alpha 4$ - $\beta 5$ interface mediates dimerization of the receiver domain after the phosphorylation-induced conformational change of the contact area. The triangles point to the active site and the symbol at the $\alpha 4$ - $\beta 5$ interface represents the invariant aromatic residue (Phe101 in FixJ). It is oriented towards the solvent in each unphosphorylated FixJN protomer and towards the protomer core in the phosphorylated species.

The $\beta 3$ - $\alpha 3$ loop (γ loop, residues 55-61) is edged by two invariant residues, the phosphorylatable Asp54 and Gly62, and the residues found at positions 55 and 57 are hydrophobic in all response regulator proteins [19]. This loop delineates one side of the acidic pocket, and provides one of the three protein ligands (the mainchain oxygen atom of residue 56) to the essential metal ion. The conformation of the γ loop observed in the self-inhibited FixJN

structure is unprecedented with respect to the location of the Leu55 and Arg56 residues. The Arg56 sidechain enters the active site and the strong polar interactions that are established suggest that the $\beta 3$ - $\alpha 3$ loop may act as a molecular switch for auto-inhibition of the receiver domain. Reports on other response regulator proteins indicate that this property may not be unique to FixJ. The flexibility of the $\beta 3$ - $\alpha 3$ loop in CheY from *E. coli* and in Spo0F from *Bacillus subtilis* was observed by nuclear magnetic resonance (NMR) spectroscopy studies [31,34]. Two conformations of this loop were also observed in the crystal structures of the metal-free PhoB from *E. coli* [26] and CheY from *Thermotoga maritima* [35]. In the latter protein, one conformation of the $\beta 3$ - $\alpha 3$ loop brings the Thr56 sidechain within hydrogen-bonding distance of Asp54 and precludes phosphorylation. The same consequence holds in CheB and NarL where the sidechains of the equivalent residues, Glu58 and Asn61, respectively, are hydrogen bonded to the phosphorylatable aspartic acid [23,25]. From the collective X-ray and NMR data it would seem that receiver domains may present a significant population of conformers impaired to different extents and in different ways for catalysis. Their occurrence may account, for example, for the high dissociation constant for divalent cations in Spo0F, which led to the proposal that the uncomplexed form might be the predominant biological state [24]. The stability of these metal-free conformers may possibly depend on the residue type within this loop region and contribute, in each system, to a subtle tuning of the regulation of signal transduction.

In the canonical structure of FixJN, the conformation of the $\beta 3$ - $\alpha 3$ loop not only excludes the Arg56 sidechain from the active site but also allows the formation of a salt-bridge

Figure 8



Stereoview of the $\beta 1$ - $\alpha 1$ - $\beta 2$ - $\alpha 2$ interface found in the FixJN crystals. The C_{α} traces of two protomers are shown in green and black, respectively. The FixJN molecules are related by a twofold axis nearly horizontal in the plane of the figure. Two symmetrical hydrogen bonds are exchanged between the mainchain atoms of residues 30 in the adjacent $\beta 2$ strands. Two hydrogen bonds are formed between the amino group of Lys31 and the mainchain oxygen atom of residue 27. The PEG fragment (see Figure 2) is bound in the vicinity of this interface region.

interaction between Arg56 and Asp86 from the $\beta 4$ - $\alpha 4$ loop. This interaction stabilizes the conformation of the $\beta 4$ - $\alpha 4$ loop and, in turn, the structure of the pre-phosphorylation state of the receiver domain [13]. This interaction is broken in phospho-FixJN as a consequence of the phosphorylation-induced large conformational change of the $\beta 4$ - $\alpha 4$ loop [13]. It therefore seems that the conformations of both the $\beta 3$ - $\alpha 3$ and $\beta 4$ - $\alpha 4$ loops may act as molecular indicators of the FixJN receiver domain to define the self-inhibited, the canonical unphosphorylated or the phosphorylated states. In these three structures, the fold of the $\beta 1$ - $\alpha 1$ loop and of the N-terminal part of helix $\alpha 1$ are identical (Figure 3,5) [13].

