Conformational dynamics are a key factor in signaling mediated by the receiver domain of a sensor histidine kinase from *Arabidopsis thaliana*

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Multistep phosphorelay (MSP) cascades mediate responses to a wide spectrum of stimuli, including plant hormonal signaling, but several aspects of MSP await elucidation. Here, we provide first insight into the key step of MSP-mediated phosphotransfer in a eukaryotic system, the phosphorylation of the receiver domain of the histidine kinase CYTOKININ-INDEPENDENT 1 (CKI1RD) from *Arabidopsis thaliana*. We observed that the crystal structures of free, Mg\(^{2+}\)-bound, and berylliofluoridated CKI1RD (a stable analogue of the labile phosphorylated form) were identical and similar to the active state of receiver domains of bacterial response regulators. However, the three CKI1RD variants exhibited different conformational dynamics in solution. NMR studies revealed that Mg\(^{2+}\) binding and berylliofluoridation alter the conformational equilibrium of the β3–α3 loop close to the phosphorylation site. Mutations that perturbed the conformational behavior of the β3–α3 loop while keeping the active-site aspartate intact resulted in suppression of CKI1 function. Mechanistically, homology modeling indicated that the β3–α3 loop directly interacts with the ATP-binding site of the CKI1 histidine kinase domain. The functional relevance of the conformational dynamics observed in the β3–α3 loop of CKI1RD was supported by a comparison with another *A. thaliana* histidine kinase, ETR1. In contrast to the highly dynamic β3–α3 loop of CKI1RD, the corresponding loop of the ETR1 receiver domain (ETR1RD) exhibited little conformational exchange and adopted a different orientation in crystals. Biochemical data indicated that ETR1RD is involved in phosphorylation-independent signaling, implying a direct link between conformational behavior and the ability of eukaryotic receiver domains to participate in MSP.

The growth and development of plants are regulated by a variety of hormones and environmental factors, as detected by specific receptors, including membrane hybrid histidine kinases (HKs). Following ligand recognition by the input domain, a histidine residue in the cytosolic HK domain of the hybrid HK receptor is autophosphorylated. The phosphate group is subsequently transferred to the conserved aspartate of the receiver domain (RD), which initiates the downstream (trans)phosphorylation cascade. The phosphorelay is triggered by the RD-mediated phosphorylation of small soluble histidine-containing phosphotransfer proteins that transfer the phosphates to the nucleus. Here, the transphosphorylation of the aspartate-containing response regulators (RRs), the final phosphaceptor, takes place, regulating different cellular events, in most cases the transcription of target genes (1, 2). This pathway, known as multistep phosphorelay (MSP), is unique to plants, lower eukaryotes, and bacteria. In the model plant *Arabidopsis thaliana*, eight canonical hybrid HK sensors involved in MSP (AHK1/AtHK1, AHK2, AHK3, AHK4/CRE1, AHK5/CKI2, ETR1, ERS1, and CKI1), five histidine-containing phosphotransfer proteins (AHP1–AHP5), and 23 RRs were identified. The plant MSP seems to have developed from its bacterial ancestor, two-component signaling. In two-component pathways, the signal-activated HK directly activates its component, whereas in the canonical RR via phosphorylation of its RD, thereby (in most cases) controlling the gene expression.

CKI1RD is structurally similar to RRs of bacterial RRs and studied thoroughly by analyzing crystal and solution structures.

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This article contains supplemental Tables S1–S4, Figs. S1–S10, and Refs. 1–4.

The atomic coordinates and structure factors (codes 5N2N, SLNN, and SLNM) have been deposited in the Protein Data Bank (http://wwpdb.org/).

The assigned chemical shifts and relaxation rates of free, Mg\(^{2+}\)-bound, and berylliofluoridated CKI1RD, respectively, were deposited in the Biological Magnetic Resonance Data Bank under codes 16917, 16918, and 26737.

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4 The abbreviations used are: HK, histidine kinase; RD, receiver domain; RR, response regulator; MSP, multistep phosphorelay; AHP, *Arabidopsis* histidine-containing phosphotransfer protein; CPMMG, Carr-Purcell-Meiboom-Gill; PDB, Protein Data Bank; r.m.s.d., root mean square deviation; HSQC, heteronuclear single quantum coherence; AIC, Akaike’s Information Criterion.
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Figure 1. A, secondary structure representation of the activated CKI1RD structure. The helices (α1–α5, cyan), β-strands (β0–β5, magenta), and loops (β3–α3 in dark green, other loops in pink) are numbered sequentially. The active-site residues of CKI1RD are shown in stick representation (showing the chemistry). Polar interactions are highlighted by dashed lines, including the coordination sphere of a Mg2+ ion (orange sphere) with water molecules (red spheres). B, detailed structural description of the active site of CKI1RD (stick representation). The coordination sphere of the Mg2+ ion and the polar interactions of Lys-1105 with Asp-992 and F3 of BeF3 are highlighted by dashed lines. C, active site of CKI1RD defined by the omit (2mFo–FoFc) electron density map at 1.0 σ.

NMR relaxation, and computer simulations. However, the knowledge of the bacterial systems cannot be readily extrapolated to the signaling mediated by CKI1RD. RDs of plant hybrid HKs and of bacterial RRs fulfill very different physiological functions in different molecular contexts. CKI1RD is a C-terminal domain of a large transmembrane protein, but bacterial RDs are N-terminal portions of RRs or separate domains. RDs of RRs involved in the highly specific bacterial two-component systems do not transfer phosphate to downstream acceptors, but directly activate their effector domains, interact with separate effector proteins, or dimerize (oligomerize) using the α4–β5–α5 face, distant from the phosphorylation site. In contrast, CKI1RD (and other RDs of hybrid HKs involved in MSP) transfers phosphate from the donating CKI1HK domain to several accepting AHP proteins, using the binding interface surrounding the phosphorylated aspartate. Interactions involving the α4–β5–α5 face have not been reported for CKI1RD.

Therefore, we decided to investigate the molecular mechanism underlying the CKI1RD-mediated phosphorelay experimentally by comparing all three relevant forms of this protein: free CKI1RD, its complex with Mg2+; and a stable analogue of the phosphorylated form, a beryllofluoride complex. We solved crystal structures of two CKI1RD active-site mutants and of the beryllofluoridated CKI1RD wild type, monitored conformational behavior of the individual CKI1RD forms using NMR spectroscopy, and showed functional relevance of the observed changes of the conformational ensembles in vivo and in vitro.

Results

Crystal structure of beryllofluoridated CKI1RD is identical with those of the free and Mg2+-bound forms

To mimic the formation of the active state of CKI1RD, a beryllofluoride (BeF3) moiety was used as a phosphate group analogue. It has been reported previously that this analogue can mimic the phosphorylation-induced structural changes of bacterial RDs (3–5).

The crystal structure of beryllofluoridated CKI1RD (PDB code 5N2N; Fig. 1 and Table 1) adopts a typical (α/β)5 fold of the CheY-like protein superfamily (6). Two residues in the N-terminal sequence preceding Met-944 of CKI1 form an additional β-strand (β0) that is antiparallel to β5 of the central β-sheet, as observed in the crystal structures of free and Mg2+-bound CKI1RD (7).

The beryllofluoridated CKI1RD structure revealed an octahedral coordination sphere of Mg2+ that is formed by the main chain carbonyl oxygen of Gln-1052, the side chain carboxyl oxygen of Asp-1050 and Asp-993, a fluorine atom of BeF3, and the oxygens of two water molecules (Fig. 1, A and B). Smaller differences in the distances for Mg2+ coordination indicate a slight deformation of the octahedral arrangement. Furthermore, the side chain of Lys-1105 is involved in the formation of a salt bridge with a fluorine atom of BeF3 (distance N–F3, 2.39 Å), which is additionally stabilized by polar contact with Asp-992 (distance N–O, 2.9 Å; Fig. 1B). The 2F0–F, and the lack of F, density maps confirmed the presence of Mg2+ and BeF3 complex non-covalently bound to the active site Asp-1050 (distance O–Be, 1.76 Å; Fig. 1C).

A comparison with the previously determined structures of metal-free (PDB code 3MM4) and Mg2+-bound (PDB code 3MMN) CKI1RD structures (7) revealed that the backbone conformation of beryllofluoridated CKI1RD remained intact (Fig. 2). The overall r.m.s.d. (Cα) between beryllofluoridated CKI1RD and the metal-free and Mg2+-bound forms of this protein were
shown to be 0.184 and 0.182 Å, respectively. Additionally, the orientations of side chains not interacting with Mg$^{2+}$ or BeF$_3$ are very similar in all three forms of CKI$_{RD}$ (Fig. 3). The orientation of the Phe-1102 side chain corresponds to the conformation typical for activated bacterial RRs (8, 9). Conformation of the Lys-1105 side chain depends on the formation of a polar contact with either Asp-1050 (in metal-free or Mg$^{2+}$/bound CKI$_{RD}$) or a fluorine atom of beryllofluoride (Fig. 3). This suggests that the reorientation of the carboxylic group of Asp-1050 in the Mg$^{2+}$/bound form induces the reorientation of Lys-1105 needed for an effective stabilization of the RD phosphorylation.

