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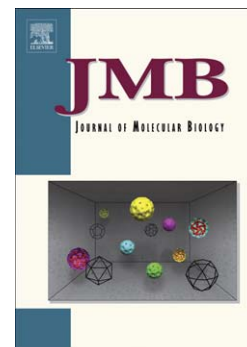
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## Snapshots of conformational changes shed light into the NtrX receiver domain signal transduction mechanism

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**Short title:** Mechanism of activation of NtrX receiver domain from *Brucella abortus*

**ABSTRACT**

*Brucella abortus* is an important pathogenic bacterium that has to overcome oxygen deficiency in order to achieve a successful infection. Previously, we proved that a two-component system formed by the histidine kinase NtrY and the response regulator NtrX is essential to achieve an adaptive response to low oxygen tension conditions. Even though the relevance of this signaling pathway has already been demonstrated in other microorganisms, its molecular activation mechanism has not yet been described in detail. In this article, we report the first crystal structures from different conformations of the NtrX receiver domain from *Brucella abortus*, and propose a sequence of events to explain the structural rearrangements along the activation process. The analysis of the structures obtained in the presence of the phosphoryl group analog beryllofluoride led us to postulate that changes in the interface formed by the  $\alpha 4$  helix and the  $\beta 5$  strand are important for the activation, producing a reorientation of the  $\alpha 5$  helix. Also, a biochemical characterization of the NtrX receiver domain enzymatic activities was performed, describing its autophosphorylation and autodephosphorylation kinetics. Finally, the role of H85, an important residue, was addressed by site-directed mutagenesis.

Overall, these results provide significant structural basis for understanding the response regulator activation in this bacterial two-component system.

**KEY WORDS**

*Brucella abortus*, REC domain, response regulator, two-component system, NtrY, X-ray crystallography

**INTRODUCTION**

Bacteria use two-component signal transduction pathways to sense environmental stimuli and generate adaptive responses<sup>1</sup>. These systems are formed by two conserved components, a histidine kinase (HK) and a response regulator (RR). The HK is typically the input component designed to sense stimuli and correspondingly regulate the signaling pathway. On the other side, the RR is the output of the system, regulated by the HK, and effecting the cellular response. External signals modulate the activity of the HK, which autophosphorylates at a particular histidine residue, creating a high-energy phosphoryl group that

is transferred to a conserved aspartate residue of the RR, inducing a conformational change that activates it.

The RR superfamily has a modular architecture and is defined by a conserved regulatory or receiver (REC) domain<sup>2</sup>, which participates in catalysis of phosphoryl transfer from the HK to itself and modulates the activity of an effector domain in a phosphorylation-dependent manner. Using the same active site, the REC domain has also enzymatic activity to promote its autophosphorylation using high-energy small molecule phosphodonors (such as acetyl phosphate, phosphoramidate, carbamyl phosphate, etc.)<sup>3, 4, 5</sup>. Despite the rate of this reaction is typically ~100-fold slower than those observed with cognate HKs as donors, most of the *in vitro* analysis of RR phosphorylation has been conducted with small phosphodonors. REC domains are also able to catalyze their autodephosphorylation. Acyl phosphates are inherently unstable, which hinders structural studies of RRs in their phosphorylated active forms. Given this handicap, several *in vitro* approaches have been developed to create stable phosphoryl group analogs, such as beryll fluoride, which forms a tightly bound complex with many RR proteins, mimicking the phosphorylated state<sup>6</sup>.

Our laboratory is interested in the signaling pathways involved in the virulence of *Brucella abortus*, an intracellular Gram-negative bacterium that causes abortion and sterility in domestic animals and undulant fever in humans<sup>7</sup>. Given the reported relevance of low oxygen tension during *Brucella* infection<sup>8, 9, 10</sup> we are particularly interested in two-component systems that could participate in an adaptive response to such environmental signal<sup>11, 12</sup>. In this regard, we described that the NtrY HK is activated upon reduction and is necessary for induction of adaptive pathways to cope with low oxygen availability. We also demonstrated that after NtrY autophosphorylation, the phosphoryl group is transferred to NtrX, its cognate response regulator<sup>11</sup>. Using genetic approaches, orthologues of the NtrY/X two-component system have been studied in other microorganisms and have been involved in nitrogen fixation and metabolism in *Azorhizobium caulinodans* (where the system was described for the first time)<sup>13</sup>; inhibition of lysosomal fusion in *Ehrlichia caffeensis*<sup>14</sup>; expression of respiratory enzymes in the oxidase-positive proteobacteria *Neisseria gonorrhoeae*<sup>15</sup>; and succinoglycan production, motility, and symbiotic nodulation in *Sinorhizobium meliloti*<sup>16</sup>. Noteworthy, the *ntrX* gene was reported to be conditionally essential in *Caulobacter crescentus*<sup>17</sup>.

NtrX comprises three domains: (1) an N-terminal REC domain (residues 1-135), (2) a central AAA+ domain (residues 147-370) and (3) a C-terminal helix-turn-helix DNA binding domain (residues 407-450) (Fig. 1a). This organization and sequence similarity classifies NtrX among the members of the NtrC subfamily. Many RRs from this group can act as bacterial enhancer binding proteins (bEBP), that is, transcriptional factors that hexamerize and regulate  $\sigma^{54}$  promoters (for example NtrC, NtrC1, NtrC4 DctD, ZraR) <sup>18</sup>. Sequence analysis shows that NtrX has a major difference with typical NtrC subfamily members: it lacks a GAFTA motif which is considered a bEBP-specific structural element. Some unusual variants of the bEBP subfamily with an absent GAFTA motif have been reported (*Rhodobacter capsulatus* NtrC, HupR) and regulate transcription at  $\sigma^{70}$  promoters <sup>19, 20</sup>. Even though it has not been clearly established which family of promoters is regulated by NtrX, the interaction of this RR with the *putA* and *glnA* promoters was reported in *E. chaffensis*, a microorganism that encodes only  $\sigma^{70}$  and  $\sigma^{32}$  factors <sup>21</sup>. Since no homologue of NtrX has been previously characterized in detail, we decided to study the structural changes in its receiver domain upon phosphorylation in order to gain insight into the activation mechanism of this RR. Here, we report the first crystal structure of the NtrX receiver domain in its nonphosphorylated state and when bound to beryll fluoride. The models indicate that phosphorylation leads to characteristic conformational changes and tightens an interface formed by the  $\alpha 4$  helix and the  $\beta 5$  strand, promoting dimerization of the REC domain. Furthermore, it was possible to crystallize the native REC domain in three conformations, one of which resembles the active state despite the absence of a phosphate analog. We also established the importance of the H85 residue in different catalytic activities of the receiver domain. Altogether, our results provide structural and biochemical information that contributes to understanding how phosphorylation at the REC domain is transduced to the other domains of NtrX and represent the first steps to comprehend the molecular activation mechanism of this RR.

## **RESULTS**

### **Crystal structure of the NtrX REC domain. Overall description**

In order to examine the structural changes linked to the inactive and activated states in the NtrX REC domain, we performed crystallization experiments on a construct encompassing residues 1-126, lacking the last 9 amino acids of the predicted  $\alpha 5$  helix (residues 109-135). We decided to work with a construct bearing a truncated  $\alpha 5$  helix to increase the chances of crystallization, since structural evidence noted in other RR domains showed the projection of this helix outside the protein globular fold (HupR, DctD, NtrC1)<sup>22; 23; 24</sup>. We solved the three-dimensional structures of this construct in its native state (REC-N), and bound to the phosphorylation mimic  $\text{BeF}_3^-$  plus  $\text{Mg}^{2+}$  (REC-A). REC-N and REC-A crystal structures were solved at medium-high resolutions using the molecular replacement method in the orthorhombic ( $P2_12_12_1$ ) and triclinic ( $P1$ ) space groups, respectively (Fig. 1, and Table 1). The good resolution and the reduced noise in the 2mFo-DFc electron density maps allowed tracing the complete protein backbone with no chain breaks in both crystal forms. The final refined models show very good stereochemistry, with more than 98.5% of the residues in the favored region of the Ramachandran plot (Table 1).

The NtrX REC domain consists of a five-stranded parallel  $\beta$ -sheet surrounded by five  $\alpha$ -helices, forming the canonical  $(\beta\alpha)_5$  topology, as found in other structurally solved homologous domains<sup>25</sup> (Fig. 1b). The secondary structure elements are comprised of the residues  $\beta 1$  (5-8),  $\alpha 1$  (12-24),  $\beta 2$  (29-32),  $\alpha 2$  (35-44),  $\beta 3$  (49-52),  $\alpha 3$  (63-73),  $\beta 4$  (79-83),  $\alpha 4$  (88-96),  $\beta 5$  (101-104) and  $\alpha 5$  (109-123). All structural states have a similar global conformation (RMSD < 0.91 Å, calculated between the  $\alpha$ -carbons of matched residues), with overall dimensions of 28 Å x 25 Å x 31 Å, and a solvent accessible surface area of approximately 6,300 Å<sup>2</sup>.

