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Phytosulfokine (PSK) is a phytohormone responsible for cell-to-cell communication in plants, playing pivotal role in plant development and growth. The binding of PSK to its cognate receptor, PSKR1, is modulated by the formation of a binding site located between leucine-rich repeat (LRR) domain of PSKR1 and the loop located in the receptor’s island domain (ID). The atomic resolution structure of the extracellular PSKR1 bound to PSK has been reported, however, the intrinsic dynamics of PSK binding and the architecture of PSKR1 binding site remain to be understood. In this work, we used atomistic molecular dynamics (MD) simulations and free energy calculations to elucidate how the PSKR1 island domain (ID) loop forms and binds PSK. Moreover, we report a novel “druggable” binding site which could be exploited for the targeted modulation of the PSKR1-PSK binding by small molecules. We expect that our results will open new ways to modulate the PSK signalling cascade via small molecules, which can result in new crop control and agricultural applications.

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Controlling the heterodimerisation of the phytosulfokine receptor 1 (PSKR1) via island loop modulation

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Abstract

Phytosulfokine (PSK) is a phytohormone responsible for cell-to-cell communication in plants, playing a pivotal role in plant development and growth. The binding of PSK to its cognate receptor, PSKR1, is modulated by the formation of a binding site located between a leucine-rich repeat (LRR) domain of PSKR1 and the loop located in the receptor’s island domain (ID). The atomic resolution structure of the extracellular PSKR1 bound to PSK has been reported, however, the intrinsic dynamics of PSK binding and the architecture of the PSKR1 binding site remain to be understood. In this work, we used atomistic molecular dynamics (MD) simulations and free energy calculations to elucidate how the PSKR1 island domain (ID) loop forms and binds PSK. Moreover, we report a novel “druggable” binding site which could be exploited for the targeted modulation of the PSKR1-PSK binding by small molecules. We expect that our results will open new ways to modulate the PSK signalling cascade via small molecules, which can result in new crop control and agricultural applications.

Introduction

Molecular signalling exerted through peptide hormones plays a key part in regulating plant growth and reproduction[1–3]. These phytohormones are also responsible for an environmental response crucial to plant survival and adaptation to different conditions[2,4]. One of such hormones is phytosulfokine (PSK): a sulfated peptide which is biologically active in nanomolar concentrations[5,6]. Comprised of five residues (Tyr(SO₃H)-Ile-Tyr(SO₃H)-Thr-Gln)[7,8], of which both tyrosines are sulfated, PSK has been classified as a crucial plant growth factor[3,9,10].

PSK is matured by the proteolytic cleavage of its precursors and then goes through a post-translational sulfanation of its tyrosine residues[11]. Its main binding partners are the phytosulfokine receptors (PSKR)[12,13]. These receptors were first identified on Daucus carota, and its gene is conserved among different plants species[7,10]. One of
these is *Arabidopsis thaliana*, which expresses two different orthologues: PSKR1[14] and PSKR2[15], with the PSK sensing occurring mainly through PSKR1[15].

PSKR1 belongs to the leucine-rich repeat receptor kinases (LRR-RK) family. It is comprised of two large domains: residues 75 to 619 contain the leucine-rich repeat (LRR) domain, and the region located within residues 734 to 1005 comprises the protein kinase domain. In 2015, Wang and coworkers solved the crystal structure of a series of different PSKR1 LRR domain configurations[16]. They showed that the PSKR1 LRR domain is comprised of a large right-handed superhelical region, which is comprised of 20 LRRs, and an island domain (ID)[16]. They also solved the structure of the PSKR1 LRR domain bound to PSK and the PSKR1/LRR-PSK complex bound to the somatic embryogenesis receptor-like kinase 1 (SERK1).

The PSKR1 LRR domain binds its partner through a different mode than other hormone-dependent LRR-RK. For example, the brassinosteroid-insensitive 1 (BRI1) [17,18] and the flagellin insensitive 2 (FLS2)[19] are LRR-RKs which are also comprised of a right-hand LRR superhelix. These three proteins are structurally similar and their signalling pathways are highly related[20]. Both BRI1 and FLS2 bind their respective hormone (brassinolide or flg22) as heterodimers, with the binding site occurring between LRR-RK and their respective heterodimerisation partner. They are receptor kinases for BRI1-associated kinase 1 (BAK1) and have a role in mediating the LRR–BAK1 interaction[18,21]. The signalling events mediated by these two receptors require their ligands to be bound within the dimerisation interface, as shown in Figure 1A and Figure 1B. In the event of a PSK-PSKR1-SERK1 complex forming, the PSK interacts solely with PSKR1. The binding event stabilises the loop within the island domain on PSKR1, without any direct interactions between PSK and SERK1[16] (Figure 1C), but the details of the formation of this complex remain poorly understood.