**Dynamics of CKI$_{RD}$ from NMR relaxation is dominated by conformational exchange in the β3–α3 loop**

The backbone motions of CKI$_{RD}$ were studied at the physiological temperature of 25 °C using standard $^{15}$N NMR relaxation experiments (10). The measured relaxation rates were analyzed in terms of the model-free approach (11–13), as implemented in the program Relax. The obtained parameters describing the fast internal motions (order parameters $S_2$ and $S_3$ and correlation times $\tau_e$ and $\tau_s$) were very similar for all three forms of CKI$_{RD}$ (Fig. 4). The flexible residues Met-944–Gly-985 form a linker that connects the receiver domain to CKI1 and is expected to be disordered in the native protein. The receiver domain itself (residues Lys-986–Glu-1116) exhibited dynamics typical for well-structured proteins, with the higher flexibility in loops resulting in

**Table 1**

Data collection and refinement statistics

|                          | BeF$_3$-activated CKI$_{RD}$ | D1050A mutant | D1050E mutant |
|--------------------------|-----------------------------|---------------|---------------|
| PDB code                 | 5N2N                        | SLNN          | SLNM          |
| Space group              | C222 (1)                    | C222 (1)      | C222 (1)      |
| Wavelength (Å)           | 0.91841                     | 0.97522       | 0.97522       |
| Unit-cell parameters     |                             |               |               |
| $a$, $b$, $c$ (Å)        | 52.85, 100.15, 80.14         | 53.18, 101.00, 79.46 | 54.43, 98.73, 79.90 |
| Resolution range (Å)     | 46.74–2.05 (2.16–2.05)      | 47.05–1.60 (1.69–1.60) | 47.60–1.95 (2.06–1.95) |
| Reflections measured     | 89,719                      | 182,010       | 90,844        |
| Unique reflections       | 13,705                      | 28,315        | 15,893        |
| Completeness (%)         | 99.9 (99.9)                 | 98.9 (95.3)   | 99.2 (94.8)   |
| $CC_{1/2}$               | 100.0 (94.5)                | 100.0 (98.6)  | 98.9 (94.6)   |
| $R_{merge}$              | 0.050 (0.470)               | 0.023 (0.175) | 0.115 (0.664) |
| Multiplicity             | 6.5 (6.8)                   | 6.4 (3.6)     | 5.7 (4.0)     |
| $R(f_i)$                  | 20.5 (4.1)                  | 37.8 (5.3)    | 11.2 (1.6)    |
| Reflections used         | 13,028                      | 26,733        | 13,110        |
| Reflections used for $R_{free}$ | 626                          | 1351          | 660           |
| $R_{free}$ (%)           | 18.18/22.51                 | 19.24/22.81   | 20.72/23.38   |
| r.m.s.d.                 |                             |               |               |
| Bond lengths (Å)         | 0.020                       | 0.026         | 0.019         |
| Bond angles (°)          | 1.858                       | 2.377         | 2.037         |
| No. of water molecules   | 53                          | 137           | 94            |
| No. of non-H atoms (total) | 1218                         | 1346          | 1294          |
| Average B factor (Å$^2$) | 46.1                        | 31.6          | 34.3          |
| Protein                 |                             |               |               |
| Mg$^{2+}$ atom           | 48.0                        | 50.3          | 41.9          |
| BeF$_3$ compound         | 50.3                        | 42.7          |               |
| Water                   | 49.0                        |               |               |
| Ramachandran plot        |                             |               |               |
| Residues in most favorable regions (%) | 99.0 | 99.4 | 97.0 |
| Residues in additional allowed regions (%) | 1.0 | 0.0 | 2.4 |

* $R_{merge} = \Sigma I_i - \langle I \rangle / \Sigma I_i$, where $I_i$ is the intensity of observation and $\langle I \rangle$ is the mean value.

* $R = \Sigma |F_o(h)| - |F_c(h)|/\Sigma |F_o(h)|$, where $F_o$ and $F_c$ are the observed and calculated structure-factor amplitudes, respectively. The free $R$ factor was calculated while excluding 5% of the data from the refinement. Glu-1055 in the D1050A mutant and Ser-935 in the D1050E mutant of CKI1$_{RD}$ were highlighted by Molprobity as having unusual $\phi/\psi$ angles.

Figure 2. Structural alignment (secondary structure representation) of the metal-free (blue), Mg$^{2+}$-bound (red), and beryllofluoridated (green) forms of CKI$_{RD}$

Dynamics of CKI$_{RD}$ from NMR relaxation is dominated by conformational exchange in the β3–α3 loop

The backbone motions of CKI$_{RD}$ were studied at the physiological temperature of 25 °C using standard $^{15}$N NMR relaxation experiments (10). The measured relaxation rates were analyzed in terms of the model-free approach (11–13), as implemented in the program Relax. The obtained spectra (supplemental Fig. S1) allowed us to analyze relaxation of 74, 78, and 69 residues of the free, Mg$^{2+}$-bound, and beryllofluoridated forms of CKI$_{RD}$ (consisting of 127 non-proline amino acids), respectively (supplemental Table S2 and supplemental Fig. S2; see under “Experimental procedures” for details).

The obtained parameters describing the fast internal motions (order parameters $S_2$ and $S_3$ and correlation times $\tau_e$ and $\tau_s$) were very similar for all three forms of CKI$_{RD}$ (Fig. 4). The flexible residues Met-944–Gly-985 form a linker that connects the receiver domain to CKI1 and is expected to be disordered in the native protein. The receiver domain itself (residues Lys-986–Glu-1116) exhibited dynamics typical for well-structured proteins, with the higher flexibility in loops resulting in
somewhat lower order parameters. In the most flexible \(2\beta_3\) loop, the model-free analysis preferred two modes of the internal motion with well-defined correlation time of the slow motion close to 1 ns. The binding of \(\text{Mg}^{2+}\) cations did not significantly change the fast dynamics, and only a small \(S_2\) increase in loops close to the phosphorylation site was observed after beryllofluoridation, indicating that the presence of \(\text{BeF}_3\) makes the structure slightly more compact.

The most significant differences between the CKI1RD samples were observed on the microsecond to millisecond time scale. These slower events are considered biologically more important (14–16). The effect of a slow exchange was included into the model-free analysis as an additional parameter \(R_{ex}\) (Fig. 5A) (10) and analyzed more precisely using \(^{15}\text{N}\) Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion experiments (17–19).

The analysis of free CKI1RD revealed only a few residues with the exchange contribution \(R_{ex}\) lower than 5 s\(^{-1}\) at 600 MHz. However, the broadening of peaks of residues in the \(2\beta_3\) loop provides strong evidence of an extensive conformational exchange in this region (supplemental Fig. S3).

The binding of \(\text{Mg}^{2+}\) was shown to have a dramatic effect on the slow dynamics of the \(\beta_3-\alpha_3\) loop (Fig. 5B–D). The peaks of the \(\beta_3-\alpha_3\) loop residues of \(\text{Mg}^{2+}\)-bound CKI1RD were observable in the \(^{15}\text{N}\)-relaxation spectra, indicating the reduction of the conformational exchange of the loop. The model-free analysis showed that the \(\beta_3-\alpha_3\) loop is the only region of \(\text{Mg}^{2+}\)-bound CKI1RD with significant slow dynamics (\(R_{ex}\) between 15 and 20 s\(^{-1}\) at 600 MHz). Peak heights of Met-1053, Met-1056, and Asp-1057 were fitted to a two-state exchange model using the Relax program. The Met-1053 data showed, with a good precision, that the exchange rate was 6000 s\(^{-1}\) and the minor state population was 2% (Table 2). The rate of formation of the minor state \(k_{A\rightarrow B}\) calculated from these values is \(~120\) s\(^{-1}\), comparable with rate constants of phosphotransfer to receiver domains of the yeast osmoregulatory system; phosphate is transferred from histidine transfer protein YPD1 to the receiver domains of hybrid histidine kinase SLN1 and of response regulator SSK1 with rate constants of 230 and 160 s\(^{-1}\), respectively (20). The value of \(k_{A\rightarrow B}\) cannot be currently correlated with the rate of phosphorylation of CKI1RD by CKI1\_HK because the kinetics of this signaling step are not known. The fits for Met-1056 and Met-1057 peaks were less precise, but showed the same time scale of the exchange (Table 2 and supplemental Fig. S5). It should be noted that the calculated exchange rates are relatively high, which makes the separation of the exchange parameters difficult. Additionally, the actual slow dynamics of the \(\beta_3-\alpha_3\) loop may be more complex than described by the two-state models. Data obtained at 1 mM MgCl\(_2\) concentration (data not shown) reveal that the chemical dynamics of the receiver domain of CKI1

**Figure 3.** A, active site (stick representation) of CKI1RD wild-type in the metal-free (blue), \(\text{Mg}^{2+}\)-bound (red), and beryllofluoridated (green) forms. B, D1050A mutant (gray), C, D1050E mutant (yellow). D, alignment of the mutant and beryllofluoridated structures. The residue colors represent the respective forms/mutants of CKI1RD. The Mg\(^{2+}\) ion is shown as a brown sphere in the Mg\(^{2+}\)-bound form and as an orange sphere in the beryllofluoridated form.
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Figure 4. Dynamics of CKI1<sub>re</sub>. Order parameters S<sub>2</sub> (A), S<sub>2</sub>' (B), effective correlation times τ<sub>e</sub> (C), and τ<sub>s</sub> (D), plotted for free (blue), Mg<sup>2+</</sup>-bound (red), and beryllofluoridated (green) CKI1<sub>re</sub>. Cyan and magenta bars and pink lines above the plots represent α-helices, β-sheets, and loops, respectively. The β3–α3 loop is highlighted in dark green. Prolines, unassigned residues, and residues that could not be analyzed quantitatively are indicated above the plots by letters “P”, crosses, and dots (color-coded form individual CKI1<sub>re</sub> forms), respectively. Parameters estimated with the relative error greater than 50% are not shown.

Figure 5. Exchange contributions to the relaxation at 600 MHz (A), plotted for free (blue), Mg<sup>2+</</sup>-bound (red), and beryllofluoridated (green) CKI1<sub>re</sub>, and results of CPMG experiments at 950 MHz (apparent rates plotted as a function of the CPMG frequency) shown for Met-1049 (B), Met-1053 (C), and Met-1056 (D) of free (blue), Mg<sup>2+</</sup>-bound (red), and beryllofluoridated (green) wild type, and of the D1050A (gray), D1050E (yellow), and D1057E,G1058N (slate blue) mutants. Cyan and magenta bars and pink lines above the plots represent α-helices, β-sheets, and loops, respectively. The β3–α3 loop is highlighted in dark green. Prolines, unassigned residues, and residues that could not be analyzed quantitatively are indicated above the plots by letters “P”, crosses, and dots (color-coded form individual CKI1<sub>re</sub> forms), respectively.

exchange (dissociation and association of Mg<sup>2+</</sup>) does not contribute significantly to the relaxation dispersion obtained at 30 mM MgCl<sub>2</sub> concentration.

The exchange contribution in the β3–α3 loop disappeared after beryllofluoridation, suggesting that the loop conformation was constrained (Fig. 5, C and D). However, several residues in the C-terminal region showed an increase in R<sub>ex</sub> (Fig. 5A). The most affected residue was shown to be Lys-1105 (R<sub>ex</sub> = 16 s<sup>-1</sup> at 600 MHz), whose side chain directly coordinates BeF<sub>3</sub><sup>−</sup> in the X-ray structure (Fig. 1B). The intensity of the Lys-1105 peak
was too low to analyze the relaxation dispersion curves quantitatively (supplemental Figs. S4–S6). An increased exchange contribution at a beryllium fluoride concentration equal to its dissociation constant (5 mM, data not shown) indicated that the other residues are influenced by a chemical exchange because beryllium fluoridated CKI1RD cannot be studied at complete saturation due to the high dissociation constant of the phosphate analogue.