The REC-N crystal arrangement bears four molecules in the asymmetric unit (A, B, C, D; with a highest RMSD value of 0.90 Å between them), with noteworthy differences at the  $\beta 3$ - $\alpha 3$  and  $\beta 5$ - $\alpha 5$  loops, where  $A=B=C\neq D$ , and at the loop  $\beta 4$ - $\alpha 4$  along with the orientation of the helix  $\alpha 4$ , where  $A=B\neq C=D$  (as described below in detail and Figs. 1c, 1e and Sl. 1a). On the other hand, REC-A shows two identical molecules per asymmetric unit (E, F; with a marginal RMSD value of 0.06 Å) (Sl. 1b), which remarkably exhibit a similar conformation with the REC-N molecules C (RMSD = 0.43 Å), and D (RMSD = 0.67 Å, mainly due to differences at the  $\beta 5$ - $\alpha 5$  loop) (Fig. 1c).

A PDBeFold server search<sup>26</sup> revealed that the best fits correspond to the RRs from the *Streptococcus pneumoniae* YycF homologue (PDB codes 2A90, 2A9P and 2A9R, unpublished results), *Escherichia coli*

ArcA (PDB codes 1XHF and 1XHE)<sup>27</sup>, *E. coli* PhoB (PDB code 1ZES)<sup>28</sup>, among others. The number of aligned residues of REC-N and REC-A with these proteins ranges from 116 to 119, the RMSD ranges from 0.9 to 1.5 Å, and the sequence identity is below 32%.

### **The native state of the NtrX receiver domain consists of an ensemble of different conformers**

The crystal structure of REC-N shows three conformers, with the main differences localized in loops surrounding the phosphorylation site. Specific open-closed states for the  $\beta 3$ - $\alpha 3$  and  $\beta 4$ - $\alpha 4$  loops are noted, according to their orientation (pointing away from the globular fold or partially covering the active site, respectively) (Figs. 1c, 1e). The molecules A and B from REC-N display their  $\beta 3$ - $\alpha 3$  and  $\beta 4$ - $\alpha 4$  loops in open-open states where S83 and F102 (from  $\beta 4$  and  $\beta 5$ , respectively) are pointing away of the active site (Fig. 1d), a characteristic conformation of response regulators in their inactive state<sup>2; 25</sup>. The molecule C from REC-N shows its  $\beta 3$ - $\alpha 3$  and  $\beta 4$ - $\alpha 4$  loops in open-closed states, and the molecule D has these loops in a closed-closed configuration (Figs. 1c, 1e). In both cases, the S83 and F102 side chains are turned towards the active site (Fig. 1d). A rearrangement on loop  $\beta 4$ - $\alpha 4$  gives rise to a slight twist into strand  $\beta 4$  (clockwise from the N-terminal point of reference) and, consequently, a displacement of helix  $\alpha 4$  (see description below).

Molecules C and D from REC-N remarkably mimic REC-A in terms of the position of the  $\beta 4$ - $\alpha 4$  loop, orientation of helix  $\alpha 4$ , and the inward configuration of S83 and F102 (RMSD = 0.43 Å) (as described below and Fig. 1c, 1d). Based on these observations, the molecules A and B from REC-N may correspond to an inactive state, whereas molecules C and D may represent a meta-active conformation (a conformation that resembles the active state despite the absence of a phosphate analog), as has been already reported for CheY and DesR<sup>29; 30</sup>.

### **Activation of the receiver domain involves conformational changes at the $\beta 4$ - $\alpha 4$ loop and reorientation of the $\alpha 4$ helix.**

Analysis of REC-A shows that its active site, formed by conserved residues important for the phosphorylation process, shares a similar architecture with other RR domains<sup>25</sup>. The active site is located at the C-terminal region of the parallel  $\beta 1$ ,  $\beta 3$  and  $\beta 4$  strands, and is partially covered by the loops  $\beta 1$ - $\alpha 1$ ,

$\beta 3\text{-}\alpha 3$ , and  $\beta 4\text{-}\alpha 4$  (Fig. 2a). Here, a crucial aspartate residue (D53) lying down at the C-terminal end of  $\beta 3$  is found to be responsible for making a nucleophilic attack on the beryllium atom of  $\text{BeF}_3^-$ . This residue is stabilized through a salt-bridge interaction between K105 and one  $\text{Mg}^{2+}$  ion. The side chains from residues D9, D10 ( $\beta 1\text{-}\alpha 1$  loop), W55 ( $\beta 3\text{-}\alpha 3$  loop) and H85 ( $\beta 4\text{-}\alpha 4$  loop) encircle the pocket on its top. The  $\text{BeF}_3^-$  ligand forms an acyl phosphate analogue with the carboxylate of D53 from the active site (Fig. 2a). The three fluoride ions are stabilized by interactions with S83, K105 and main chain nitrogen atoms of I54, W55, G84, the  $\text{Mg}^{2+}$  ion and a water molecule. In addition, a van der Waals contact with the H85 side chain is observed.

When comparing the structures of the inactive and active states (molecules A vs. E, respectively), significant changes within the active site are noted (Fig. 2b). In the active state, the  $\beta 4\text{-}\alpha 4$  loop is reoriented towards the  $\beta 3\text{-}\alpha 3$  loop (around 6.0 Å displacement), which creates a  $\pi$ -stacking interaction between the side chains of W55 and H85 (which are 12.6 Å away from each other in the native state). This is accomplished by the formation of a new interaction between the D53 and K105 side chains (from 4.3 Å in the native state down to 3.3 Å in the active state). This is a consequence of two new salt-bridge interactions between the donor atom N $\zeta$  of K105 and the acceptor atoms O $\delta 1$  of D9, and a fluorine atom of the  $\text{BeF}_3^-$  ligand. In addition, the S83 and F102 side chains make a flip, pointing now towards the active site (Fig. 1d and Fig. 2b), allowing the interaction between the serine side chain and a fluoride ion, as well as the burying of the phenylalanine. Those changes lead to a movement of the N-terminal region of the  $\alpha 4$  helix, which protrudes 2.3 Å outside the protein globular fold (Fig. 2b). It is important to state that loops  $\beta 3\text{-}\alpha 3$  and  $\beta 4\text{-}\alpha 4$ , despite being exposed on the protein surface, are not involved in major intermolecular interactions.

### **Active NtrX REC domains exhibit a more extensive interface between monomers**

The structural analysis of REC-N reveals a dimeric interface formed by the  $\alpha 4$  helix and  $\beta 5$  strand of combined monomers (Fig. 3). This interface can adopt two possible conformations, A-B and C-D. The first one has a buried surface area of 431 Å<sup>2</sup> and is observed between molecules from the same asymmetric unit, but the C-D conformer is assembled by chains belonging to adjacent asymmetric units with a buried surface area of 546 Å<sup>2</sup> (Fig. SI 2). In all cases, the two monomers forming the dimer are positioned in the



same orientation. The mentioned interface involves mainly a salt bridge interaction between R96 (N-terminus of the  $\beta 5$  strand) and D101 (C-terminus of the  $\alpha 4$  helix) and hydrophobic interactions between I95 from both monomers (Fig. 3a, left). The presence of F102 pointing away from the protein core prevents the assembly of a tighter interface (Fig. 3b). Noteworthy, the C-D dimer interface observed in REC-N shows an identical orientation and interaction in comparison with the interface noticed in E-F conformers from REC-A (Fig. SI 2).

As mentioned before, in REC-A the helix  $\alpha 4$  protrudes outside the protein globular fold, allowing for a more extensive interface in the activated state achieved by additional hydrophobic residues, such as I88, A91 and V92 from this helix (Fig. 3a, right). The salt bridge interaction between R96 and D101' is preserved, as well as the hydrophobic contacts between I95-I95' (Fig. 3a). In conclusion, despite that the secondary structural elements involved in this interface are the same as those in REC-N, the comparison between the dimer interface from the A-B conformers in REC-N and E-F from REC-A reveals a distorted area, where the rearrangement into helix  $\alpha 4$  orientation (Fig. 3b, bottom) causes a greater buried surface area ( $600 \text{ \AA}^2$ ) (Fig 3a, top).

### **The NtrX REC domain is under monomer-dimer equilibrium and phosphorylation stabilizes the dimeric form**

It has been well established that small molecule phosphodonors such as acetyl phosphate (AcP) and phosphoramidate represent valuable tools to generate a phosphorylated response regulator *in vitro*<sup>31</sup>. Moreover, in RRs that bear a tryptophan residue close to the phosphorylatable aspartate (such as W55 in NtrX), the phosphorylation reaction can be studied using fluorescence spectroscopy<sup>31</sup>.

