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Crystal Structure of a Complex between the Phosphorelay Protein YPD1 and the Response Regulator Domain of SLN1 Bound to a Phosphoryl Analog

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The crystal structure of the yeast SLN1 response regulator (RR) domain bound to both a phosphoryl analog [beryllium fluoride (BeF_3^-)] and Mg^{2+}, in complex with its downstream phosphorelay signaling partner YPD1, has been determined at a resolution of 1.70 Å. Comparisons between the BeF_3^--activated complex and the unliganded (or apo) complex determined previously reveal modest but important differences. The SLN1-R1•Mg^{2+}•BeF_3^- structure from the complex provides evidence for the first time that the mechanism of phosphorylation-induced activation is highly conserved between bacterial RR domains and this example from a eukaryotic organism. Residues in and around the active site undergo slight rearrangements in order to form bonds with the essential divalent cation and fluorine atoms of BeF_3^-. Two conserved switch-like residues (Thr1173 and Phe1192) occupy distinctly different positions in the apo *versus* BeF_3^--bound structures, consistent with the "Y–T" coupling mechanism proposed for the activation of CheY and other bacterial RRs. Several loop regions and the α4–β5–α5 surface of the SLN1-R1 domain undergo subtle conformational changes (~1–3 Å displacements relative to the apo structure) that lead to significant changes in terms of contacts that are formed with YPD1. Detailed structural comparisons of protein–protein interactions in the apo and BeF_3^--bound complexes suggest at least a two-state equilibrium model for the formation of a transient encounter complex, in which phosphorylation of the RR promotes the formation of a phosphotransfer-competent complex. In the BeF_3^--activated complex, the position of His64 from YPD1 needs to be within ideal distance of and in near-linear geometry with Asp1144 from the SLN1-R1 domain for phosphotransfer to occur. The ground-state structure presented here suggests that phosphoryl transfer will likely proceed through an associative mechanism involving the formation of a pentacoordinate phosphorus intermediate.

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Introduction

In two-component regulatory systems,[1] phosphoryl transfer between a sensor histidine kinase (HK) and its cognate response regulator (RR) is the chemical basis for signal transduction. Multistep

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Abbreviations used: RR, response regulator; BeF_3^-, beryllium fluoride; HK, histidine kinase; HPt, histidine-containing phosphotransfer; PEG, polyethylene glycol.

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His-Asp phosphorelay signaling systems have evolved from the simpler two-component systems and are typically composed of a hybrid HK containing a C-terminal RR domain that transmits signals to a histidine-containing phosphotransfer (HPt) protein, which then relays phosphoryl groups to downstream RRs.[1–3] Phosphorylation of cytoplasmic RRs results in activation of either associated effector domains or downstream signaling partners.

While structures of RR proteins (or domains) in their unphosphorylated inactive states have been well documented, structural studies of phosphorylated RRs have been somewhat limited due to the intrinsic lability of phosphoaspartyl linkage. However, in recent years, there have been numerous reports describing the use of beryllium fluoride (BeF_3^-), a noncovalent mimic of the phosphoryl group, in order to obtain NMR or X-ray structures of stably activated RR domains (reviewed by West and Stock,[3] Stock and Guhaniyogi[4] and Gao *et al.*[5]). These have shown that phosphorylation of a conserved aspartate residue and binding of an essential divalent cation induce localized structural rearrangements within the active site of RR domains that lead to subtle long-range conformational changes affecting primarily one surface of the protein. RR domains have a central five-stranded β-sheet surrounded by five α-helices (overall α_5/β_5 fold). Three carboxylate-containing residues, including the aspartic acid that is the site of phosphorylation, and an invariant lysine residue comprise the active site located at the C-terminal edge of the central β-sheet. Two additional highly conserved residues in the vicinity of the active site (Thr/Ser from β4, and Tyr/Phe from β5) have been implicated as switch-type residues that occupy distinctly different positions in the phosphorylated RR in comparison to the unphosphorylated state. Hence, the orientation of these two residues is highly indicative of whether the RR is in active or inactive conformation. The combined effect of the active site rearrangement on phosphorylation and the so-called "Y–T" conformational coupling of the switch residues results in a modest alteration of the α4–β5–α5 surface of the protein.[6–8] Despite the growing numbers of RR domain structures that support a simple two-state model, there are two notable exceptions[9,10] suggesting the possibility that multiple conformational states occur along the pathway to RR activation.