The discussed results of the NMR relaxation and relaxation dispersion analyses do not correlate with the crystallographic B factors (supplemental Fig. S7), indicating that the crystal structures do not reflect the dynamics of CKI1RD in solution.

In summary, both Mg$^{2+}$ and BeF$_3^-$ greatly affect slow the conformational dynamics of the β3–α3 loop in solution, but they do not influence the backbone conformation in the crystal structures.

**Mutations of the β3–α3 loop termini change conformation of the loop in solution**

To further probe dynamics of the β3–α3 loop and its dependency on the phosphorylation state of CKI1RD, we perturbed the loop by mutating the phosphoaccepting Asp-1050 to non-phosphorylatable alanine and phosphorylation-mimicking glutamic acid. The effects of the mutation were local, and the number of assigned peaks in NMR spectra was similar to the wild-type samples. Selective methionine labeling confirmed that the mutations of Asp-1050 affected only the methionines of the loop. CPMG experiments showed that conformational exchange of Met-1053 was eliminated, and the exchange of Met-1056 was reduced to the extent observed in Mg$^{2+}$-bound wild-type CKI1RD (Fig. 5, C and D).

Another mutation probing conformational behavior of the β3–α3 loop was based on a comparison with the RD of the ethylene receptor ETR1 (ETR1RD). ETR1RD is structurally similar to CKI1RD except for the β3–α3 loop. The loop adopts different orientations in the crystal structure (21) from those observed in the CKI1RD X-ray structures. Furthermore, no severe NMR line-broadening was reported, suggesting its lower conformational flexibility in comparison with RDs of bacterial HKs (22). We confirmed the published results by acquiring CPMG data for ETR1RD at 950 MHz. In our experiment, none of the signals exhibited conformational exchange comparable with CKI1RD. Among the well-resolved peaks of Gly-664, Val-665, Glu-666, Asn-667, and Gln-669 of the β3–α3 loop, the highest exchange contribution at 950 MHz did not exceed values corresponding to 5 s$^{-1}$ at 600 MHz (supplemental Fig. S8).

Comparison of the β3–α3 loop sequences reveals that the β3–α3 loop of ETR1 (VCMPGVEN) differs from the consensus sequence XXMPXΦDG, where X is a variable amino acid and Φ represents a bulky hydrophobic residue (supplemental Table S3). A highly conserved pair DG at the C terminus of the loop is replaced by EN in ETR1RD. Substitution of the conformationally least restricted amino acid, glycine, by asparagine, is expected to influence conformational dynamics of the β3–α3 loop significantly. We mutated the corresponding residues in CKI1RD to mimic the ETR1 sequence. NMR spectra showed that the mutation of Asp-1057 and Gly-1058 perturbed the conformation in the region surrounding the β3–α3 loop, including the α2 and α3 helices, the β3 strand, and the β2–α2 loop (cf. Fig. 6 and supplemental Fig. S9A). Met-1049 (preceding the active site) and Met-1099 in the α4–β5 loop were affected in spectra of the selective methionine-labeled double mutant, in addition to the methionines in the β3–α3 loop (data not shown). Weak peak intensities in spectra of the uniformly labeled sample did not allow us to assign residues most affected by the mutation, whereas more distant regions were assigned reliably. Signs of two conformers in a slow exchange were also observed, most obviously in the methionine methyl region of $^{1}$H–$^{13}$C HSQC spectra but also for some well-resolved peaks in the $^{1}$H–$^{15}$N spectra (supplemental Fig. S9, B–E). Changes in NMR spectra observed during titration with MgCl$_2$ and NaF/BeCl$_2$ indicate that the Asp-1057 and Gly-1058 mutant is able to bind Mg$^{2+}$ ($K_a = 6$ mM) but not BeF$_3^-$.

Altogether, mutations of phosphoaccepting Asp-1050 influence conformational exchange of the β3–α3 loop with marginal effect on other regions of CKI1RD. Replacing Asp-1057 and Gly-1058 by the corresponding residues of ETR1 results in much more dramatic changes of the β3–α3 loop conformations, affecting regions surrounding the β3–α3 loop but not perturbing the overall fold of CKI1RD.

**Mutation of the active site Asp-1050 shifts Phe-1102 to the inactive orientation in crystal structures of CKI1RD**

Screening of crystallization conditions was performed for the point mutants D1050A and D1050E and for the double mutant D1057E/G1058N. The double mutant did not crystallize under
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Figure 7. Radiograms showing incorporation of $^{32}$P into ETR1$_{HK}$ (HK$_P$), CKI1$_{RD}$ (RD$_P$), and AHP2 (AHP2$_P$) in the presence (A) and absence (B) of AHP2. Coomassie Blue-stained gels showing the amount of loaded ETR1$_{HK}$ (HK), CKI1$_{RD}$ (RD), and AHP2 (AHP2) are displayed below the radiograms. PageRuler Prestained Protein Ladder (Thermo Fisher Scientific) was used as a molecular weight marker (M).

the conditions tested, presumably due to the mentioned conformational heterogeneity, but well-diffracting crystals of the point mutants were obtained and X-ray structures solved (PDB code 5LNN, 5LMN; Table 1). A structural comparison of the mutants with the beryllofluoridated CKI1$_{RD}$ revealed that the backbone conformation remained intact. The overall r.m.s.d. (C$\alpha$) between beryllofluoridated CKI1$_{RD}$ wild type and D1050A and D1050E mutants were 0.237 and 0.190 Å, respectively. With the exception of the mutated residues, the mutant structures differed from the wild type in the orientation of the Phe-1102 side chain that was rotated by $\sim$80° and exposed as inactive RDs of bacterial RR. Furthermore, we detected slight displacements of the backbone atoms of residues 1104–1106 of $\sim$2 Å and variations of some torsion angles between Lys-1105 and Phe-1102 ($\phi$ and $\psi$ of Asp-1104 and $\psi$ of Leu-103 changed by $\sim$25° and $\psi$ of Phe-1102 changed by 15° by the mutations, Fig. 3, B–D). The $\epsilon$ amine of Lys-1105 directly interacts with the active site in the crystal structures. Orientations of the side chains of residues surrounding Phe-1102 in the crystal were not changed by the mutations. Presumably, minor perturbations introduced by mutating or coordinating Asp-1050 are propagated through the Lys-1105 side chain and backbone of residues Asp-1104 and Leu-1103 and alter the populations of the Phe-1102 rotamers in favor of the buried state in the mutants.

To sum up, whereas wild-type CKI1$_{RD}$ exhibits the “active” side-chain orientation of Phe-1102, mutations in phosphoaccepting Asp-1050 change its orientation to the position found in most bacterial RR in their inactive state.

Chemical shifts indicate that Phe-1102 is reoriented in solution upon phosphorylation

In contrast to RDs of bacterial RR, no biological role has been assigned to the reorientation of Phe-1102 in CKI1$_{RD}$. No significant changes in fast or slow dynamics were observed in the vicinity of Phe-1102 upon Mg$^{2+}$ binding or beryllofluoridation, and the same (active) orientation of Phe-1102 was found in crystal structures of all CKI1$_{RD}$ forms. In contrast, crystal structures of the D1050A and D1050E mutants revealed that the Phe-1102 orientation is sensitive to the active-site state. It should be stressed that none of the above-mentioned observations directly answers the question whether phosphorylation changes orientation of Phe-1102 in solution or not. We addressed this question by directly comparing chemical shifts of residues in various regions of CKI1$_{RD}$ mutants and forms, to avoid difficulties of NMR structure determination in the presence of multiple conformational states. Residues close to the active site are influenced by both chemical modifications and conformational changes (e.g. Asp-993 in Fig. 6 and Asn-994 in supplemental Fig. S9A, directly coordinating Mg$^{2+}$). Chemical shifts of such residues are mostly influenced by the presence of Mg$^{2+}$. Distant residues are not affected by direct chemical perturbations, and their chemical shifts reflect ensemble-averaged conformational changes. Analysis of the spectra revealed that chemical shifts of the residues distant from the active site, but close to Phe-1102 (e.g. Leu-103 in Fig. 6 and Asn-994 in supplemental Fig. S9A, and Gly-1098 in supplemental Fig. S9B), are similar for active-site mutants, free, and Mg$^{2+}$-bound wild-type, but greatly changed by beryllofluoridation. Taken together, our data suggests that Phe-1102 is reoriented in solution upon phosphorylation, presumably to its active orientation.

Functional studies show that perturbation of the conformational equilibria of the $\beta_3$–$\alpha_3$ loop suppresses the CKI1$_{RD}$ function

To prove the functional importance of the aforementioned structural changes, we performed a series of functional studies, both in vitro and in vivo. The histidine kinase domain of ETR1 (ETR1$_{HK}$) was used to monitor the ability of the wild-type CKI1$_{RD}$ and its mutants to mediate phosphorelay in vitro. Although phosphorylation of the D1050A and D1050E CKI1$_{RD}$ mutants was not observed, as expected (data not shown), phosphorylation of wild-type CKI1$_{RD}$ and CKI1$_{RD}$-mediated phosphate transfer to AHP2 was detected (Fig. 7, A and B). Importantly, the phosphorylation or phosphate transfer to AHP2 was not observed for the D1057E/G1058N CKI1$_{RD}$ mutant, with the
intact active-site Asp-1050 but perturbed conformational equilibrium of the $\beta_3-\alpha_3$ loop by the mutation resembling the ETR1$_{RD}$ sequence.

The effect of mutations in the $\beta_3-\alpha_3$ loop on the in vivo CKI1 activity was monitored in the Arabidopsis protoplast assay. CKI1 and its mutants were coexpressed together with the cytokinin-responsive MSP reporter TCS/LUC (23). CKI1, shown previously to activate MSP independently of the cytokinin treatment (24), triggered the expression of the TCS reporter independently of cytokinin treatment (Fig. 8), as reported previously (24, 25). Mutation of Phe-1102 to alanine did not have any significant effect. However, similarly to the mutations in the active-site aspartate (D1050A and D1050E), the double mutation D1057E/G1058N suppressed the cytokinin-independent CKI1 activity to the control level (Fig. 8).