A recent report used this approach to propose a model in which the phosphorylation of PhoB is coupled to its dimerization<sup>32</sup> by the formation of a heterodimer between a phosphorylated and a non-phosphorylated monomer. Also, allosteric coupling between dimerization and phosphorylation has been proved for the DesR RR<sup>30</sup>. Then, since autophosphorylation kinetics might provide a functional tool to assess the quaternary structure of the protein, we decided to study the phosphorylation of the NtrX REC domain using AcP. To this end, different concentrations of the receiver domain were incubated with a fixed amount of AcP and  $\text{Mg}^{2+}$ , and the changes in fluorescence intensity were recorded during a certain

period of time to determine the fluorescence quenching of W55. Incubation with AcP leads to a time-dependent fluorescence quenching (Fig. SI 3), indicating that the reaction with the small phosphodonor occurs and showing that the reaction rate is dependent on the protein concentration. This is consistent with the proposed model for PhoB since higher concentration of the REC domain would favor the formation of the heterodimer, whose dimerization constant has been proved to be in the same range as the protein concentration used in our assays<sup>32</sup>.

We also performed kinetic experiments with a fixed concentration of the REC domain (10  $\mu$ M) and increasing amounts of AcP. The fluorescence curves were fitted to a single exponential decay function and the derived constants were plotted against the concentration of AcP. The results indicate that the autophosphorylation rate varies sigmoidally with the concentration of phosphodonor (Fig. 4a). This apparent positive cooperativity indicated communication between NtrX REC active sites, such that autophosphorylation of one active site led to enhanced autophosphorylation kinetics at other active sites. Altogether, this result, along with the protein concentration dependence, is in agreement with the dimerization of the REC domain associated with its autophosphorylation, as has been described for PhoB<sup>32</sup>.

To directly address the oligomeric state of the REC domain in solution, we performed size exclusion chromatography coupled to static light scattering (SEC-SLS). The chromatograms show that the elution volume was smaller for increasing concentrations of protein (Fig. 4b and Fig. SI 4). The molecular weight (MW) estimated in each case (Table 2) depend on the REC domain concentration, being higher when the protein is more concentrated. For example, when injecting the protein at 500  $\mu$ M into the column, the MW obtained was 25.3 kDa, which is between that expected for a monomer (16 kDa) and a dimer (32 kDa). This indicates that the REC domain is under a rapid monomer-dimer equilibrium that depends on protein concentration and that in these conditions the SEC is not able to resolve both populations.

When the REC domain was preincubated with AcP, the observed peak shifted to lower elution volumes and the estimated MW agrees better with that expected for the dimeric form (Table 2). This indicates that phosphorylation stabilizes the dimer of the REC domain, which is in agreement with the more extensive interface observed in the crystallographic structures with beryll fluoride. At low protein concentration (4  $\mu$ M) the MW of the phosphorylated REC was 27 kDa (Table 2) and showed a marked tailing profile. This

can be explained by an incomplete phosphorylation since, as we reported before, the phosphorylation reaction depends on protein concentration.

### **H85 influences the rate of autophosphorylation with AcP, the phosphotransfer efficiency and determines the half-life of the activated REC domain**

Previous reports on CheY have demonstrated that its residue E89 (equivalent to H85 in NtrX) modulates the autophosphorylation kinetics, phosphodonor preference<sup>33</sup> and dephosphorylation rate<sup>34</sup>. In the NtrX REC domain structures, H85 is located in the  $\beta$ 4- $\alpha$ 4 loop (the region with the highest structural plasticity in the transition from the inactive to active states) and interacts with W55 in the active conformation (Fig. 2b). Those observations led us to investigate the role of H85 and for this purpose we decided to perform site-directed mutagenesis experiments in which we replaced this amino acid with alanine (REC-H85A). Using fluorescence spectroscopy, we determined the kinetics of phosphorylation of REC-H85A with AcP. The mutant domain is severely impaired to autophosphorylate, as can be deduced from the markedly reduced rate constants obtained at different AcP concentrations when compared to those obtained for REC-WT (Table 3).

Given that result, and taking into consideration that autophosphorylation with small molecule phosphodonors proceed through the same chemistry that the phosphotransfer reaction<sup>2</sup>, we aimed to determine whether H85 could be important for the efficiency of phosphoryl transfer using the NtrY phosphohistidine as a substrate. We performed phosphotransfer experiments from phosphorylated NtrY<sup>11</sup> to either REC-WT or REC-H85A and found that the mutant domain is phosphorylated to a lesser extent than the wild type (Fig. 5a). Despite this result was obtained in several independent experiments, the differences were subtle in comparison with the severe impairment in autophosphorylation with AcP. In consequence, we decided to perform further experiments adding a molar equivalent of full-length NtrX, which has a wild type receiver domain to compete for the phosphohistidine substrate. As expected, in the competition experiment approximately 50% of the radioactive phosphate was detected in NtrX and the other 50% in REC-WT (Fig. 5b). However, when the assay was performed using REC-H85A there was a marked preference towards NtrX, with approximately 80% of the label incorporated to the full length protein (Fig. 5b). This indicates that H85 is involved in the phosphotransfer reaction and that mutation of

this residue affects the ability of the REC domain to phosphorylate using the phosphohistidine as a substrate, generating a kinetic disadvantage during the competition experiments.

We also established the autodephosphorylation rate constant of wild type and mutated REC-P domains. Fig. 5c shows a representative experiment in which it is evident that the amount of phosphorylated REC-H85A is considerably lower at the beginning of the experiment (time 0) compared to REC-WT, confirming the phosphorylation defect in the mutant protein. Dephosphorylation rate constants were obtained from plots of phosphorylated fraction against time (Fig. 5d) and confirmed that the REC-WT domain dephosphorylates significantly more slowly ( $k_{deph}= 0,006 \text{ min}^{-1}$ ) than the REC-H85A mutant ( $k_{deph}= 0,018 \text{ min}^{-1}$ ). The rate constant value for NtrX REC domain is comparable to those of FixJ<sup>35</sup> and DctD<sup>23</sup> (0,005 and 0,007  $\text{min}^{-1}$ , respectively), both of which have a histidine residue at position 85.

To determine whether H85 influences the oligomeric state of the NtrX REC domain, we performed SEC-SLS experiments. The elution profile of REC-H85A (Fig. SI 5a) indicates that in the inactive state the protein is in monomer-dimer equilibrium, with estimated MWs close to those of REC-WT (Table 2). This indicates that the substitution of the histidine residue does not influence significantly the quaternary structure in the inactive state. When REC-H85A was phosphorylated with AcP, the domain eluted in two peaks (Fig. SI 5b) that were not completely resolved. As the concentration of the mutant domain increased, the MW corresponding to the first peak agreed better with that expected for a dimer (Table 2) and the second peak eluted in a lower volume with higher estimated MW. These results are consistent with partial phosphorylation of REC-H85A and indicate that despite the mutation, REC-H85A is still able to dimerize.

## **DISCUSSION**

The prevailing conceptual framework for receiver domain activation has been that the protein exists in a dynamic equilibrium that accesses both an inactive and active conformations<sup>36</sup>. Both states are populated in unphosphorylated REC domains, and phosphorylation shifts the equilibrium towards the active species. However, several crystal structures of the CheY response regulator show intermediate configurations<sup>29, 37, 38</sup> and recent NMR data supported a model of segmental motions underlying the activation of CheY, instead of a preexisting, concerted, two-state switch<sup>39</sup>. In the middle of this conceptual

landscape, the structures of the isolated NtrX receiver domain argues against a simple equilibrium between inactive and active conformations, since it was possible to crystallize “meta-active” conformations in the absence of the phosphorylation mimic  $\text{BeF}_3^-$ . Even more, among those active-like species, different positions in the  $\beta 3$ - $\alpha 3$  loop were observed, accounting for conformational diversity in the native state. Therefore, our results support that phosphorylation stabilizes a pre-existing fraction of the REC domain population that samples the active conformation (rather than a phosphorylation-induced conformational change) which was achieved through segmental motions of different residues.

The crystallographic structures presented in this work allow us to postulate a possible conformational pathway to comprehend the allosteric changes of NtrX REC domain in which the  $\beta 3$ - $\alpha 3$  loop plays a central role (Fig. 6a). Most of the RRs have a proline residue that generates a kink at this loop. However, that position is occupied by a glutamine (Q57) in NtrX and the loop is extended by two amino acids (S59 and R60). NMR studies on NtrC REC domain<sup>40</sup> and molecular dynamics simulations on CheY have suggested that the  $\beta 3$ - $\alpha 3$  loop is flexible<sup>41</sup>, and in fact two conformations were found in our REC-N structures. The conformational pathway that we propose starts from a ground state with both loops  $\beta 3$ - $\alpha 3$  and  $\beta 4$ - $\alpha 4$  opened (inactive state, molecules A and B from REC-N). Fluctuations in the  $\beta 3$ - $\alpha 3$  loop position would lead to a closed state in which R60 is able to interact with backbone atoms from the  $\beta 4$ - $\alpha 4$  loop (N87 and H85), stabilizing the closed conformation of both loops (molecule D from REC-N). This conformation also presents the  $\beta 5$ - $\alpha 5$  loop in an altered position with K105 pointing away of the protein core and interacting with the E104 side chain, providing space for the displacement of the  $\beta 4$ - $\alpha 4$  loop. Also, E104 interacts with S83, stabilizing the inward orientation of this key residue, which must flip in order to achieve an active state. Therefore, the molecule D from REC-N might represent an intermediary conformation where the closed state of loop  $\beta 3$ - $\alpha 3$  contributes to an initial closure of the  $\beta 4$ - $\alpha 4$  loop, which is also allowed by the movement of the loop  $\beta 5$ - $\alpha 5$ . Subsequently, the loop  $\beta 4$ - $\alpha 4$  adopts an even more closed position and its conformation might be maintained by the interaction between W55 and H85, allowing the loop  $\beta 3$ - $\alpha 3$  to return to an opened conformation (molecule C from REC-N) sustained by the interaction between R60 and D36 (from  $\alpha 2$ ). In this state, K105 adopts a buried location and interacts with D53. Finally, the binding of beryllofluoride stabilizes this conformation through the interaction of the ligand with S83 and K105 and the parallel  $\pi$  stacking between W55 and H85. The proposed sequence of

conformational transitions is supported by the fact that among the meta-active structures reported, molecule C is the one that best resembles the active state, leaving conformation D as an intermediate state. Also, a previous article postulated that non-native interactions are used to “hold on” to the free energy until the final new native contacts of the active state are built<sup>42</sup> and the analyzed interactions (Fig. 6a) might account for this role.