Protein interfaces as modulators of the functional states

The transition from the canonical to the self-inhibited structure, induced by the binding of PtCl_4^{2-} 30 Å away from the active site, cannot be predicted from the structure. It nevertheless suggests that the conformational state (self-inhibited or canonical) of the unphosphorylated receiver domain may be influenced by binding interactions in the receiver domain, at a remote distance from the active site. This transition between two conformational states and the solving of a self-inhibited structure of FixJN offer a possible explanation for the 44-fold inhibition of phosphorylation of the receiver domain by the C-terminal domain in the full-length response regulator [12]. The protein-protein interface between these domains is known to mediate the inhibition of the DNA-binding properties of the C-terminal domain [11]. The hypothesis that this protein-protein interface may have a dual function and favor the self-inhibited structure of the receiver domain is attractive because it would be a means of protecting the response regulator, *in vivo*, against fortuitous phosphorylation by small phosphodonnors [36] and unrelated kinases.

Another modulation of the function in response regulator proteins is the formation of oligomeric species induced by phosphorylation but also favored upon binding to the DNA targets, which may potentiate protein-protein interactions by increasing the local concentration of the proteins [37]. Crystal-packing constraints, by promoting intermolecular associations, may suggest protein-protein interfaces of biological significance. Two contact areas between FixJN protomers were found in both monoclinic and triclinic crystals. The first protein-protein interface excludes 700 Å² from the solvent, and involves residues from helix $\alpha 4$ and strand $\beta 5$ from each protomer. This interface does not lead to dimeric FixJN species in solution but the phosphorylation-induced conformational changes in this region restructure the protomer surface, allow a new orientation of the molecules and increase the contact area to 900 Å² (Figure 7). This $\alpha 4$ - $\beta 5$ interface mediates dimerization of phosphorylated FixJN as well as that of the phosphorylated full-length protein, which

significantly increases the binding of the response regulator to the *fixK* promoter [12,13].

A second interface between FixJN molecules is located 30 Å away and involves 20 residues from helix $\alpha 1$, strand $\beta 2$ and helix $\alpha 2$ from each protomer. It is mostly hydrophobic and excludes 1,700 Å² from solvent (Figure 8). This value is significant and, in the crystal, this interface associates FixJN molecules independent of their phosphorylation state. This favorable area may promote the formation of oligomeric forms of FixJ, *in vivo*, as was suggested to occur for NarL, PhoP, OmpR, and VirG [38–41] and shown for UphA and NtrC [42,43] when these proteins bind to their target promoters.

Four significantly different conformations of the receiver domain of FixJ are now documented. One of them illustrates the phosphorylated state, and the three others were found for the unphosphorylated protein. These structures differ by the conformation of all regions lining the active site, the $\beta 1$ - $\alpha 1$ loop and the N-terminal part of helix $\alpha 1$, the $\beta 3$ - $\alpha 3$ and the $\beta 4$ - $\alpha 4$ loops. Interestingly, alanine-scanning mutagenesis on Spo0F recently showed that three groups of residues located in these regions define the molecular surfaces for the specific recognition of this response regulator with its phosphorelay proteins [33,44,45]. Mutational, biochemical and genetic studies are underway to identify the residues essential for the FixL/FixJ interactions and to address the biological significance of the $\alpha 1$ - $\beta 2$ - $\alpha 2$ interface.