In conclusion, D1057E/G1058N CKI1$_{RD}$ perturbed in conformational equilibrium of the $\beta_3-\alpha_3$ loop by the mutation resembling the ETR1$_{RD}$ sequence associates with suppression of biological activity in both the ability of CKI1$_{RD}$ to mediate transphosphorylation to the downstream CKI1 signaling partner and activate MSP signaling.

Discussion

Structural changes of CKI1$_{RD}$ and bacterial RDs upon activation

Several hundred RD structures are available in the PDB, including mostly the structures of bacterial RRs. The X-ray structures of bacterial (and yeast) RDs exhibit changes upon phosphorylation (berylliofluoridation) and divalent cation binding. The extent of the structural rearrangement varies among the RRs, but three general conformational changes can be identified as follows: rearrangement of the $\beta_3-\alpha_3$ loop; reorientation of the $\beta_4-\alpha_4$ loop; and rotation of a hydrophobic (typically aromatic) side chain in the $\beta_5$ strand. For the sake of simplicity, we limit the discussion of the mentioned structural changes to two prototypic bacterial RDs, chemotaxis RR CheY and Thermotoga maritima RR468.

The $\beta_3-\alpha_3$ loop of both phosphorylated and unphosphorylated RDs adopts an open “γ-turn loop” conformation (26) in the presence of divalent cations (Fig. 9). In this conformation, the phosphorylation site is quite accessible, and the conserved hydrophobic residue (typically methionine) in the position +3 (relative to the phosphorylation site) is buried in the interior of the domain. In the absence of divalent cations,
“closed” conformations of the β3–α3 loop have been observed, with the active-site aspartate buried, with the hydrophobic "+3" side chain exposed, and with the "+2" side chain oriented so that it precludes phosphorylation of the active-site aspartate (21, 27, 28). Although the metal ions seem to play a major role among factors determining the structure of the β3–α3 loop, the “open” conformation was also found in crystals grown in the absence of divalent cations (29).

Structural changes following the activation of metal-bound CheY are limited to Thr-87 and the β4–α4 loop (residues 88–91) and to the side-chain rotation of Tyr-106 in the β5 strand (corresponding to Phe-1102 in CKI1RD). The comparison of the structures of inactive and active wild-type CheY, and of a series of CheY mutants, led to the suggestion that the molecular mechanism underlying the RD activation, dubbed Y-T coupling (8, 9), represents a correlated motion of Thr-87 (forming a hydrogen bond with the phosphate group), β4–α4 loop (being pulled to the phosphate group), and Tyr-106 (rotating from the exposed g− to the buried g+ orientation). However, not all structural data support the Y-T coupling mechanism. For example, a mixture of inactive and active-like conformations of the β4–α4 loop and Tyr-106 were observed in a high-resolution crystal structure of unphosphorylated CheY (30).

Conformations observed in unphosphorylated crystal structures of AHK5RD and ETR1RD from A. thaliana seem to be determined by the same principles as outlined for bacterial RDs. The conformation of the β3–α3 loop (Fig. 9) is open in Mg2+-bound AHK5RD (31) and similar to the closed conformations of CheY and RR468 in metal-free ETR1RD (21). The residues corresponding to CheY Tyr-106 (Val-711 in ETR1RD and Phe-903 in AHK5RD) are exposed as in inactive bacterial RDs. The β4–α4 loop of ETR1RD resembles the inactive conformation of bacterial RDs. The structure of AHK5RD is known only in complex with AHP1, where the β4–α4 loop, close to the interaction surface, adopts a conformation different from any of those discussed above.

Unlike the bacterial RDs, all three forms of CKI1RD (metal-free, Mg2+-bound, and beryllofluoridated) crystallized with the open β3–α3 conformation, buried (active) Phe-1102, and the conformation of the β4–α4 loop and Ser-1082 (corresponding to T87 of CheY) resembling the inactive state of bacterial RDs. The determined structure can be viewed as the energetically most favorable state of CKI1RD in the crystal, adopted at low temperature (100 K), and possibly influenced by crystal packing. Although the wild-type CKI1RD crystal structures represent excellent evidence of the conformational selection mechanism (14–16, 32, 33), the conformational behavior of CKI1RD had to be derived from additional data. Analysis of NMR spectra revealed conformational changes in the β3–α3 loop and shift of populations of the rotameric states of Phe-1102 by beryllofluoridation. Analysis of the wild-type and mutant crystal structures suggests that the phosphorylation site and Phe-1102 are not coupled through Ser-1082 and the β4–α4 loop, but via Lys-K1105, as proposed in earlier studies of CheY (34).

Conformational dynamics of CKI1RD and bacterial RDs from NMR relaxation

The body of literature dedicated to the dynamics of the different forms of RDs is less extensive than structural reports. Among the bacterial RR0s, CheY (15, 35, 36) and nitrogen regulatory protein NtrC (NtrC\textsuperscript{B}) from Salmonella enterica (14, 16, 37) are the most studied ones, including studies of relaxation dispersion. NMR relaxation experiments showed that all investigated RDs exhibit similar dynamics on a fast, sub-nanosecond time scale. Metal binding, phosphorylation, or beryllofluoridation did not result in a dramatic change of the fast backbone dynamics in the β3–α3 loop in any of the studied RDs (14, 36, 38, 39). However, beryllofluoridation of CheY changed fast dynamics of side chains in the same regions where the slow conformational exchange (see below) was observed (36). In our study, we observed a small increase in the rigidity of the β3–α3 loop and in the adjacent loops upon beryllofluoridation of CKI1RD (Fig. 4), in agreement with the data published for other RDs.

Slow conformational dynamics results in the exchange contribution, given by exchange rates $k_{\text{ex}}$, populations $p_{\alpha}$, $p_{\beta}$ and chemical shift difference $\Delta \omega$ of the states. Exchange was observed in the β3–α3 loop, and in other regions that show structural differences between the inactive and active forms: the β4–α4 loop and the vicinity of Thr-87 and Tyr-106 in CheY (15, 36), and a much larger region between the β3–α3 loop and β5-sheet in NtrC\textsuperscript{B} (14, 16, 40). Beryllofluoridation largely suppresses the conformational exchange in CheY (15, 36) and NtrC\textsuperscript{B} (40).

The analysis of the relaxation dispersion data provided an opportunity to test the Y-T coupling model of bacterial RD activation, predicting the correlation of movements in the β4–α4 loop with the rotation of critical aromatic side chain in the β3 strand (Tyr-101 in CheY and Tyr-106 in NtrC\textsuperscript{B}). The variations in exchange rates and populations (supplemental Table S4), together with the lack of correlation between chemical shift changes upon beryllofluoridation and chemical shift differences obtained from the local fits of relaxation dispersion data, were interpreted as evidence for a role of segmental motions in the activation of CheY (15). Relaxation dispersion of NtrC\textsuperscript{B} revealed much faster exchange in its unphosphorylated form and partially activated mutants (40). In comparison, beryllofluoridated NtrC\textsuperscript{B} together with its transition-state mutants revealed exchange rates similar to the values reported for CheY (16, 40). In addition, a much faster dynamic process was detected and attributed to the side-chain rotation of Tyr-101 (16), indicating that the rotameric exchange is not correlated with the backbone rearrangement. To sum up, despite the significant differences between CheY and NtrC\textsuperscript{B}, relaxation dispersion experiments showed that both RR\textsubscript{0}s exhibit multiple uncorrelated modes of motions. This is in contrast to the previously proposed Y-T coupling model of activation.

Our NMR relaxation data revealed that conformational exchange in CKI1RD is strongly manifested in the β3–α3 loop but is negligible in other regions. It shows that CKI1RD differs in conformational dynamics from the bacterial RDs, discussed above (exhibiting strong conformational exchange also in other regions). The observed conformational dynamics of CKI1RD is
Conformational dynamics of the receiver domain of CKI1

also substantially different from another plant hybrid HKs, ETR1\textsubscript{RD}, crystallizing with the inactive closed conformation of the β3–α3 loop and exhibiting negligible conformational exchange in solution. This is in line with the recently described phosphorylation-independent mode of action of ETR1\textsubscript{RD} (22).

Computational studies of conformational changes of bacterial RDs

The potential role of the β3–α3 loop in the allosteric activation of bacterial RDs has been addressed in several computational studies. The conclusions mostly depend on the starting structures used. When the models of the inactive and active states were derived from structures with the open β3–α3 loop, dynamics in this loop did not play any significant role in the activation (40–43). The transitions between the active and inactive states occurred through multiple pathways, with intermediates stabilized by hydrophobic packing and non-native hydrogen bonds.

However, simulations started from the inactive state with the closed conformation of the β3–α3 loop indicated that conformational changes of this loop dominate the transition to the active state (44, 45). Our experimental observation of the conformational exchange in the β3–α3 loop suggests that similar transitions may occur also in CKI1\textsubscript{RD}.

Relation of the β3–α3 loop dynamics to the function of CKI1\textsubscript{RD}

Signal transduction mediated by RDs relies on interactions with a broad spectrum of proteins. Not surprisingly, the regions of conformational changes associated with RD activation (namely the α4–β5–α5 face and loops in the vicinity of the phosphorylation site) are also important for the interactions.

The β4–α4 loop and the residue corresponding to Tyr-106 of CheY are located in the α4–β5–α5 face involved in dimerization of RDs of bacterial RRs and their interactions with the effector domains/proteins. The rotameric state of Tyr-106 is influenced by some, but not all, proteins interacting with the α4–β5–α5 face of CheY. Tyr-106 of unphosphorylated CheY remains exposed in the complexes with the CheA P2 domain (46, 47) but is buried (as in the active form) in complexes with short fragments of its interacting partners, CheZ (48) and FltM (9). In CKI1\textsubscript{RD}, no conformational exchange influenced by beryllofluoridation was observed in the α4–β5–α5 face, but mutagenesis and chemical shift changes revealed that Phe-1102 of CKI1 undergoes similar allosteric changes as Tyr-106 of CheY. Biological relevance of this observation is unclear because proteins interacting with the α4–β5–α5 face of CKI1\textsubscript{RD} are not known.