Even though the same secondary structure elements are involved in REC-N and REC-A dimer interfaces, the binding of beryll fluoride favors the displacement of the  $\alpha 4$  helix to a position closer to the  $\beta 5$  strand from the opposite monomer. Also, an activated interface is created by opening a hydrophobic pocket at the N-terminal end of helix  $\alpha 4$  and strand  $\beta 5$  with a specific T-G-X-G-X-Hyd-X3-Hyd-X2-Hyd motif that is frequently present in this assembly (Hyd: I88, V92, I95 in NtrX)<sup>24</sup>. Those changes lead to a more extensive interface (the buried surface area increases from 431 Å<sup>2</sup> in REC-N to 600 Å<sup>2</sup> in REC-A) that stabilizes the dimeric form of the receiver domain, as confirmed by the SEC-SLS experiments. Preliminary experiments indicate that non phosphorylated full length NtrX is a stable dimer in solution, therefore the dimerization of the REC domain does not provide a mechanism for transmitting the phosphorylation signal to the rest of the NtrX molecule. The fact that the receiver domain is in monomer-dimer equilibrium in the native state might indicate that the dimerization of full length NtrX depends on interactions between the remaining domains. A similar behavior was observed in NtrC4 from *Aquifex aeolicus*, which is a dimer in solution but its isolated REC domain is a monomer<sup>43</sup> as a consequence of using truncated domains rather than the full length protein.

Superposition of dimeric structures from REC-N and REC-A indicates that the relative orientations of the  $\alpha 5$  helices change drastically upon activation (Fig. 3b). Members of the NtrC subfamily of response regulators usually have an extended  $\alpha 5$  helix and NtrX is not an exception, since secondary structure predictions indicate that this helix holds approximately 26 residues (in contrast to FixJ's which has 15 amino acids). To gain insight into the changes that could take place in the full-length  $\alpha 5$  helix, we traced the potential projection of this helix by molecular modelling of the dimeric structures of REC-N and REC-A (Fig. 6b). In this model it is possible to observe that those helices from opposite monomers do not interact (then, they cannot form a coiled coil) and the superposition also indicates that in REC-N those helical segments are pointed away, whereas in the active form they point to a similar direction. Therefore, the

projection of the  $\alpha 5$  helices allows us to postulate that the changes in the active-state dimer interface would lead to a relative reorientation of these helices, which might contribute to the signal transduction towards the neighboring AAA+ domain. A previous article<sup>43</sup> analyzed the sequences of linker regions of NtrC-like RRs whose REC domains, as NtrX, contain the GHG motif. The authors found that 12% have a predicted coiled coil (like NtrC1 and DctD), 22% have a predicted unstructured linker (NtrC4) and the majority (65.5%) have variable amounts of helical structure but are not predicted to form a coiled coil. NtrX belongs to the latter group and the proposed reorientation of  $\alpha 5$  helices as a transduction mechanism might be extended to a majority of proteins within this subfamily.

The activity of NtrC-like proteins is tightly regulated by either one of two postulated mechanisms<sup>18</sup>: (1) positive regulation, as seen in NtrC of enteric bacteria, in which phosphorylation of the receiver domain brings about a new interaction with the central domain, leading to the assembly of an active oligomer; (2) negative regulation, as seen in DctD and NtrC1, where the phosphorylation of the receiver domain leads to rearrangements that release the AAA+ domain so they can assemble an oligomer. A hint on the possible mechanism of regulation can be obtained by studying the isolated receiver domain of a response regulator because it has been proved that the NtrC REC domain is a monomer in solution despite its phosphorylation status, whereas DctD and NtrC1 receiver domains are dimers in solution and phosphorylation selects an alternative dimeric state. We proved that the NtrX REC domain is under monomer-dimer equilibrium in solution and phosphorylation promotes dimerization, as has been reported for the HupR receiver domain<sup>22</sup>. HupR is another unusual response regulator among the NtrC-like family that lacks a functional GAFTGA motif, as does NtrX<sup>20</sup>. Therefore, this behavior of the isolated REC domains from NtrX and HupR in solution is neither consistent with the described positive regulation mechanism (NtrC) nor with the negative one (DctD or NtrC1) and may reflect an alternative activation mechanism valid for atypical members among the NtrC family that are not proper activators of  $\sigma^{54}$  promoters.

Our work provides, for the first time, insights into the activation mechanism of the NtrX response regulator. We conclude that phosphorylation selects pre-existing dimeric states, which would lead to signal transduction from the REC domain to the rest of the molecule. Also, the different conformations observed in the crystals allowed us to postulate a pathway to comprehend the allosteric activation of RRs.

Finally, by means of a mutant construct we could identify H85 as an important residue for different catalytic activities performed by this REC domain. Even though further studies are required to evaluate possible changes in the oligomeric state of full length NtrX and the relevance of different residues in the activation of the NtrY/X pathway *in vivo*, our work is an important contribution to understand the NtrY/X signaling system.

## **MATERIALS AND METHODS**

### **DNA manipulations**

DNA manipulations were performed according to standard techniques. DNA encoding NtrX REC domain was amplified by PCR from the existing pET24d-NtrX construct<sup>11</sup> using the primers 5'-AAGGATCCATGGCGGCCGATATTCTTGTGT-3' and 5'-TTCTCGAGCTTGAGCTTGGAGGTTTCCAGC-3'. The purified product was inserted into the pET24a vector (Novagen) using the restriction enzymes BamHI and XhoI. The final pET24a-REC(NtrX) construct, the quality of which was checked by DNA sequencing, includes the coding region of the REC domain (residues 1-126), ten additional N-terminal residues (MASMTGGQQMGRGS) from the cloning procedure, two residues (LE) after the coding region and a six-residue long C-terminal histidine tag.

The plasmid encoding the mutant protein REC-H85A was obtained from pET24a-REC(NtrX) by PCR amplification using the primers 5'-GTGATGATTTCCGGTGCTGGCAATATCGAAACCG-3' and 5'-CGGTTTCGATATTGCCAGCACCGGAAATCATCAC-3', followed by digestion with the enzyme DpnI. All products are under control of the T7 promoter of the vector.

The plasmids encoding NtrY (soluble construct encompassing the PAS and histidine kinase domain, pET24d-PAS-HK-NtrY) and NtrX (full length, pET24d-NtrX) were prepared before<sup>11</sup>.

### **Protein overexpression and purification**

BL21(DE3)pLys cells were transformed with pET24a-REC or pET24a-REC-H85A and grown at 37 °C in 1 l of LB media with the appropriate antibiotic until absorbance (at 600 nm) reached 0.6. At this point,  $\beta$ -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the culture was



incubated for another 16 h at 28 °C with agitation. The bacteria were centrifuged at 7000 rpm for 10 minutes at 4 °C. Pellets were resuspended in lysis buffer [20 mM Tris-HCl, 200 mM NaCl, 30 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH=8], sonicated and then centrifuged at 45000 rpm in a Beckman Coulter L7-65 ultracentrifuge for 45 minutes at 4 °C. The supernatant was loaded into a HisTrap HP column (all columns were from GE Healthcare) in a high performance liquid chromatography apparatus (GE). Elution was performed with a linear gradient of buffer B [20 mM Tris-HCl, 200 mM NaCl, 500 mM imidazole, pH=8]. A major peak was observed at around 20% buffer B. The appropriate fractions were pooled and further purified by gel filtration chromatography on a Superdex 75 column with isocratic elution in Buffer I [20 mM Tris-HCl, 200 mM NaCl, pH=8], yielding a major peak at around 70 ml. For fluorescence, light scattering and activity experiments, a fraction of the total volume collected was directly concentrated by centrifugation in Amicon Ultra-4 devices (Millipore) and stored at -70 °C, while the rest was concentrated and simultaneously exchanged into crystallization buffer [10 mM Tris-HCl, 25 mM NaCl, pH=7.5] and finally stored at -70 °C.

PAS-HK (NtrY) and NtrX were expressed and purified as reported previously <sup>11</sup>.