![Figure 1: A) BRI-BAK2 complex: BAK1 is coloured blue, BRI1 is coloured orange, the brassinolide is coloured green (PDB Code: 4M7E). B) FLS2-BAK1 complex: BAK2 is coloured blue, FLS2 is coloured orange, the flg22 is coloured green (PDB Code: 4MN8). C) PSKR1-SERK1 complex: the SERK1 is coloured blue, the PSKR1 is coloured orange, the PSK is coloured green.](https://example.com/figure1.png)
In this work, we used atomistic molecular dynamics (MD) simulations and enhanced sampling techniques to study how the PSKR1-PSK binding event occurs at the nanoseconds to microseconds time scale. We have proposed a sequential model of PSKR1-PSK binding, which goes through a transient, local minimum denoted as state B. We have shown that state B creates a novel allosteric “druggable” binding site, the formation of which is dependent on PSK. This binding site shows characteristics which can be used for structure-guided design of small molecular modulators of PSK-PSKR1 signalling, which may prove useful for agricultural applications[16].

Methodology

Molecular modelling

The holo A. thaliana PSKR1-PSK complex simulation started from the experimental structure (PDB code: 4Z63). The unstructured apoPSKR1 from Daucus Carota started from the structure reported in the PDB code: 4Z62. Both models have been checked for any missing residues or double-occupancy atoms, which were amended. Any missing segments were modelled using MODELLER[22] interface in UCSF Chimera[23]. All glycosylated residues were kept.

Equilibrium molecular dynamics (MD) simulations

All simulations were performed using GROMACS 2019[24]. The equilibrium simulations were run in 3 sets: (1) an apo unstructured loop from Daucus Carota (DcPSKR1), (2) a holo PSKR1-PSK from Arabidopsis thaliana (PSKR1), and (3) an apo configuration generated after removing the PSK from A. thaliana holo-PSKR1. All three systems were parametrised using the CHARMM36m[25] force field with automated identification of the glycans[26–28] and immersed in the cubic box of TIP3P[29] water model using CHARMM-GUI web server[30]. Box distance was set to 1 nm and standard 3D periodic boundary conditions (PBC) were applied. The box was solvated and Na+ and Cl- ions were added to achieve a 0.1 M/L concentration and to maintain charge neutrality of the unit. The solvated systems were energy minimised and equilibrated. The minimisation ran using steepest descent for 1,000 cycles followed by the conjugate gradient. Energy step size was set to 0.001 nm and the maximum number of steps was set to 50,000. The minimisation was stopped when the maximum force fell below 1000 kJ/mol/nm using the Verlet cutoff scheme. Treatment of long-range electrostatic interactions was set to Particle Mesh-Ewald (PME)[31], and the short-range electrostatic and van der Waals cutoff set to 1.0 nm. After the energy minimisation, heating to 300 K was performed for 20 ps with a time step of 2 fs and position restraints applied to the backbone in an NVT ensemble. The constraint algorithm used was LINCS[32], which was applied to all bonds and angles in the protein. With the Verlet cut-off scheme and the non-bonded short-range interaction, the cut-off was set to 1.0 nm. Long-range electrostatics were again set to PME. The temperature coupling was set between the protein and the non-protein entities by using a Berendsen thermostat, with a time constant of 0.1 ps and the temperature set to reach 300 K with the pressure coupling off. Pressure equilibration
was run at 300 K with a Parrinello-Rahman pressure coupling on and set to 1 bar[33] in an NPT ensemble. The equilibration trajectories were set to three triplicates of 400 ns, with the first 10 ns discarded from the analysis.

Analysis of the trajectories was performed using GROMACS tools, including root-mean-square deviation (RMSD) to assess overall stability, per-residue root-mean-square fluctuation (RMSF) to assess the local flexibility, and solvent-accessible surface areas.

**Island domain loop well-tempered metadynamics**

To evaluate the folding/unfolding of the island domain (ID) loop, well-tempered metadynamics[34] were performed using PLUMED[35–37]. Starting from the equilibrated crystal structure after the NPT equilibration, 5x10^7 simulations steps were made. All MD parameters were the same as in the previous section. The well-tempered metadynamics used the radius of gyration (RG_ID) for the atoms 7194-7760 (the ID loop) as the collective variable. The gaussian sigma was set to 0.1 nm, with a height of 1 kJ/mol with a deposition every 500 steps. The bias factor was set to 20, at a constant temperature of 300 K. A lower wall boundary was set for RG_ID at 1.27 nm (its initial value), with a height of 2000 kJ/mol. The mapping of potential novel binding spots was performed using FTMap[38], a well-established web server for mapping of allosteric binding sites.