The β3–α3 loop, exhibiting the most significant changes of dynamics upon beryllofluoridation of CKI1\textsubscript{RD}, marginally contributes to the binding site of downstream signaling partners (histidine-containing phosphotransfer proteins) and is the central element of the kinase/phosphatase binding face of RDs, crucial for the interactions with the ATP-binding domain of the upstream HKs. It seems natural that a region that is involved in interactions with different proteins (HKs and AHPs, with different binding interfaces) is dynamic, so that it can accommodate both the upstream and downstream interacting partners. It has been observed for a homologous yeast RD, SLN1-R1, that phosphorylation of RD leads to a conformational change optimal for phosphate transfer to the phosphotransfer protein YPD1 (49). Crystal structures of the eukaryotic RDs interacting with the HK domains are not available, but comparison with the SpoOF–SpoOB and HK853–RR468 co-crystals provides valuable homology models. The β3–α3 loop in its open conformation interacts with the ATP lid of the kinase, gluing together the assembly of the RR, catalytic domain, and dimerization domain of HK (28). High structural homology of individual bacterial and plant domains suggests similar interactions for CKI1 (supplemental Fig. S10). Remarkably, the complex of RR468 with HK853, with the open conformation of the RR468 β3–α3 loop, is formed even in the absence of divalent cations (28), implying that the interaction of the RR with its cognate HK might replace the effect of Mg\textsuperscript{2+} and stabilize the β3–α3 loop in the open conformation. Thus, the changes of the conformational exchange in the β3–α3 loop observed in CKI1\textsubscript{RD} may reflect shifts of conformer populations important for interactions of CKI1\textsubscript{RD} with the ATP-binding sub-domain of CKI1\textsubscript{HK}. It has been shown previously (14, 40) that phosphorylation shifts populations of already existing inactive and active conformations of NtrC\textsuperscript{b} by stabilizing the active state. Our NMR data indicate that a similar conformational selection takes place also in the case of CKI1\textsubscript{RD} and that the conformational exchange in the β3–α3 loop of CKI1\textsubscript{RD} is not an accident but manifestation of conformational equilibrium optimal for the proper function of CKI1. The conformational energy landscape of the loop is such that phosphorylation is able to stabilize the active state, and interactions with the kinase domain and AHPs presumably select conformations optimal for accepting and donating the phosphate group, respectively. The conformational freedom can be viewed as a mechanism allowing the unphosphorylated/phosphorylated β3–α3 loop to be involved in interactions with multiple proteins, with distinct affinities and slightly different binding surfaces. This hypothesis also provides a physical explanation of results of the in vitro phosphorylation assay and of the (in)ability of CKI1 and its mutants to activate the cytokinin-responsive reporter TCS/LUC in plant protoplasts. I both experiments, the activities exhibited by the wild-type were eliminated by mutating not only Asp-1050 but also the pair Asp-1057 and Gly-1058 (Figs. 7 and 8). These mutations suppress the activities in different ways. Although Asp-1050 is directly phosphorylated and coordinates the Mg\textsuperscript{2+} ion, Asp-1057 and Gly-1058 are relatively distant from the phosphorylation site (over 6 Å) and do not coordinate Mg\textsuperscript{2+} or BeF\textsuperscript{3-} in the wild type (Fig. 1 and supplemental Table S1). However, the D1057E/G1058N mutations strongly affect conformational energy landscape of the β3–α3 loop (the mutated residues have very different energetically favored areas in the Ramachandran diagram). The conformation ensemble of the loop in the Asp-1057 and Gly-1058 mutant thus seems to be affected in such a way that interactions with HK and/or phosphorylation of Asp-1050 no longer able to stabilize sufficiently the conformation(s) needed for the transfer of the phosphate group to CKI1\textsubscript{RD}, preventing the CKI1-mediated signaling downstream through MSP, as evidenced by both in vitro and in vivo assays.

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**Experimental procedures**

**CKI1<sub>RD</sub> constructs**

The CKI1<sub>RD</sub> construct containing the receiver domain and an N-terminal 60-amino acid region mostly invisible in crystal structures (7) was prepared as described earlier (50). Additionally, a shorter construct, containing only the receiver domain was tested as well, but, even though it was expressed as a well-folded protein sufficiently stable to perform backbone NMR assignment, the degradation of the sample was observed several days after the expression and crystallization attempts failed. This suggests that the N-terminal sequence plays an important stabilizing role in vitro. This conclusion is supported by an observation that a stretch of residues SGLVP, in the N-terminal (met to 10 mg/ml, and a reservoir solution containing 0.1M MES (Duchefa), 2.54M (NH₄)₂SO₄ (Lachner), 11.8% glycerol (Sigma), 70 mM NaF (Sigma), and 10 mM MgCl₂ (Merck) to 30 mM MgCl₂.

The crystallization of the beryllofluoridated form and D1050A mutants of CKI1<sub>RD</sub> was performed as described for free and Mg<sup>2+</sup>-bound CKI1<sub>RD</sub> (50). Following the optimization, the crystals were obtained by the hanging drop method, by mixing equal volumes of protein solution at the concentrations of 7.5 to 10 mg/ml, using 0.5-ml Eppendorf tube with Nanosep<sup>®</sup> centrifugal filters, with the molecular mass cutoff of 3 kDa (Pall Corp.). The crystallization of the beryllofluoridated form and D1050A and D1050E mutants of CKI1<sub>RD</sub> was performed as described for free and Mg<sup>2+</sup>-bound CKI1<sub>RD</sub> (50). Following the optimization, the crystals were obtained by the hanging drop method, by mixing equal volumes of protein solution at the concentrations of 7.5 to 10 mg/ml, using 0.5-ml Eppendorf tube with Nanosep<sup>®</sup> centrifugal filters, with the molecular mass cutoff of 3 kDa (Pall Corp.). The crystallization of the beryllofluoridated form and D1050A and D1050E mutants of CKI1<sub>RD</sub> was performed as described for free and Mg<sup>2+</sup>-bound CKI1<sub>RD</sub> (50). Following the optimization, the crystals were obtained by the hanging drop method, by mixing equal volumes of protein solution at the concentrations of 7.5 to 10 mg/ml, using 0.5-ml Eppendorf tube with Nanosep<sup>®</sup> centrifugal filters, with the molecular mass cutoff of 3 kDa (Pall Corp.). The crystallization of the beryllofluoridated form and D1050A and D1050E mutants of CKI1<sub>RD</sub> was performed as described for free and Mg<sup>2+</sup>-bound CKI1<sub>RD</sub> (50). Following the optimization, the crystals were obtained by the hanging drop method, by mixing equal volumes of protein solution at the concentrations of 7.5 to 10 mg/ml, using 0.5-ml Eppendorf tube with Nanosep<sup>®</sup> centrifugal filters, with the molecular mass cutoff of 3 kDa (Pall Corp.). The crystallization of the beryllofluoridated form and D1050A and D1050E mutants of CKI1<sub>RD</sub> was performed as described for free and Mg<sup>2+</sup>-bound CKI1<sub>RD</sub> (50). Following the optimization, the crystals were obtained by the hanging drop method, by mixing equal volumes of protein solution at the concentrations of 7.5 to 10 mg/ml, using 0.5-ml Eppendorf tube with Nanosep<sup>®</sup> centrifugal filters, with the molecular mass cutoff of 3 kDa (Pall Corp.).

**X-ray crystallography**

CKI1<sub>RD</sub> was beryllofluoridated by the addition of 9 mM BeCl₂ (Sigma), 70 mM NaF (Sigma), and 10 mM MgCl₂ (Merck) to ~1.0 mg/ml of the protein solution (51). The sample was equilibrated for 45 min at room temperature and concentrated to 7.5 mg/ml, using 0.5-ml Eppendorf tube with Nanosep<sup>®</sup> centrifugal filters, with the molecular mass cutoff of 3 kDa (Pall Corp.). The crystallization of the beryllofluoridated form and D1050A and D1050E mutants of CKI1<sub>RD</sub> was performed as described for free and Mg<sup>2+</sup>-bound CKI1<sub>RD</sub> (50). Following the optimization, the crystals were obtained by the hanging drop method, by mixing equal volumes of protein solution at the concentrations of 7.5 to 10 mg/ml, using 0.5-ml Eppendorf tube with Nanosep<sup>®</sup> centrifugal filters, with the molecular mass cutoff of 3 kDa (Pall Corp.). The crystallization of the beryllofluoridated form and D1050A and D1050E mutants of CKI1<sub>RD</sub> was performed as described for free and Mg<sup>2+</sup>-bound CKI1<sub>RD</sub> (50). Following the optimization, the crystals were obtained by the hanging drop method, by mixing equal volumes of protein solution at the concentrations of 7.5 to 10 mg/ml, using 0.5-ml Eppendorf tube with Nanosep<sup>®</sup> centrifugal filters, with the molecular mass cutoff of 3 kDa (Pall Corp.). The crystallization of the beryllofluoridated form and D1050A and D1050E mutants of CKI1<sub>RD</sub> was performed as described for free and Mg<sup>2+</sup>-bound CKI1<sub>RD</sub> (50). Following the optimization, the crystals were obtained by the hanging drop method, by mixing equal volumes of protein solution at the concentrations of 7.5 to 10 mg/ml, using 0.5-ml Eppendorf tube with Nanosep<sup>®</sup> centrifugal filters, with the molecular mass cutoff of 3 kDa (Pall Corp.). The crystallization of the beryllofluoridated form and D1050A and D1050E mutants of CKI1<sub>RD</sub> was performed as described for free and Mg<sup>2+</sup>-bound CKI1<sub>RD</sub> (50). Following the optimization, the crystals were obtained by the hanging drop method, by mixing equal volumes of protein solution at the concentrations of 7.5 to 10 mg/ml, using 0.5-ml Eppendorf tube with Nanosep<sup>®</sup> centrifugal filters, with the molecular mass cutoff of 3 kDa (Pall Corp.).

**NMR spectroscopy**

The uniformly <sup>15</sup>N- and <sup>13</sup>C,<sup>15</sup>N-labeled and selectively <sup>18</sup>O-labeled CKI1<sub>RD</sub> samples were prepared according to Ref. 7, using M9 minimal medium supplemented with [1<sup>3</sup>C]glucose (Cambridge Isotope Laboratories) and/or [1<sup>5</sup>N]NH₄Cl (Cambridge Isotope Laboratories) for uniform labeling, and M9 medium supplemented with 100 mg of each unlabeled amino acid except methionine, for the selective labeling. One hour after the induction, 2 g of glutamine (Sigma) and glutamic acid (Sigma), 1 g of other unlabeled amino acids (Sigma) except methionine, and 100 mg of [1<sup>5</sup>N]methionine (Cortecnet) were added. Cells were harvested after 2 h and purified as described above.