### Crystallization

Initial crystallization trials were made on 96-well plates using a Honeybee 963 robot (Digilab, Marlborough, MA, USA) and commercial crystallization kits from Jena Bioscience (Jena, Germany) and Hampton Research (Aliso Viejo, CA, USA). Optimized REC-N crystals were obtained by the hanging drop method by mixing 2 µl of the concentrated protein stock with an equal amount of a crystallization solution consisting of 22% (w/v) PEG 550 monomethyl ether, 0.15 M malic acid and 0.1 M imidazole (pH=7). Bar-shaped crystals with an approximate size of 0.1 mm x 0.1 mm x 0.3 mm appeared after three days of equilibration. For REC-A, freshly prepared 10X BeF<sub>3</sub><sup>-</sup> solution (50 mM BeCl<sub>2</sub>, 300 mM NaF and 50 mM MgCl<sub>2</sub>) was mixed with the protein solution in 1:10 proportion and then this sample was used for the crystallization trials. The best crystals grew within three days with the same method and volumes using 22% (w/v) PEG 400 and 0.1 M MES (pH=6.9) as precipitating agent, showing a significant smaller size (0.05 mm x 0.1 mm x 0.1 mm). Samples were cryoprotected in their respective mother liquors added with PEG 400 and then cryocooled in liquid nitrogen.

### **X-ray data collection and processing**

Native datasets were collected at 100 K on single crystals at the PROXIMA 1 protein crystallography beamline at the SOLEIL Synchrotron (France), which is equipped with a PILATUS 6M detector (Dectris, Baden, Switzerland). X-ray diffraction data were processed with XDS<sup>44</sup> running in a semi-automated mode using the xdsme package (<http://code.google.com/p/xdsme/>). 5% of the reflections were set aside for cross validation. Detailed information on data collection parameters and processing statistics are presented in Table 1.

### **Structure resolution, model building and refinement**

The REC-N structure was solved by the molecular replacement method using the program Mr. Bump<sup>45</sup> from the CCP4 package<sup>46</sup>. The search model corresponded to the receiver domain of the *Sinorhizobium meliloti* DctD response regulator (PDB code 1L5Z;<sup>47</sup>). The procedure included automatic trimming of the search probe with Sculptor<sup>48</sup>, molecular replacement search with Phaser<sup>49</sup> and initial model building with Buccaneer<sup>50</sup>. A total of four copies of the molecule were successfully located in the asymmetric unit. Manual model building and refinement were performed with Coot<sup>51</sup> and Buster<sup>52</sup>, respectively. REC-A was then solved by molecular replacement using REC-N as model, with two copies in the asymmetric unit. Detailed statistics on the refinement process and PDB deposition are found in Table 1.

### **Validation and analysis of the models**

Both crystallographic models were validated with MolProbity<sup>53</sup>. The interface areas were obtained with PDBePISA<sup>26</sup> and the superpositions and RMSD calculations were performed with PDBeFold<sup>54</sup>. Sequence alignments were done with ClustalW2<sup>55</sup>. Figures were prepared using PyMOL (Schrödinger, New York, NY, USA).

### **Autophosphorylation with AcP followed by fluorescence spectroscopy**

All fluorescence measurements were made using a Jasco FP-6500 spectrofluorimeter. Tryptophan fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 351 nm. Sample temperature was held at 25 °C by a circulating water bath.

Autophosphorylation reactions of REC-WT and REC-H85A were carried out in quartz cuvettes with manual mixing to initiate the reaction, and tryptophan fluorescence was continuously monitored. The beginning of the time traces was at 20 s after mixing. To establish protein concentration dependence, different concentration of REC domain (3, 5, 10, 20, 30  $\mu$ M) were prepared [20 mM Tris-HCl, 200 mM NaCl, pH=8], separately incubated with 50 mM AcP and fluorescence recording began when 50 mM  $MgCl_2$  was added to the cuvette. To determine the influence of AcP concentration, the REC domain was diluted [20 mM Tris-HCl, 200 mM NaCl, pH=8] to a final concentration of 10  $\mu$ M, incubated in different reactions with increasing amounts of the phosphodonor (5, 10, 20, 30, 40, 50, 60, 80  $\mu$ M for REC-WT; 10, 20, 40  $\mu$ M for REC-H85A) and fluorescence recording began when 20 mM  $MgCl_2$  was added to the cuvette. Reactions were performed in triplicate for each experimental condition.

### **Analysis of kinetic data**

Autophosphorylation reactions were performed under pseudo-first order conditions, with a large molar excess of AcP over protein and were analyzed as reported elsewhere<sup>32</sup>. All time traces were normalized by dividing the fluorescence intensities by the initial fluorescence intensity to generate relative fluorescence intensity (RFI). Initial curve fits were made to a single exponential decay (GraphPad Prism).  $R^2$  values of the fittings were greater than 0.9972 but systematic variations were observed during the first 150 s of reaction so the rate constants derived were termed apparent rate constants ( $k_{app}$ ). Plots of  $k_{app}$  versus the concentration of AcP for REC-WT appeared sigmoidal and were thus fit using a Hill equation (GraphPad Prism).

### **Static Light Scattering**

The average molecular weights of REC-WT or REC-H85A were determined by static light scattering (SLS) using a Precision detector PD2080 light scattering instrument connected in tandem to a high-performance liquid chromatography system and an LKB 2142 differential refractometer. In each case, 500

$\mu\text{l}$  of protein solution was injected into an analytical Superdex 75 column and eluted with a buffer consisting of 20 mM Tris-HCl, 200 mM NaCl (pH=8). The 90° light scattering and refractive index signals of the eluted protein were analyzed with the Discovery32 software supplied by Precision Detectors. The 90° light scattering detector was calibrated using bovine serum albumin (MW: 66.5 kDa) as a standard. To achieve the *in vitro* phosphorylation of the samples, 500  $\mu\text{l}$  of protein solution with 30 mM AcP and 30 mM  $\text{MgCl}_2$  were prepared, incubated at 30 °C for 1 h and then injected into the Superdex 75 column.

### Phosphoprotein affinity gel electrophoresis

Phos-tag<sup>TM</sup> acrylamide running gels contained 15% (w/v) 29:1 acrylamide/N'N'-methylenebisacrylamide, 375 mM Tris (pH= 8.8), and 0.1% (w/v) SDS. Gels were copolymerized with 75  $\mu\text{M}$  Phos-tag<sup>TM</sup> and 150  $\mu\text{M}$   $\text{ZnCl}_2$ . Stacking gels contained 4% (w/v) 29:1 acrylamide/N'N'-methylenebisacrylamide, 125 mM Tris (pH= 6.8), and 0,1% (w/v) SDS. All Phos-tag<sup>TM</sup> acrylamide containing gels were run with standard denaturing running buffer at 4 °C under constant voltage (150 V). The fraction of phosphorylated protein in each gel lane was determined by quantifying the Coomassie Blue staining intensity (Quantity One, BioRad) for the upshifted (phosphorylated) protein band in each lane relative to the total staining intensity for both bands (total protein) in the lane.

### Receiver domain autodephosphorylation analysis

REC-WT and REC-H85A were separately phosphorylated as follows: 500  $\mu\text{l}$  solutions of 100  $\mu\text{M}$  protein were prepared in reaction buffer [20 mM Tris-HCl, 200 mM NaCl, pH=8] containing 30 mM AcP and 30 mM  $\text{MgCl}_2$ . The reaction was allowed to proceed for 2 h at 30 °C and then the 500  $\mu\text{l}$  were loaded into a G25 desalting column (previously equilibrated in reaction buffer) to remove the excess of AcP and  $\text{MgCl}_2$ . 800  $\mu\text{l}$  of eluate were recovered, quantified, diluted to 20  $\mu\text{M}$  and after 20 mM  $\text{MgCl}_2$  was added, 50  $\mu\text{l}$  aliquots were removed at different times (0, 30, 60, 120, 180 min) and quenched by the addition of 10  $\mu\text{l}$  of 6X SDS loading buffer. Quenched samples were kept on ice until the last time point was obtained and finally the extent of protein phosphorylation was determined using phosphoprotein affinity gel electrophoresis. The rate of autodephosphorylation was determined from fits of the phosphorylated

fraction (an average of three independent experiments) vs. time using a single exponential decay function (GraphPad Prism).

### **Phosphotransfer from [<sup>32</sup>P]NtrY~P to NtrX receiver domain**

PAS-HK (NtrY) (10 μM) was reduced<sup>11</sup> and incubated with either 10 μM REC-WT or REC-H85A in reaction buffer [20 mM Tris-HCl, 200 mM NaCl, 5 mM MgCl<sub>2</sub>, pH=8]. The reaction was started by addition of 0.1 mM of ATP containing 0.5 μCi of [γ-<sup>32</sup>P] ATP (3000 Ci/mmol, Perkin Elmer) at room temperature and 20 μl samples were removed and added to an equal volume of 2X SDS-PAGE buffer at the following time points: 0, 5, 15, 30, 60 min. Samples were electrophoresed on 15% polyacrylamide gels. The gels were dried and exposed to a Store Phosphor Screen (GE Healthcare). The screen was scanned using a Storm Image and Detection system (Molecular Dynamics).