Biological implications

Two-component systems mediate a large variety of adaptive cellular responses to environmental stimuli in bacteria. The FixL/FixJ system is responsible for the regulation of nitrogen-fixation genes in *S. meliloti*, symbiotic bacteria that are responsible for the formation of, and reside in, nodules on plant roots that enable nitrogen to be provided to the plant. In microaerobic conditions, autophosphorylation of the kinase FixL and phosphotransfer to the receiver domain of FixJ activates the transcription of more than twenty genes. Activation of the response regulator proteins by the kinases in two-component systems involves a metal-ion-dependent mechanism and a specific recognition of the two protein partners in order to minimize detrimental cross-talk between unrelated signaling cascades. The seven X-ray structures of the unphosphorylated N-terminal receiver domain of FixJ (FixJN) solved in this study, with and without bound metal cations, illustrate three conformations of the unphosphorylated receiver domain and shed some light on possible regulatory aspects of this activation.

One of these structures is impaired for catalysis. This feature is not unique to this receiver domain but in FixJ it involves a major conformational change of the $\beta 3$ - $\alpha 3$

loop. It may be favored in the full-length FixJ protein by interactions or topological constraints between the receiver domain and the C-terminal DNA-binding domain. This hypothesis would provide an explanation for the kinetics of phosphorylation of FixJ, and, *in vivo*, would constitute an attractive way of minimizing fortuitous activation of the response regulator protein by phosphodonors other than the cognate kinase.

The second FixJN structure displays the standard geometry of the active site. This canonical form was found in the absence and in the presence of bound catalytic metal ions, suggesting that the canonical form would be a precursor rather than a product of metal binding. From this set of structures and the X-ray and NMR data on homologous proteins, it would seem that factors modulating the stability of the metal-free conformers of response regulator proteins may provide a subtle tuning for the regulation of their activation.

In the structures of unphosphorylated FixJN reported here, significant conformational changes are observed in the $\beta 1$ - $\alpha 1$ loop, the N-terminal part of helix $\alpha 1$, and the $\beta 3$ - $\alpha 3$ loop. The third loop in the active-site area, $\beta 4$ - $\alpha 4$, undergoes a large movement upon phosphorylation of the receiver domain [13]. This ensemble of crystal structures corresponds to two signaling states (unphosphorylated and phosphorylated) and, within the unphosphorylated state, to apparently different functional states — impaired and ready for catalysis. Each of these structures offers different potential recognition surfaces to the FixL protein because of the conformations of these loops.

The intermolecular associations found between FixJN protomers in these crystal structures involve two distinct regions, the $\beta 4$ - $\alpha 5$ and the $\beta 1$ - $\alpha 2$ - $\beta 2$ surfaces. Dimerization of FixJ via the $\beta 4$ - $\alpha 5$ region [13] and further oligomerization, possibly mediated by the $\beta 1$ - $\alpha 2$ - $\beta 2$ interface, would be in line with the protection of about 50 base pairs on the DNA promoter region of *fixK* [15].

Materials and methods

Crystallization and data collection

The FixJN protein (residues 1–126) was expressed from the pTF7-7 plasmid in the *E. coli* strain BL21(DE3)(pLysS). The protein was purified to homogeneity using chromatographic methods [13]. FixJN monomer in 20 mM Tris-HCl (pH 7.0), 0.1 mM EDTA, 1 mM dithiothreitol (DTT) was concentrated to 12 mg ml⁻¹ using Centricon 10. Crystals were grown at 4°C using the hanging-drop vapor-diffusion method.

Triclinic crystals were obtained by mixing the protein solution (2 μ l) with an equal volume of the reservoir solution, which contained 40% (w/v) polyethylene glycol (PEG) 1500 in 20 mM Tris buffer (pH 7.0). After three months, the crystal dimensions were about 150 \times 50 \times 20 μ m³. The crystals have unit-cell dimensions $a = 31.7$ Å, $b = 42.2$ Å, $c = 44.6$ Å, $\alpha = 93.2^\circ$, $\beta = 104.5^\circ$ and $\gamma = 101.9^\circ$, and contain two molecules per asymmetric unit for a solvent content of 30%. Monoclinic crystals were obtained by mixing the protein solution (2 μ l) with an equal