Samples consisting of 0.6 mM uniformly <sup>15</sup>N-labeled CKI1<sub>RD</sub> in 20 mM Tris-HCl buffer (Serva), pH 7.5, containing 150 mM sodium chloride (Lachner), 1 mM EDTA (Lachner), and 10% deuterium oxide (Lachner) were used for the NMR relaxation studies of metal-free Mg<sup>2+</sup>-, Mg<sup>2+</sup>-bound CKI1<sub>RD</sub> samples, used in the relaxation experiments and for Be<sup>2+</sup> titration, were prepared by adding 30 mM MgCl₂ to the free CKI1<sub>RD</sub> sample. The dissociation for Mg<sup>2+</sup>- was determined previously to be (0.43 ± 0.06) mM (7). Titration with beryllium chloride and sodium fluoride, up to 50 mM concentration of Be<sup>2+</sup>, was performed. The dissociation constant, determined from the chemical shift changes observed in the H<sup>1</sup>-<sup>15</sup>N HSQC spectra (60, 61), was shown to be (4.8 ± 0.8) mM. The beryllofluoridated samples, used for chemical shift assignment and relaxation studies, contained 50 mM MgCl₂ and 150 mM NaF in addition to 30 mM MgCl₂.

The sample of 0.6 mM uniformly <sup>15</sup>N-labeled ETR1<sub>RD</sub> was prepared according to Ref. 62, using M9 medium supplemented with [1<sup>5</sup>N]NH₄Cl and pYRSF53LA (University of Helsinki) as a vector. The sample used in the relaxation dispersion experiment consisted of 0.6 mM <sup>15</sup>N-ETR1<sub>RD</sub>, 50 mM sodium phosphate (Merck) buffer, pH 6.0, 1 mM dithiothreitol (Duchefa), and 10% deuterium oxide.

NMR spectra were acquired at 25 °C using a Bruker Avance III HD 600 MHz spectrometer, equipped with the cryogenic (H<sup>1</sup>-<sup>31</sup>P–<sup>13</sup>C–<sup>15</sup>N) inverse probe head (Bruker), a Bruker Avance III HD 700 MHz spectrometer equipped with cryogenic (H<sup>1</sup>-<sup>15</sup>N) probe head optimized for T<sup>1</sup>C detection, a Bruker Avance III HD 850 MHz spectrometer equipped with the cryogenic (H<sup>1</sup>-<sup>31</sup>P–<sup>13</sup>C–<sup>15</sup>N) inverse probe head, and a Bruker Avance III HD 950 MHz spectrometer equipped with the cryogenic (H<sup>1</sup>-<sup>15</sup>N) inverse probe head.

(56) using the metal-free CKI1<sub>RD</sub> (PDB code 3MM4) as a structural template (7). Crystallographic refinement was carried out with the Refmac program (57), and manual model building was achieved using Coot (58). The incorporation of BeF<sub>3</sub> and Mg<sup>2+</sup> was performed following the inspection of mFo – DF<sub>o</sub>-weighted maps. Water molecules were introduced automatically using Coot and inspected manually. Alternative conformations were constructed where necessary (with occupancies estimated from the refined relative B-factors of the conformations). The stereochemical quality of the models was assessed with the Molprobity program (59). Protein structure images were prepared using PyMOL (Schrödinger, Inc.).
**NMR assignment**

Backbone amide signals of two methionine residues in the β3–α3 loop in free CKI1RD, broadened beyond the detection limit in standard NMR experiments (7), were identified by performing an 1H–15N HSQC experiment, with a large number of scans and with a reduced length of the polarization transfer periods. The relaxation-optimized 2D 1H–15N HSQC experiment (60, 61) of the free [15N]Met-labeled CKI1RD was recorded at 600 MHz with 96 increments in the indirect dimension and with 1024 scans per increment. The lengths of the increment and polarization transfer delay were set to 0.2 and 2.8 ms, respectively. Under these conditions, two additional broad signals were observed (supplemental Fig. S3B). The comparison with the chemical shift changes during the last steps of the Mg2+-titration allowed us to assign these signals to Met-1053 and Met-1056. Six of eight non-proline residues in the β3–α3 loop of Mg2+-bound CKI1RD were assigned recently using amino acid selective labeling and triple resonance experiments with relatively short polarization transfer periods (63).

HNCA, HN(CO)CA, HNCACB, and CBCA(CO)NH experiments (64–66) were used to assign backbone resonances of 13C,15N-labeled beryllofluoridated CKI1RD. The same experiments were applied to assign 1H,15N spectra of the CKI1RD mutants.

**NMR relaxation**

Standard experiments (10) were used for the measurements of $R_1$ at 600 MHz, with relaxation delays of 22.4*, 112, 268.8, 470.4*, 761.6*, 1120*, 1792, 2688*, 3696, and 4704* ms, and of $R_2$ at 600 MHz and 850 MHz, with relaxation delays of 0, 14.4*, 28.8, 43.2, 57.6*, 72, 86.4*, 100.8, 115.2*, 144*, and 172.8 ms (the asterisk denotes the spectra recorded twice). The $R_2$ rates were measured with the delay between the 180° pulses in the CPMG train equal to 0.9 ms. The heating of the sample was compensated for by lowering the temperature setting by a value determined using the NMR thermometer function of the Bruker Topspin software. Relaxation rates measured by varying relaxation delays were obtained by fitting the peak intensities to a mono-exponential decay using the Relax program, version 3.3.8 (67, 68). Bootstrap re-sampling was used to estimate the experimental errors of $R_1$ and $R_2$ (69). The 1H,15N steady-state nuclear Overhauser effect (70) was measured at 600 MHz with a 12-s inter-scan relaxation delay; the steady state was achieved using 226 repeats of 180° 1H pulses, separated by 22.22-ms delay. The reference spectra were measured interleaved together with the spectra under the steady-state conditions. All experiments were performed at 25 °C. The experimental error was obtained from three independent measurements.

The complete model-free (11–13, 71) analysis was performed using the Relax program, version 3.3.8 (67, 68). Briefly, the protocol started by optimizing parameters $\tau_m$ (a local correlation time approximating the effect of the overall rotational diffusion individually for each residue), $S_m^0$ (Lipari and Szabo generalized order parameter), $\tau_e$ (effective correlation time in the original model-free formula), $S_m^0$ and $\tau_e$ (amplitude and effective correlation time of the faster motion in the extended model-free formula, respectively), $\tau_f$ (effective correlation time of the slower motion in the extended model-free formula), and $R_{ex}$ (slow exchange contribution), defining models $tm0$ to $tm9$ as follows: $tm0 = \{\tau_m; \tau_f, R_{ex}\}$; $tm2 = \{\tau_m, S_m^0, \tau_e\}$; $tm3 = \{\tau_m, S_m^0, S_m^2, R_{ex}\}$; $tm4 = \{\tau_m, S_m^0, \tau_f, R_{ex}\}$; $tm5 = \{\tau_m, S_m^0, S_m^2, \tau_f, R_{ex}\}$; $tm6 = \{\tau_m, S_m^0, S_m^2, \tau_f, \tau_e, R_{ex}\}$; $tm7 = \{\tau_m, S_m^0, S_m^2, \tau_f, \tau_e, \tau_f, R_{ex}\}$; and $tm8 = \{\tau_m, S_m^0, S_m^2, \tau_f, \tau_e, \tau_f, \tau_e, R_{ex}\}$. Then, failed models were eliminated, and the best model was selected using Akaike’s Information Criterion (AIC). Then, $\tau_m$ was removed from the models; the remaining parameters were fixed; and parameters describing the overall rotational diffusion (isotropic, axial, and rhombic components of the diffusion tensor and Euler angles defining its orientation) were optimized. In the next step, the parameters of the overall rotational diffusion were fixed and the internal parameters $S_m^0$, $\tau_f$, $\tau_e$, and $R_{ex}$ were optimized for models $tm0$ to $tm9$, corresponding to models $tm0$ to $tm9$, respectively, without $\tau_m$. The best model was identified using AIC. The last two steps were repeated iteratively, until the convergence was reached. Finally, AIC was applied to find the model of the overall rotational diffusion (ellipsoid, prolate spheroid, oblate spheroid, or local $\tau_m$ fit), which provided the best description of the dynamics. Prolate, ellipsoid, and oblate models of rotational diffusion were selected by the Relax program for free, Mg2+-bound, and beryllofluoridated CKI1RD, respectively. However, the estimated parameters of the ellipsoid model ($D_1/D_3$ of 1.21, 1.13, and 1.10, and asymmetry of 0.28, 0.34, and 0.06, for free, Mg2+-bound, and beryllofluoridated CKI1RD, respectively) revealed that the rotational diffusion of all three forms was close to isotropic. Small differences between the isotropic components of the rotational diffusion tensor (12.1, 12.0, and 12.8 ns$^{-1}$ for free, Mg2+-bound, and beryllofluoridated CKI1RD, respectively) most likely reflect the variations in viscosity and heating of the samples of different ionic strengths, rather than biologically relevant changes. The model-free parameters were obtained for 74 (93), 78 (97), and 69 (90), respectively, and backbone NH groups of the free, Mg2+-bound, and beryllofluoridated forms of CKI1RD consisting of 127 non-proline amino acids (the numbers in parentheses refer to the whole construct containing 198 backbone NH groups). 10 (36), 10 (37), and 8 (32) residues, respectively, were not assigned; 43 (69), 39 (64), and 50 (76) residues could not be analyzed due to peak overlaps and/or low signal intensity or because the model $m0$ was selected during the model-free protocol.

**NMR relaxation dispersion**

The slow conformational exchange was studied using the relaxation-compensated CPMG relaxation dispersion experiment (18, 19), with the CPMG frequency ranging from 50 to 1000 Hz and the relaxation delays of 0, 18, and 36 ms. The relaxation delays were shortened to 0, 9, and 18 ms, and the CPMG frequency was extended to 2000 Hz in some experiments, which did not lead to a significant sample heating. The relaxation dispersion analysis of free wild-type CKI1RD was performed at 600 MHz; experiments in the presence of 30 mM MgCl$_2$ and of the selectively [15N]-Met-labeled D1050A and D1050E mutants were run at 600, 700, 850, and 950 MHz; the experiment in the presence of 1 mM MgCl$_2$ was run at 600 MHz; the experiments in the presence of 30 mM MgCl$_2$, 150 mM NaF, and 50 mM BeCl$_2$ were performed at 600 and 950 MHz; and the experiment in the presence of 30 mM MgCl$_2$, 15 mM NaF, and 5
The CPMG experiment at 950 MHz. The CPMG experiment at the concentration of 30 mM MgCl₂, at 950 MHz, was repeated with selectively [¹⁵N]Met-labeled CKI1RD to resolve the overlap of Met-1056 with Val-1013. All experiments were performed at 25 °C.