For competition assays, 10 μM PAS-HK was reduced and incubated simultaneously with 10 μM REC-WT (or REC-H85A) and 10 μM NtrX (full length) in reaction buffer. The rest of the protocol was followed as detailed previously. Finally, the intensity of the bands was quantified using Quantity One (BioRad) and the percentage of radioactive label corresponding to NtrX~P or REC~P was obtained as the intensity of the selected band relative to the total intensity in that lane.

### **Modeling of NtrX REC domain with full length α5 helices**

NtrX REC domain sequence (residues 1-135) were introduced in the SWISS-MODEL server<sup>56; 57; 58</sup>, and homology models were obtained using DctD REC domain in their inactive state (PDB code 1QKK) or bound to beryllofluoride (PDB code 1L5Y) as templates. The modeled structures were then aligned to each monomer in either REC-N (molecules A and B) or REC-A (molecules E and F), respectively, to generate the dimeric state.

### **ACCESSION NUMBERS**

The coordinates of NtrX REC domain structures in the absence of ligand (REC-N) and bound to beryllofluoride and magnesium (REC-A) have been deposited in the PDB with accession codes 4D6X and 4D6Y, respectively.

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## **FIGURE LEGENDS**

**Figure 1.** Structural states from NtrX REC domain. (a) NtrX architecture. The REC, AAA+, and DB domains are depicted in red, blue and yellow, respectively. The phosphorylatable aspartate residue (D53) is highlighted on the REC domain. The numbers indicate each domain boundaries. (b) Cartoon representation of the NtrX REC domain structure in the native state (REC-N) with the secondary structure elements labeled. (c) Structural comparison of molecule A (red), C (green) and D (wheat) from REC-N, along with molecule E (light blue) from REC-A. The position of the key loops  $\beta 3\text{-}\alpha 3$ ,  $\beta 4\text{-}\alpha 4$ ,  $\beta 5\text{-}\alpha 5$  is labeled and the beryllofluoride moiety ( $\text{BeF}_3^-$ ) is represented with yellow and violet spheres. (d) Close-up view of the aligned structures as reported in (c), indicating the position of the residues D53 (bound to  $\text{BeF}_3^-$ , yellow and violet sticks), S83 ( $\beta 4$  strand) and F102 ( $\beta 5$  strand). The different positions of  $\beta 3\text{-}\alpha 3$ ,  $\beta 4\text{-}\alpha 4$ ,  $\beta 5\text{-}\alpha 5$  loops as well as  $\alpha 4$  helix reorientation are shown. (e) Schematic representations of the different molecular conformations found in the REC domain crystals. The  $\beta 3\text{-}\alpha 3$  and  $\beta 4\text{-}\alpha 4$  loops are drawn in brown and blue respectively. The open (dark colors) or closed (light colors) states are highlighted. In the schemes, the position of D53 is indicated with a red asterisk. The table also informs whether  $\text{BeF}_3^-$  is present in the structure, the buried surface area (B.S.A.) between the indicated molecules, and their respective conformational state.

**Figure 2.** NtrX REC domain active site. (a) Close-up view of the active site from the REC-A structure (active state). Relevant amino acids are shown in sticks, with carbons in light blue, oxygens in red, and nitrogens in blue. Beryllofluoride is drawn as yellow and violet sticks, magnesium as green spheres, and water molecules as red spheres. Polar interactions are depicted as dashed lines. The most relevant loops are labeled. (b) Structural contrast between the active sites from the inactive (red, molecule A from REC-N) and active states (light blue, molecule E from REC-A). The positions of the crucial residues involved in the activation (S83 and F102) are illustrated.

**Figure 3.** NtrX REC domain dimer interface. (a) Solvent-accessible surface representation of the dimeric structure of the REC domain in its inactive (red, left panel) and active (light blue, right panel) states. Below these illustrations, the interface formed by  $\alpha 4$  and  $\beta 5$  from opposite monomers is depicted, showing polar and hydrophobic contacts. (b) Cartoon representation of the overlay between the dimers A-

B from REC-N (red) and E-F from REC-A (light blue). On the active state,  $\alpha 4$  helix is displaced tightening the interface (inset). The different conformations of S83 and F102 are also indicated.

**Figure 4.** Biochemical and biophysical assessment of the REC domain quaternary structure. (a) Fluorescence spectroscopy was used to determine the kinetics of NtrX REC domain autophosphorylation with different concentrations of AcP. The rate constant of this reaction performed in triplicate is plotted against the concentration of the phosphodonor with the best-fit sigmoid curve overlaid. (b) Size-exclusion chromatography profiles of the REC domain in its native state (dashed lines) or phosphorylated by previous incubation with AcP (solid lines). The SLS signal at  $90^\circ$  was also recorded (not shown) and used, along with the corresponding absorbance at 280 nm, to determine the average MW of the different samples, as expressed in Table 2.

**Figure 5.** Biochemical characterization of the NtrX REC domain and the effect of the H85A mutation. (a) A representative experiment of a phosphotransfer assay is shown. NtrY (PAS-HK construct) was incubated simultaneously with the NtrX REC domain (wild-type or REC-H85A) and  $\gamma$ - $^{32}\text{P}$ -ATP. Aliquots were taken at different times and subjected to SDS-PAGE. The bands corresponding to radioactive REC domain or residual autophosphorylated NtrY are indicated. (b) Representative phosphotransfer experiment performed as described previously, but adding an equimolar amount of full length NtrX. The bands corresponding to radioactive REC domain or NtrX are indicated (c) Phosphoprotein affinity gel electrophoresis was used to assess the autodephosphorylation activity of wild-type and H85A-mutant REC domains. Each protein was phosphorylated with AcP and then the excess of phosphodonor was removed using a desalting column. Then,  $\text{Mg}^{2+}$  was added and aliquots were removed at different times and subjected to SDS-PAGE in gels prepared with Phos-tag<sup>TM</sup>. In the representative experiment shown, the upper band corresponds to the phosphorylated domain. (d) From the phosphoprotein affinity gels, the phosphorylated fraction (intensity of the phosphorylated band with respect to the total intensity in the same lane) was obtained and the average of three independent experiments was plotted against time. The best-fit single decay function was fitted to obtain the dephosphorylation rate constant.

**Figure 6.** Proposed model for activation and signal transduction. (a) Cartoon representation of the REC domain under different conformations, ordered according to the proposed conformational pathway that might explain the activation. Important residues for each structure are shown in sticks and the interactions between them are indicated with dashed lines. The capital letter under the cartoon denotes the molecule designation used along this article. (b) Superposition of the dimeric structure of the inactive (red, left) and active (light blue, right) states of the REC domain that were modeled including their extended  $\alpha 5$  helix. Phosphorylation reorients the  $\alpha 4$  helix, leading to a rearrangement of the interface and a reorientation of the  $\alpha 5$  helices.