**Results**

To investigate the events behind the PSK-PSKR-SERK1 complex formation, equilibrium simulations were made based on the available crystal structures. Simulations for the apo DcPSKR1 were performed, with the island loop (IL) of the island domain in an unstructured conformation. As shown in Figure 2, the RMSD of the DcPSKR1-apo displays higher fluctuations in comparison to the PSKR1-holo and the PSKR1-apo (Figure 2A). This difference in intrinsic flexibility arises mainly from the island loop comprised of residues 501 to 536 (Figure 2B and 2C), which regulates the binding of the PSKR1. The apoPSKR1 did not form a β-sheet, which was found in the holo-structure. Instead, the apo ID loop transitioned from a partially helical/disordered conformation to a fully disordered configuration, which resulted in a higher radius of gyration for the apo ID loop than its holo counterpart (Supporting Figure S1).
Figure 2: A) Calculated RMSD for the whole structured with the LRR domain fitted. B) Calculated RMSD for the ID loop with the LRR domain fitted. C) Ensemble from the DcPSKR1 dynamics; Grey: the LRR domain and transparent grey: the overlaid configurations; the rainbow gradient: the disordered conformations of the ID loop from DcPSKR1.

Analysis of the total number of hydrogen bonds (H-bonds) shows a decrease through the simulation time, reaching a peak of six H-bonds formed (Figure 3), indicating that the DcPSKR1-apo configuration of the island domain does not interact tightly with the LLR domain. The average number of H-bonds between LRR and island domain was three, resulting in a higher solvent accessible surface area (SASA) for the 501-536 loop (Figure S2). This increase in the SASA is due to the high net charges located within this loop, such as the poly-lysine region (residues 517-519), and a high prevalence of disorder inducing residues like the G524 and G525. In comparison, the PSKR1-PSK complex has a highly constrained loop, with a lower RMSF than its apo counterparts (Figure S3). The β-sheet located in the island loop interacts with the PSK backbone, stabilising the interaction. The PSK backbone acts as a third β-strand configuration and forming a series of hydrogen bonds between the F506-R509 and the peptides that comprise the PSK.
The sulphated tyrosines are fundamental for binding PSK to PSKR1. The two sulfated tyrosines of PSK create a hydrogen bond network conserved for both residues, as shown in Figure 3A and Figure 3B. The N-terminal PSK tyrosine interacted with K509 and N424 with an average heavy atom distance of 0.35±0.02 nm and 0.38±0.02 nm respectively for the first 200 ns of the simulation. Interestingly, we observed a swap of interactions: the sulfate group flipped between interactions with N424 and K508 (DS-NZ =0.36±0.02 nm in the first half of the simulation), it also found another stable interaction with R349. The starting distance between the S atom of sulphated tyrosine and the NH2/NH1 of R349 was 0.6 nm, but it has been reduced to 0.36±0.02 nm during the simulation, as shown in Figure 4B. This chain (N424 -> S-Tyr -> K506 -> S-Tyr -> R349) connected residues form the island loop to the LRR-domain via the PSK sulfates, resulting in a low RMSD value in comparison to the crystallographic structure (0.23±0.04 nm for the loop region and 0.2 ±0.03 for the whole protein). This also directly affected the PSK crystallographic pose, which has an average of 0.05±0.01 nm for its RMSD.
The simulations of apo-PSKR1 starting from the folded configuration showed an unstable folded loop. The equilibrium run for the structured apo PSKR1 showed that after 200 ns, the island loop started to acquire a different state from its initial configuration, as shown in Figure 1B and given by the fact that the RMSD before 200 ns has an average of 0.27 ± 0.05 nm and then between 200 ns to 500 ns, the value increased to 0.55±0.05 nm. Nonetheless, no equilibrium replica showed a complete unfolding of the island loop in the simulation of apo-PSKR1 (Figure S4).