The data were analyzed using the auto-analysis script in Relax, version 4.0.0 (72), modified to suit our needs. First, the R₂, eff values were obtained by fitting the peak intensities to a two-parameter exponential decay and used in subsequent analyses with various CPMG models. The R₂, eff optimization was done using Newton minimization with no constraints, and all other models were optimized with Nelder-Mead simplex method with the default constraints. The optimizations were preceded by grid search (with 11, 9, or 7 increments) with the default bounds. The following models implemented in Relax were used: R2eff; No Rex; LM63; CR72; CR72 full; IT99; B14; B14 full; NS CPMG 2-site expanded; NS CPMG 2-site 3D; NS CPMG 2-site 3D full; NS CPMG 2-site star; NS CPMG 2-site star full; (see the Relax user’s manual for detailed description). The model selection was done using AIC. The numerical reduced NS 2-site 3D CPMG model and the analytical Luz-Meiboom model LM63 (73) were selected by the Relax auto-analysis for dispersion data of Mg²⁺-bound and beryllofluoridated CKI1RD, respectively.

**In vitro phosphorylation assay**

ETR1HK used in the phosphorylation assay, was prepared as follows. A plasmid containing a DNA fragment encoding the HK domain (residues 324–604) was constructed by PCR with primers 5'-TAGGATCCATGGACGCAATGTTGCTTT-G'- (BamHI site shown in bold) and 5'-GTTCACGCATTCCCGACATTAAGCCTTTACT-3' (HindIII site shown in bold). Amplified DNA was digested with BamHI and HindIII, ligated into the vector pGRSF05, and transformed into Escherichia coli strain DH5α (New England Biolabs) and finally into E. coli expression strain ER2566 (New England Biolabs). The cells carrying the expression plasmid were cultured with shaking in TB medium, pH 8, at 37 °C until an A₉₀₀ of 0.6 was reached. The expression of ETR1HK was then induced by adding isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.5 mM. After an overnight incubation at 18 °C, the cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. The cell pellet was resuspended in lysis buffer (50 mM Tris, pH 7.9, 0.5 mM NaCl, 10 mM imidazole, 10% glycerol, 2.5 mM MgCl₂, 0.1% Triton X-100, 1 mg/ml lysozyme), left on ice for 20 min, and disrupted by sonication. The suspension was centrifuged (20,000 rpm, 35 min, 4 °C), filtered, and loaded onto the HisTrap 5-ml column (GE Healthcare) equilibrated with a buffer containing 50 mM Tris, 0.5 mM NaCl, 10 mM imidazole, 10% glycerol, 2.5 mM MgCl₂, and 1 mg/ml lysozyme, dialyzed against the PBS buffer, cleaved overnight at 4 °C by UlpI protease, purified from the cleaved SUMO protein and thioredoxin on the HisTrap 5-ml column (GE Healthcare), and concentrated on a 10-kDa Amicon Ultra concentration filter (Merck). CKI1RD wild-type and mutants were prepared as described above, and AHP2 was produced as reported earlier (74).

Prior to the phosphate transfer reaction, ETR1HK was auto-phosphorylated with 32 nm γ-[³²P]ATP (600 Ci/mmol, Hartmann Analytic GmbH) in a buffer containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 5 mM MgCl₂, 5 mM MnCl₂, and 2 mM diethiothreitol for 90 min at room temperature. Residual ATP was removed with Zeba™ spin desalting columns, 7000 MWCO, 0.5 ml. The reaction was started by adding 2 μM auto-phosphorylated ETR1HK to samples containing 6 μM CKI1RD wild-type and mutants with and without 6 μM AHP2. The total volume of the reaction mixtures was 10 μl. Reactions were stopped at various times by addition of 4× SDS loading buffer supplemented with 80 mM EDTA. Samples were applied to a 15% SDS-polyacrylamide gel and separated by electrophoresis without being boiled. Incorporation of ³²P was monitored using Typhoon Fla-7000 Scanner (GE Healthcare). Coomassie Blue staining of the same gel was performed to verify the protein loading.

**TCS/LUC Arabidopsis protoplast assay**

CKI1 gene was synthesized by GenScript Co. and cloned using GATEWAY™ Cloning Technology. CKI1 mutants were generated as described above. Protoplasts from Arabidopsis leaves of Col-0 were prepared and transformed as described by Ref. 75. Protoplasts were cotransformed with TCS/LUC (23), 35S/Renilla (76), and either with mock (35S:GUS) or 35S:CKI1. Expression of the CKI1 wild type and mutants was confirmed by Western blotting. Transformed protoplasts were incubated for 15 h on light at room temperature and treated with either DMSO or 100 nM trans-zeatin for 1 h. Luminescence measurement was performed with the Dual-Luciferase Reporter Assay System kit (Promega), and the signal emitted was recorded using a GloMax-Multi detection system (Promega). The experiment was done in three independent biological replicates.

**Homology modeling**

The model of the CKI1 region 370–1120 was constructed in two steps. First, the temporary homology model of the corresponding CKI1 region was built based on the complex of T. maritima HK853 mutants A268V, A271G, T275M, V294T, and D297E with RR468 mutants V13P, L14I, I17M, and N21V, PDB code 4JAS (77), by using Modeler version 9v8 (78) and the sequence alignment by Clustal (79). Second, the presented crystal structure of beryllofluoridated CKI1RD, PDB code 5N2N, was superposed with the corresponding region within the homology model. The resulting model of CKI1 containing HK and RD is presented in Fig. 9. Regions of individual CKI1 domains were taken from the SMART database (80), and the graphical representation of the homology model was generated in PyMOL (Schrödinger, Inc.).

**Author contributions**—O. O., G. D., M. W., J. Hejátko, and L. Z. conceived and designed the research. O. O., S. J., B. P., and L. J. prepared CKI1RD samples. Z. J., A. S., H. L., and B. P. prepared ETR1HK samples. G. D., T. K., and J. M. optimized crystallization conditions and solved the X-ray structure. O. O., P. K., P. P., Z. J., M., and L. Z. acquired and analyzed NMR data. A. S., Z. G., and B. P. performed the functional studies. J. Hritzid did the homology modeling. O. O., G. D., and L. Z. wrote the manuscript. All authors reviewed and contributed to the manuscript.
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References

1. Schaller, G. E., Shiu, S. H., and Armitage, J. P. (2011) Two-component systems and their co-option for eukaryotic signal transduction. Curr. Biol. 21, R320–R330

2. Pekárová, B., Szmikitowska, A., Dopitová, R., Degtjarik, O., Žídek, L., Hejátko, J. (2015) Structural aspects of multistep phosphorelay-mediated signaling in plants. Mol. Plant. 9, 71–85

3. Robinson, V. L., Buckler, D. R., and Stock, A. M. (2000) A tale of two components: a novel kinase and a regulatory switch. Nat. Struct. Biol. 7, 626–633

4. Yan, D., Cho, H. S., Hastings, C. A., Igo, M. M., Lee, S. Y., Pelton, J. G., Stewart, V., Wemmer, D. E., and Kustu, S. (1999) Berylfluorochrome mimics phosphorylation of NtrC and other bacterial response regulators. Proc. Natl. Acad. Sci. U.S.A. 96, 14789–14794

5. Kern, D., Volkman, B. F., Luginbühl, P., Nohaile, M. J., Kustu, S., and Wemmer, D. E. (1999) Structure of a transiently phosphorylated switch in bacterial signal transduction. Nature 402, 894–898

6. Wilson, D., Pethica, R., Zhou, Y., Talbot, C., Vogel, C., Madera, M., Chothia, C., and Gough, J. (2009) Superfamily-simplified comparative genomics, data mining, visualization and phylogeny. Nucleic Acids Res. 37, D380–D386

7. Pekárová, B., Klumperl, T., Třísková, O., Horák, J., Jansen, S., Dopitová, R., Borkovcová, P., Papousková, V., Nejedlá, E., Sklenár, V., Marek, J., Žídek, Hejátko, J. I., and Janda, L. (2011) Structure and binding specificity of the receiver domain of sensor histidine kinase CKII from Arabidopsis thaliana. Plant J. 67, 827–839

8. Stock, A. M., and Guhaniyogi, I. (2006) A new perspective on response regulator activation. J. Bacteriol. 188, 7328–7330

9. Dyer, C. M., and Dahlquist, F. W. (2006) Switched or not?: the structure of unphosphorylated CheY bound to the N terminus of FlrM. J. Bacteriol. 188, 7354–7363

10. Korzhnev, D. M., Billeter, M., Arseniev, A. S., and Orekhov, V. Y. (2001) NMR studies of Brownian tumbling and internal motions in proteins. Prog. Nucl. Magn. Reson. Spectrosc. 38, 197–266

11. Lipari, G., and Szabo, A. (1982) Model-free approach to the interpretation of nuclear magnetic-resonance relaxation in macromolecules. 1. Theory and range of validity. J. Am. Chem. Soc. 104, 4546–4559

12. Lipari, G., and Szabo, A. (1982) Model-free approach to the interpretation of nuclear magnetic-resonance relaxation in macromolecules. 2. Analysis of experimental results. J. Am. Chem. Soc. 104, 4559–4570

13. Halle, B., Andersson, T., Forsén, S., and Lindman, B. (1981) Protein hydration from water oxygen-17 magnetic-relaxation. J. Am. Chem. Soc. 103, 500–508

14. Volkman, B. F., Lipson, D., Wemmer, D. E., and Kern, D. (2001) Two-state allosteric behavior in a single-domain signaling protein. Science 291, 2429–2433

15. McDonald, L. R., Boyer, J. A., and Lee, A. L. (2012) Segmental motions, not a two-state concerted switch, underlie allostery in CheY. Structure 20, 1363–1373

16. Villali, J., Pontiggia, F., Clarkson, M. W., Hagan, M. F., and Kern, D. (2014) Evidence against the “Y-T coupling” mechanism of activation in the response regulator NtrC. J. Mol. Biol. 426, 1554–1567