volume of the reservoir solution, which contained 40% w/v PEG 1500 in 100 mM Tris buffer (pH 7.8). After five months, the crystal dimensions were about 100 \times 20 \times 20 μ m³. The space group is C2 and the cell parameters are $a = 114.4$ Å, $b = 36.2$ Å, $c = 66.4$ Å, $\beta = 117.6^\circ$. There are two molecules in the asymmetric unit for a solvent content of 40%. Heavy-atom derivatives were obtained by soaking triclinic crystals in a 4 mM Sm(NO₃)₃ solution for 48 h, or in a 8 mM K₂PtCl₄ solution for 15 h (Table 2). Mn²⁺ and Mg²⁺ derivatives were obtained by soaking monoclinic crystals in 5 mM MnCl₂ for 24 h, and triclinic crystals in 20 mM MnCl₂ for 43 h or in 20 mM MgCl₂ for 23 h.

Triclinic and monoclinic crystals were cryocooled in liquid propane and stored at liquid-nitrogen temperature. The data sets were collected at 95K on synchrotron beam lines W32 at LURE (Orsay), and D2AM, ID14-EH3 and ID14-EH4 at ESRF (Grenoble). Data processing was performed using DENZO and SCALEPACK (Table 2) [46].

Structure determinations: triclinic forms

Attempts to solve the structure by molecular replacement using the coordinates of homologous domains (CheY, SpoOF, NtrC, NarL, CheB) as a model were unsuccessful. The native, Sm³⁺ and PtCl₄²⁻ data sets were used for phasing at 2.4 Å resolution. Four heavy-atom sites were located in each derivative by inspection of Patterson and Fourier difference maps. Phasing was performed using SHARP [47] and took into account the anomalous signal of the Sm³⁺ derivative (Table 2). The initial figure of merit was 0.52. Cycles of solvent flattening and histogram matching were performed with DM [48] using a solvent mask generated automatically. The position of the noncrystallographic twofold axis, detected by calculation of a self-rotation function using the program POLARRFN of the CCP4 suite [48], was determined from the improved phases using GETAX [49]. The real-space correlation coefficient (Cc) was 34% at 4 Å resolution. Subsequent cycles of density modification using noncrystallographic-symmetry averaging improved the value of Cc to 90% at 2.4 Å resolution. The initial atomic model was generated from the 2F_o-F_c electron-density map. This model was used to calculate a new mask using MAMA [50]. Further cycles of density modification led to a map of excellent quality in which a new model containing 112 residues was built.

Refinement was performed using data at the highest available resolution for each crystal species (Table 1). Refinement included cycles of conjugate gradient minimisation and molecular dynamics using CNS [51]. Bulk-solvent correction and noncrystallographic symmetry (NCS) constraints were applied. The cross-validation factor, R_{free}, was evaluated using 10% of the data set. NCS restraints were applied when the R and R_{free} values dropped to 28% and 32%, respectively. In the last stages of refinement, the two copies in the asymmetric unit were refined independently, solvent molecules were positioned and restrained individual B factors were calculated. For the native triclinic crystals, the final R and R_{free} values at 1.6 Å resolution were 18.7% and 22.4%, respectively (Table 1). In copy A of the asymmetric unit (named self-inhibited in Table 1), no electron density could be assigned for residues 13–17, and 57–59. The second copy, B (named canonical in Table 1), is fully defined from residues 1–125. A few sidechains had no electron density from the C $_{\alpha}$ positions (Glu13A, Val15A, Met57A) or from their C $_{\beta}$ positions (Gln34A, Arg67A, Glu64B and Arg67B). The sidechains of residues Met25A, Ser78A, Ser18B, Val50B, Ser78B and Ile102B have been modeled as two-state conformers. Residue 105 is a *cis*-proline. 148 water molecules and a hydroxycarbonated chain of 19 atoms have been positioned. This chain has been interpreted as a fragment of PEG 1500. The average temperature factors are 23 Å² for copy A, 20 Å² for copy B, 30 Å² for water molecules and 22 Å² for the PEG fragment. A Ramachandran plot generated with PROCHECK [52] locates 95% of the residues in the most-favored regions.