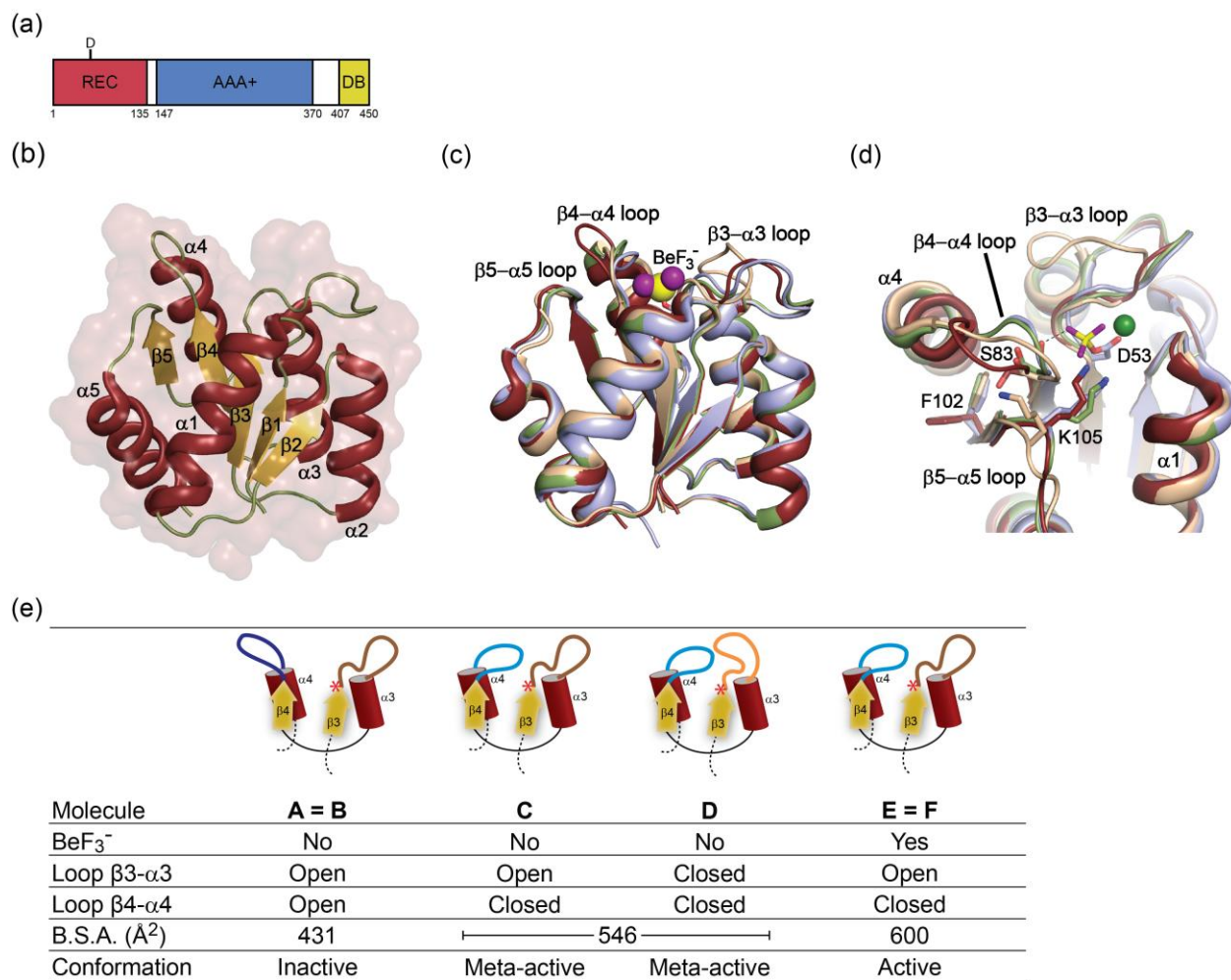


Figure 1

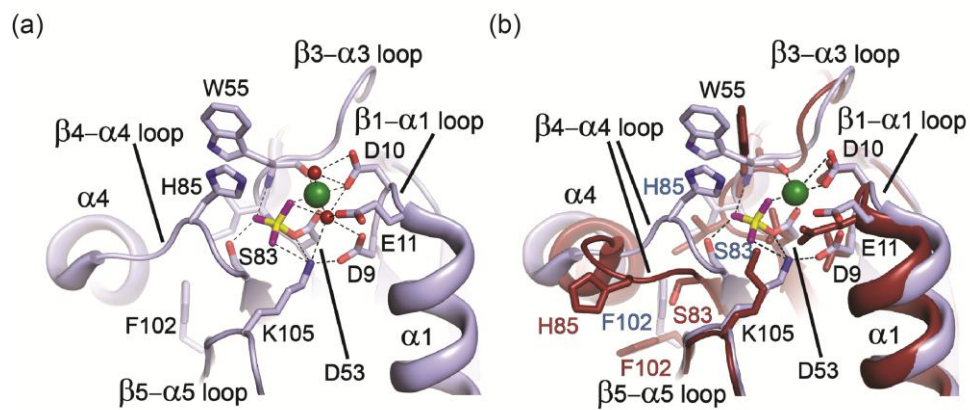


Figure 2



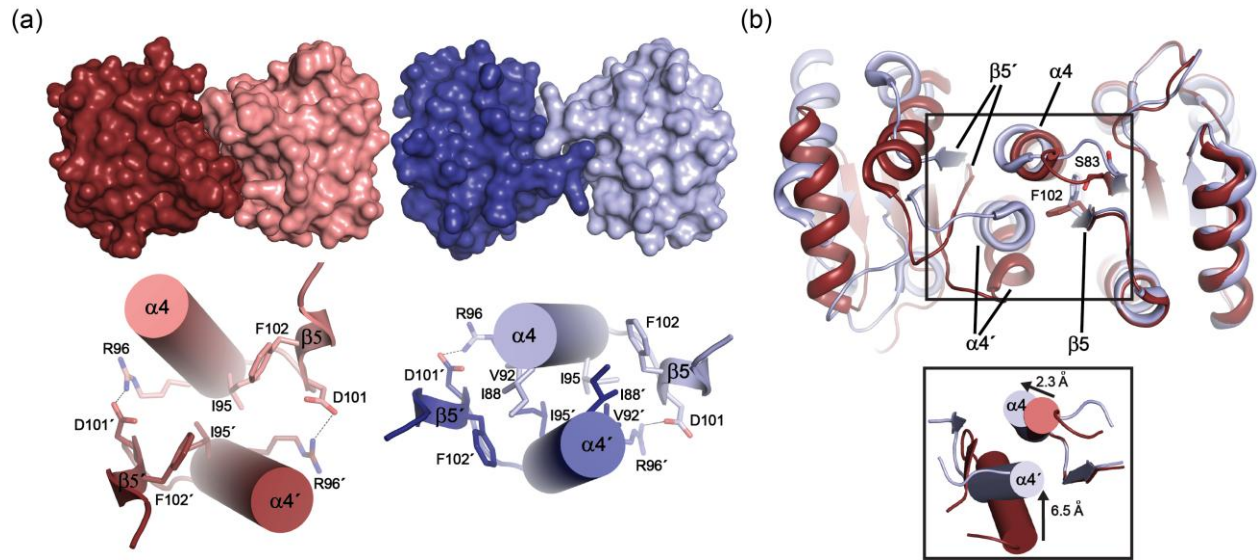


Figure 3

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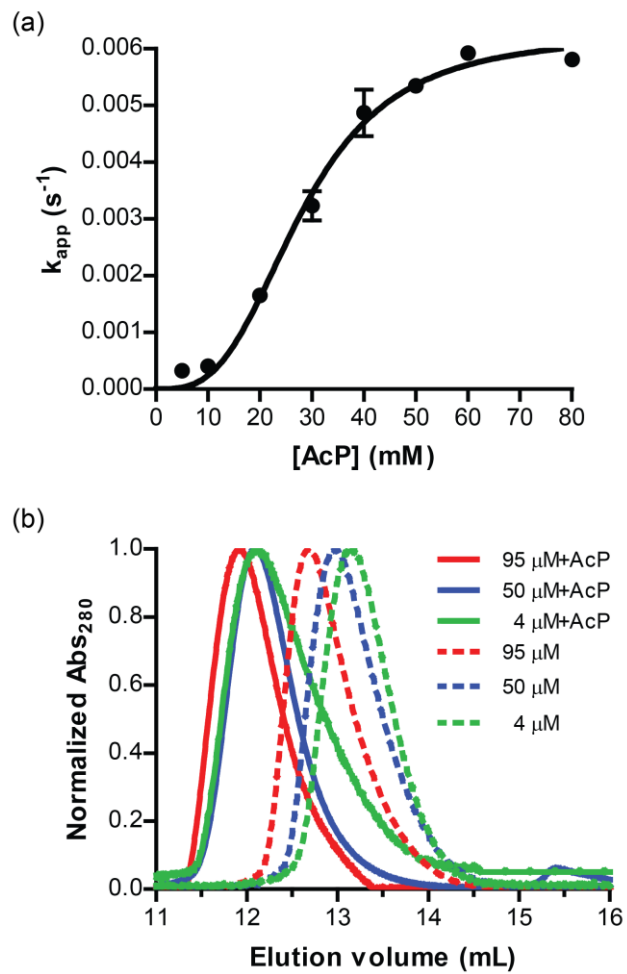