17. Loria, J. P., Rance, M., and Palmer, A. G. (1999) A relaxation-compensated Carr-Purcell-Meiboom-Gill sequence for characterizing chemical exchange by NMR spectroscopy. J. Am. Chem. Soc. 121, 1331–1332

18. Yip, G. N., and Zuidweg, E. R. (2004) A phase cycle scheme that significantly suppresses offset-dependent artifacts in the R1-CPMG 15N relaxation experiment. J. Magn. Reson. 171, 25–36

19. Long, D., Liu, M., and Yang, D. (2008) Accurately probing slow motions on millisecond timescales with a robust NMR relaxation experiment. J. Am. Chem. Soc. 130, 2432–2433

20. Janiak-Spens, F., Cook, P. F., and West, A. H. (2005) Kinetic analysis of YPD1-dependent phosphotransfer reactions in the yeast osmoregulatory phosphorelay system. Biochemistry 44, 377–386

21. Müller-Dieckmann, H. J., Grantz, A. A., and Kim, S. H. (1999) The structure of the signal receiver domain of Arabidopsis thaliana ethylene receptor ETR1. Structure 7, 1547–1556

22. Hung, Y. L., Jiang, I., Lee, Y. Z., Wen, C. K., and Sue, S. C. (2016) NMR study reveals the receiver domain of Arabidopsis ETHYLENE RESPONSOR1 ethylene receptor as an atypical type response regulator. PLOS ONE 11, e0160598

23. Müller, B., and Sheen, J. (2008) Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. Nature 453, 1094

24. Hwang, I., and Sheen, J. (2001) Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature 413, 383–389

25. Hejátko, J., Ryu, H., Kim, G. T., Dobesová, R., Choi, S., Choi, S. M., Soucek, P., Horák, J., Pekárová, B., Palme, K., Brzobohaty, B., and Hwang, I. (2009) The histidine kinases CYTOKININ-DEPENDENT1 and ARABIDOPSIS HISTIDINE KINASE2 and 3 regulate vascular tissue development in Arabidopsis shoots. Plant Cell 21, 2008–2021

26. Volz, K. (1993) Structural conservation in the CheY superfamily. Biochemistry 32, 11741–11753

27. Usher, K. C., de la Cruz, A. F., Dahlquist, F. W., Swanson, R. V., Simon, M. I., and Remington, S. J. (1998) Crystal structures of CheY from Thermotoga maritima do not support conventional explanations for the structural basis of enhanced thermostability. Protein Sci. 7, 403–412

28. Casino, P., Rubio, V., and Marina, A. (2009) Structural insight into partner specificity and phosphoryl transfer in two-component signal transduction. Cell 139, 325–336

29. Volz, K., and Matsumura, P. (1991) Crystal structure of Escherichia coli CheY refined at 1.7-Å resolution. J. Biol. Chem. 266, 15511–15519

30. Simonovic, M., and Volz, K. (2001) A distinct meta-activation conformation in the 1.1-angstrom resolution structure of wild-type apoCheY. J. Biol. Chem. 276, 28637–28640

31. Bauer, J., Reiss, K., Veerabagu, M., Heunemann, M., Harter, K., and Stehle, T. (2013) Structure-function analysis of Arabidopsis thaliana histidine kinase AHK5 bound to its cognate phosphotransfer protein AHP1. Mol. Plant 6, 959–970

32. Feher, V. A., and Cavanagh, J. (1999) Millisecond-time scale motions contribute to the function of the bacterial response regulator protein SpoF. Nature 400, 289–293

33. Formanek, M. S., Ma, L., and Cui, Q. (2006) Reconciling the "old" and "new" views of protein allosterism: a molecular simulation study of chemotaxis Y protein (CheY). Proteins 63, 846–867

34. Belsoléll, L., Prieto, J., Serrano, L., and Coll, M. (1994) Magnesium binding to the bacterial chemotaxis protein CheY results in large conformational changes involving its functional surface. J. Mol. Biol. 238, 489–495

35. Moy, F. J., Lowry, D. F., Matsumura, P., Dahlquist, F. W., Krywo, J. E., and Domaille, P. J. (1994) Assignments, secondary structure, global fold, and dynamics of chemotaxis-Y protein using three- and four-dimensional heteronuclear (13C,15N) NMR-spectroscopy. Biochemistry 33, 10731–10742

36. McDonald, L. R., Whiteley, M. J., Boyer, J. A., and Lee, A. L. (2013) Colocalization of fast and slow time scale dynamics in the allosteric signaling protein CheY. J. Mol. Biol. 425, 2327–2331

37. Otten, R., Villali, J., Kern, D., and Mulder, F. A. (2010) Probing microsecond time scale dynamics in proteins by methyl 1H Carr-Purcell-Meiboom-Gill relaxation dispersion NMR measurements. Application to activation of the signaling protein NtrC. J. Am. Chem. Soc. 132, 17004–17014

38. Sheftic, S. R., Garcia, P. P., White, E., Robinson, V. L., Gage, D. J., and Alexandrescu, A. T. (2012) Nuclear magnetic resonance structure and dynamics of the response regulator Smr0114 from Sinorhizobium meliloti. Biochemistry 51, 6932–6941
Conformational dynamics of the receiver domain of CKI1

39. Shefte, S. R., White, E., Gage, D. J., and Alexanderscu, A. T. (2014) NMR structure of the HWE kinase associated response regulator SmoI114 in its activated state. Biochemistry 53, 311–322

40. Gadino, A. K., Villal, J., Kivenson, A., Lej, M., Liu, C. F., Stiepnd, P., Eisenmesser, E. Z., Labeikovsky, W., Wolf-Watz, M., Clarkson, M. W., and Kern, D. (2009) Transient non-native hydrogen bonds promote activation of a signaling protein. Cell 139, 1109–1118

41. Vanatta, D. K., Skuda, D., Lawrenz, M., and Pande, V. S. (2015) A network of molecular switches controls the activation of the two-component response regulator NtrC. Nat. Commun. 6, 7283

42. Pontiggia, F., Pachov, D. V., Clarkson, M. W., Villal, J., Hagan, M. F., Pande, V. S., and Kern, D. (2015) Free energy landscape of activation in a signalling protein at atomic resolution. Nat. Commun. 6, 7284

43. Ma, L., and Cui, Q. (2007) Activation mechanism of a signaling protein at atomic resolution from advanced computations. J. Am. Chem. Soc. 129, 10261–10268

44. Khaliil, M., and Wales, D. J. (2008) Pathways for conformational change in nitrogen regulatory protein C from discrete path sampling. J. Phys. Chem. B 112, 2456–2465

45. Banerjee, R., Tan, H., and Cukier, R. I. (2015) Conformational transition in signal transduction: metastable states and transition pathways in the activation of a signaling protein. J. Phys. Chem. B 119, 6591–6602

46. Welch, M., Chinar, R., Mourey, L., Birck, C., and Samama, J. (1998) Structure of the CheY-binding domain of histidine kinase CheA in complex with CheY. Nat. Struct. Biol. 5, 25–29

47. Park, S. Y., Beel, B. D., Simon, M. I., Bilows, A. M., and Crane, B. R. (2004) In different organisms, the mode of interaction between two signaling proteins is not necessarily conserved. Proc. Natl. Acad. Sci. U.S.A. 101, 11646–11651

48. Guhaniyogi, J., Robinson, V. L., and Stock, A. M. (2006) Crystal structures of beryllium fluoride-free and beryllium fluoride-bound CheY in complex with the conserved C-terminal peptide of CheZ reveal dual binding modes specific to CheY conformation. J. Mol. Biol. 359, 624–645

49. Zhao, X., Copeland, D. M., Soares, A. S., and West, A. H. (2008) Crystal structures of a complex between the phosphorelay protein YPD1 and the phosphorylated phosphorelay domain of SLN1 bound to a phosphorylated analog. J. Mol. Biol. 375, 1141–1151

50. Klumpler, T., Pekárová, B., Marek, J., Borkovcová, P., Janda, L., and Héjátko, J. (2009) Cloning, purification, crystallization and preliminary X-ray analysis of the receiver domain of the histidine kinase CKI1 from Arabidopsis thaliana. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 65, 478–481

51. Barbieri, C. M., Mack, T. R., Robinson, V. L., Miller, M. T., and Stock, A. M. (2010) Regulation of response regulator autophosphorylation through interdomain contacts. J. Biol. Chem. 285, 32325–32335

52. Mueller, U., Darowski, N., Fuchs, M. R., Förster, R., Helligm, M., Paimthanka, K. S., Puhringer, S., Steffen, M., Zocher, G., and Weiss, M. S. (2012) Facilities for macromolecular crystallography at the Helmholtz-Zentrum Berlin. J. Synchrotron Radiat. 19, 442–449

53. Kabsch, W. (2010) XDS. Acta Crystallogr. D Biol. Crystallogr. 66, 125–132

54. Krug, M., Weiss, M. S., Heinemann, U., and Mueller, U. (2012) XDSAPP: a graphical user interface for the convenient processing of diffractometry data using XDS. J. Appl. Crystallogr. 45, 568–572

55. Wynn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keghan, R. M., Krissinel, E. B., Leslie, A. G., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterton, E. A., Powell, H. R., et al. (2011) Overview of the CCP4 suite and current developments. Acta Crystallogr. D Biol. Crystallogr. 67, 235–242

56. Vain, A., and Teplyakov, A. (2010) Molecular replacement with molrep. Acta Crystallogr. D Biol. Crystallogr. 66, 22–25

57. Vain, A. A., Steiner, R. A., Lebedev, A. A., Potterton, L. M., McNicholas, S., Long, F., and Murshudov, G. N. (2004) Refmac5 dictionary: organization of prior chemical knowledge and guidelines for its use. Acta Crystallogr. D Biol. Crystallogr. 60, 2184–2195

58. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501
Conformational dynamics of the receiver domain of CK11

76. Després, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., and Fobert, P. R. (2003) The Arabidopsis NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. *Plant Cell* 15, 2181–2191

77. Podgornaia, A. I., Casino, P., Marina, A., and Laub, M. T. (2013) Structural basis of a rationally rewired protein-protein interface critical to bacterial signaling. *Structure* 21, 1636–1647

78. Sali, A., and Blundell, T. L. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* 234, 779–815

79. Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* 31, 3497–3500

80. Letunic, I., Doerks, T., and Bork, P. (2015) SMART: recent updates, new developments and status in 2015. *Nucleic Acids Res.* 43, D257–D260