The structures of the PtCl₄²⁻, Sm³⁺, Mn²⁺ and Mg²⁺ derivatives of FixJN in triclinic crystals were refined as described above, using the native structure of the protein as starting model. In the PtCl₄²⁻

Table 2

Data collection and statistics.

Crystal form	Triclinic							Monoclinic	
Data	Native 1	Native 2	Merge	Sm(NO ₃) ₃	K ₂ PtCl ₄	MnCl ₂	MgCl ₂	Native	MnCl ₂
Resolution (Å)	2.4	1.6	1.6	2.4	1.9	2.0	2.0	2.9	3.0
Wavelength (Å)	0.93	0.95	-	0.93	0.93	0.95	0.95	1.07	0.95
Oscillation (step°)	180 (1)	247.5 (2.5)	-	300 (3)	180 (1)	172.5 (2.5)	240 (2.5)	180 (2.5)	196.5 (1.5)
No. of measurements	15,435	48,916	62,615	26,081	30,758	24,325	31,730	15,742	14,984
No. of uniques	8231	19,484	20,628	8265	15,980	13,062	13,586	5471	4760
Completeness (%)	98	66*	73*	98	94	87	88	97	97
I > 3σ(I) (%)	95	64	70	93	80	74	96	87	91
R _{sym} (%)	2.1	6.6	7.1†	5.0	4.0	5.1	8.7	7.7	6.0
R _{iso} (%)	-	-	-	27	29	30	25	-	47
R _{cullis} (iso/ano)	-	-	-	0.8/0.6	0.9/-	-	-	-	-
Power† (iso/ano)	-	-	-	1.5/1.8	1.1/-	-	-	-	-

R_{sym} = $\sum |I - \langle I \rangle| / \sum I$. *Completeness is 95% to 1.9 Å, †R_{merge}. †Phasing power. Unit-cell dimensions are a = 31.7 Å, b = 42.2 Å, c = 44.6 Å, α = 93.2°, β = 104.5°, γ = 101.9° for the triclinic form; and a = 114.4 Å, b = 36.2 Å, c = 66.4 Å, β = 117.6° for the monoclinic form.

derivative, the two copies in the asymmetric unit are identical and adopt the self-inhibited conformation (Table 1). The two molecules in the asymmetric units of the Sm³⁺, Mn²⁺ and Mg²⁺ derivatives are identical and adopt the canonical conformation (Table 1).

Structure determinations: monoclinic forms.

The structure of FixJN in the triclinic crystals was used to solve the structure of the protein in the native and in the Mn²⁺-soaked monoclinic crystals. A unique solution was found by molecular replacement using the program AMoRe [53] for two copies in the asymmetric unit. Refinement was conducted as described for the triclinic form but the NCS restraints (weight = 100 kcal mol⁻¹ Å⁻²) were kept throughout refinement because of the lower resolution limit. In native crystals, the two copies in the asymmetric unit are similar and are found in the self-inhibited conformation. In Mn²⁺-soaked crystals, the two copies have different conformations. One monomer adopts the canonical fold with a Mn²⁺ ion bound in the active site and the second monomer is found in a new conformation named 'atypical' (Table 1). The electron density for residues 13–17 is continuous but does not allow an accurate model building for the mainchain atoms. The average temperature factor, 59 Å², is high for this derivative, as was the value calculated from a Wilson plot (72 Å²).

Electron-density maps were displayed on a Silicon Graphics workstation using TURBO-FRODO [54]. Secondary structure assignments and solvent accessibilities were calculated with DSSP [55]. Atomic models were superposed using LSQKAB of the CCP4 suite [48].

Accession numbers

The coordinates of the self-inhibited, canonical and atypical structures have been deposited with the PDB with accession codes 1DBW, 1DCK and 1DCM, respectively.

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