Small-molecule phosphodonors, such as acetyl phosphate and phosphoramidate, have also been used to phosphorylate RRs *in vitro*.[11,12] However, the observed rate of phosphorylation is several orders of magnitude slower than if the cognate HK or HPt protein served as the phosphodonor.[13–15] A similar rate difference was exploited as a means for differentiating cognate from noncognate HK–RR and HPt–RR pairs in a phosphotransfer profiling assay.[16,17] This raises two important questions: (1) How do cognate HK, HPt and RR proteins specifically interact with each other? (2) What aspect(s) of the association contributes to phosphotransfer efficiency?

To date, there are only two examples of structures of RR domains in complex with their cognate HPt protein, namely, Spo0B/Spo0F[10,18] and YPD1/SLN1-R1.[19] No high-resolution structures have been determined, thus far, of complexes between an HK and RR. The reason for the paucity of structural and biochemical data on RR–HPt and RR–HK interactions most likely stems from the transient and presumably weak nature of the interaction, which is typical of signaling partners, and the additional influence that phosphorylation may have on recognition and/or binding.

In the yeast *Saccharomyces cerevisiae*, YPD1 functions as an HPt protein that shuttles phosphoryl groups to and from three homologous RR domains associated with SLN1, SSK1 and SKN7 (referred to as SLN1-R1, SSK1-R2 and SKN7-R3, respectively). In the osmoregulatory branch of this phosphorelay system (SLN1–YPD1–SSK1), YPD1 serves two important functions: mediating phosphoryl group transfer between the hybrid HK SLN1 and the SSK1 RR, and stabilizing the phosphorylated state of SSK1.[20,21] In its phosphorylated state, SSK1 is incapable of activating the downstream mitogen-activated protein kinase cascade and therefore inhibits signaling via the high-osmolarity glycerol response mitogen-activated protein kinase cascade under normal growth conditions.

We have previously reported the first structure of a monomeric HPt domain (YPD1) in complex with its upstream phosphodonor, the SLN1-R1 domain.[19] Two crystal forms ($P3_2$ and $P2_12_12_1$ space groups) that differed slightly in the relative orientation of YPD1 with respect to the SLN1-R1 domain were obtained. The $P2_12_12_1$ crystal form appeared to be favored when BeF_3^- was included in the crystallization condition;[22] from the structure of this complex, we observed the two active sites to be better aligned than the $P3_2$ complex, facilitating phosphoryl transfer. However, in the YPD1/SLN1-R1 complex, we did not observe electron density for the phosphoryl analog BeF_3^- or for the divalent metal ion.

Recently, Varughese *et al.* published the 3.05-Å structure of Mg^{2+}- and BeF_3^--bound Spo0F in complex with the dimeric Spo0B HPt protein.[10] This complex revealed a snapshot of a pretransition state complex just prior to phosphotransfer. Here we report the crystal structure of BeF_3^-- and magnesium ion (Mg^{2+})-bound SLN1 RR domain (SLN1-R1) in complex with the HPt protein YPD1 at 1.70 Å resolution. This structure also represents a pretransition state interaction between "activated" SLN1-R1 and YPD1, and allows for a detailed analysis of the active site configuration, protein–protein interactions and mechanism of phosphotransfer between HPt and RR proteins. Furthermore, because of the high degree of structural homology among HPt proteins and within the RR superfamily, the yeast YPD1/SLN1-R1•Mg^{2+}•BeF_3^- quaternary complex can serve as a model for

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