Figure 4

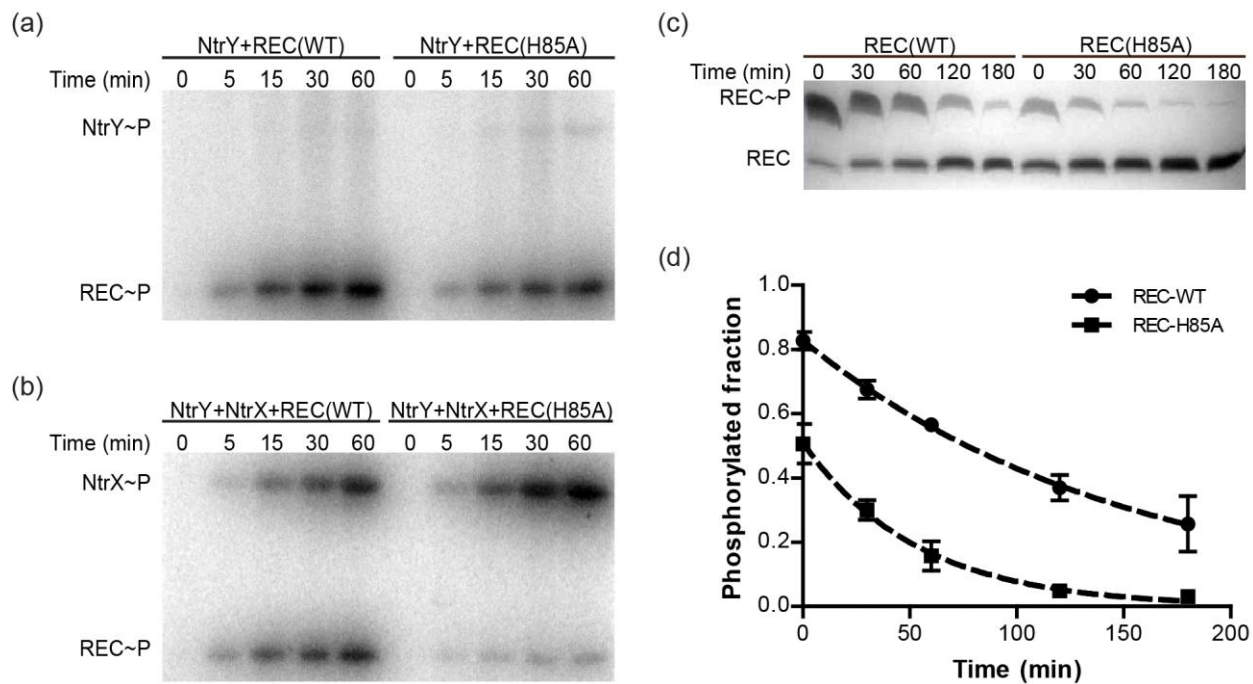


Figure 5

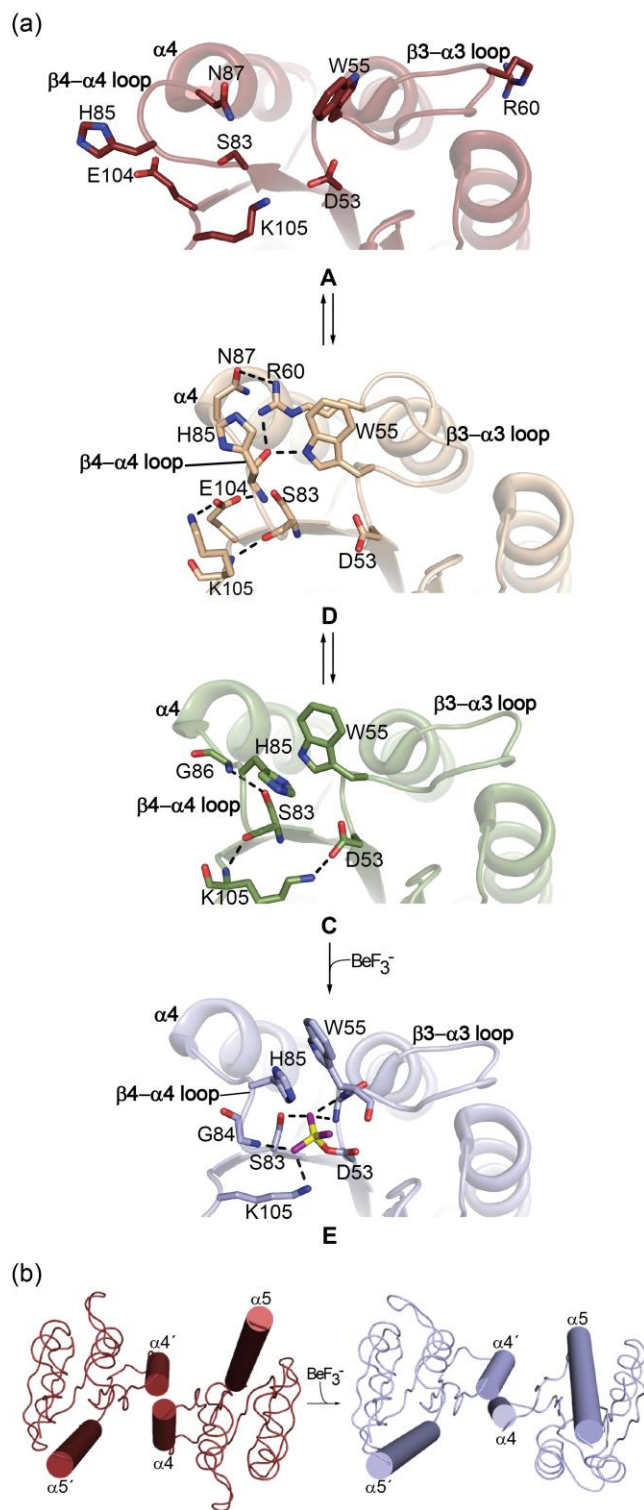


TABLE 1. Data collection and refinement statistics.

Statistics	REC-N	REC-A
PDB code	4D6X	4D6Y
<b>Data collection</b>		
Synchrotron source	SOLEIL	SOLEIL
Beamline	PROXIMA 1	PROXIMA 1
Number of frames	700	3000
Oscillation step (deg)	0.2	0.1
Detector distance (mm)	343.5	244.3
Wavelength (Å)	0.9785	0.9762
Exposure per frame (s)	0.2	0.1
<b>Indexing and scaling</b>		
Cell parameters		
a (Å)	38.67	30.39
b (Å)	110.94	39.34
c (Å)	119.43	52.96
$\alpha$ (deg)	90.00	97.30
$\beta$ (deg)	90.00	96.97
$\gamma$ (deg)	90.00	89.97
Spacegroup	$P2_12_12_1$	$P1$
Resolution limit (Å)	2.11	1.70
Number of total reflections	153925	75586
Number of unique reflections	30141	25833
Average multiplicity <sup>a</sup>	5.1 (5.3)	2.9 (2.8)
$\langle I/\sigma(I) \rangle$	16.6 (2.0)	9.9 (3.4)
$R_{\text{meas}}$	0.046 (0.651)	0.080 (0.420)
Completeness (%)	98.7 (98.1)	97.2 (95.8)
Monomers per asymmetric unit	4	2
Solvent content (%)	37	35
B-factor (Wilson plot, Å <sup>2</sup> )	57	22
<b>Refinement</b>		
Resolution range (Å)	40.6-2.11	20.0-1.70
Number of protein atoms	3712	1880
Number of ligand atoms	15	10
Number of water molecules	83	106
R	0.213	0.184
$R_{\text{free}}$	0.219	0.204
Rms deviations from ideal values <sup>b</sup>		
Bond lengths (Å)	0.010	0.010
Bond angles (deg)	1.1	1.0
B-factor (average, Å <sup>2</sup> )	75	28
<b>MolProbity validation<sup>c</sup></b>		
Clashscore	3.61	4.23
Poor rotamers (%)	8.9	4.5
Ramachandran plot		
Favored (%)	98.5	100.0

Allowed (%)	1.5	-
Disallowed (%)	-	-

<sup>a</sup> Values in parentheses correspond to the highest resolution shell: REC-N: 2.24–2.11 Å, REC-A: 1.77–1.70 Å,

<sup>b</sup> 57

<sup>c</sup> 51

TABLE 2. Molecular weight (MW) determined by SEC-SLS for wild type (REC-WT) and mutant (REC-H85A) receiver domains in the nonphospho (- AcP) and phosphorylated (+ AcP) states.

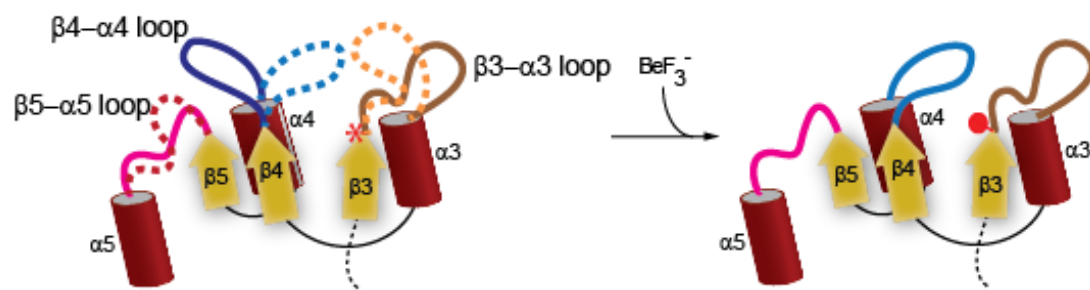
Protein	Concentration ( $\mu\text{M}$ )	MW (kDa)	
		- AcP	+ AcP
REC-WT	500	25.3	ND*
	95	21.9	31.0
	50	19.2	30.0
	4	16.8	23.1
REC-H85A	500	22.6	ND*
	95	18.9	30.6
			20.4
	50	18.2	29.6
	4	16.1	20.2
		15.2	

\*ND: not determined

TABLE 3. Autophosphorylation rate constants ( $k_{app}$ ) determined by fluorescence spectroscopy for wild type (REC-WT) and mutant (REC-H85A) receiver domains using different concentrations of acetyl phosphate (AcP).

[AcP] (mM)	$k_{app}$ ( $\cdot 10^{-4} \text{ seg}^{-1}$ )*	
	REC-WT	REC-H85A
10	$4.050 \pm 0.004$	$1.77 \pm 0.02$
20	$16.5 \pm 0.3$	$1.90 \pm 0.14$
40	$45.8 \pm 4.1$	$3.18 \pm 0.38$

\*Average rate constant  $\pm$  SD



Graphical abstract

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## Highlights

- A molecular description of the activation mechanism of NtrX is not available.
- Different crystal structures of NtrX REC domain are reported for the first time
- Phosphorylation stabilizes a conformation of the loop  $\beta$ 4- $\alpha$ 4, helices  $\alpha$ 4 and  $\alpha$ 5
- Biochemical characterization of the REC domain enzymatic activities was performed
- A mechanism for the signal transduction to the full length protein is proposed

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