**Metadynamics of island loop transition**

To evaluate the loop transition towards different local free energy minima, well-tempered metadynamics simulations were carried out. Starting from the holo configuration and using the radius of gyration of the island loop (RGID) as the collective variable, the second stable conformation was found. The metadynamics free energy surface are showed in Figure 5. A second folded state (state B, RGID = 1.75 nm) was
found as a local minimum, with a difference of 20 kcal/mol in comparison to the native state (\(\text{RG}_{1D} = 1.3\)). These 2 states are shown in Figure 6B. This energy difference arises from the partial breaking of the interactions between the loop and the PSK. Transitioning from state B, the energy barrier to folding back to the native conformation is 3 kcal/mol. The PSK-PSKR1 interactions in state B occur in three different PSK regions: the two PSK termini and the N-terminal sulfated tyrosine. While the N terminal interaction is kept between the terminus backbone and D445, the PSK C-terminus keeps its interaction with R300. The N-terminal sulfated tyrosine keeps its interaction with the N424. Nonetheless, the network of hydrogen bonds was broken, reducing the number of hydrogen bonds compared to the equilibrium run, thus disrupting the PSK-ID loop stability, as shown in Figure 6A.

While in state B, the number of interactions between PSKR1 and PSK is reduced. PSK does not fully unbind in state B; however, the average number of hydrogen bonds decreased from six in the equilibrium runs to four in state B.

The formation of state B is PSK-depandant. To better understand the formation of state B, we ran well-tempered metadynamics from the holo configuration without the presence of the PSK. As shown in Figure 5, the energy well located at \(\text{RG}_{1D} = 1.75\) nm did not appear as a local minimum. Throughout the metadynamics, the ID loop steadily unfolded towards an unstructured conformation (Figure S1), resulting in a disordered loop after 60 ns of metadynamics. This indicates that the formation of state B depends on the partial stabilisation caused by the residual interactions of PSKR1-PSK.

Figure 5: Free energy surface calculated by metadynamics for PSKR1-PSK and PSKR1-apo.
The island loop conformation for state B is different from the native PSK-PSK1-SERK1 configuration. As shown in Figure 6B, the PSKR1-PSK experimental structure complex is different from the obtained configuration in state B. The translation of the loop towards the area where SERK1 binds to PSKR1 creates a series of steric clashes, impeding the biological function of PSKR1. Hence, the allosteric binding site observed exclusively in state B may result in an antagonist modulation of the SERK1 binding, disrupting the PSKR1 signalling cascade. The convergence of the systems is shown in Figure S5.

**Evaluation of state B “druggability”**

To evaluate the possible novel binding sites observed exclusively in state B, solvent mapping by FTMAP webserver was carried out on both configurations. The results of which, shown in Figure 7, identified a novel binding site that is not found in native PSKR1. The cavity identified by FTMAP is formed in the region opposite to the PSK, between the LRR domain and the island loop.
This novel binding site is comprised of residues S320, D321 and L296 on the LRR domain and residues D502, F503, R515 and Q517 (Figure 8) of the central region of the island loop. This binding site shows a series of characteristics: first, the loop side is composed of a sequence of polar residues. Second, it has a slightly hydrophobic patch, open for apolar interactions, specifically on the LRR domain side. The small molecular probes found by FTMAP to interact with this site were acetamide, acetaldehyde, benzaldehyde, phenol, cyclohexane and benzene. Therefore, most of them can interact with both the apolar patch and the polar residues located in the island loop.

Figure 8: State B binding site represented by the red connected spheres. In focus: the residues involved in the binding pocket formation.
The existence of this binding site does not fully obstruct the main PSK binding site. This is evidenced by the fact that PSK did not fully unbind after the loop transition, strongly indicating that this is a non-competitive binding site.

Discussion

PSK directly affects the folding energy of the island loop

The PSKR1 simulations of PSKR1 showed a significant difference in dynamics for the island loop. The apo unstructured loop transitioned between a conformation with a low helical content towards a fully unstructured/disordered loop. This indicates that this region is disorder-prone, which was expected given the fact that this region has several disorder-inducing motifs, such as the triple lysine repeat. Moreover, the disordered form of the ID loop transits randomly between configurations, resulting in a series of nonspecific interactions with the LRR repeats of PSKR1.

The decrease of secondary structure content coupled to high spatial fluctuation observed in all replicas indicates that PSK binding is required to induce the secondary structure of the ID loop and adopt a β-turn-β conformation. This is consistent with findings by Wang et al. [16] on the Daucus Carota PSKR (DcPSKR1) free receptor. The crystal structure of the free DcPSKR1 does not show density for the island loop, and our simulations show that area as fully flexible and highly unstable. The PSKR1-PSK allows for heterodimerisation in a different way than brassinosteroid-insensitive 1 (BRI1) and the flagellin insensitive 2 (FLS2). The model of activation of the heterodimer and their respective kinase is different: Both FLS2 and BRI1 mediated the LRR–BAK1 interaction via a series of residues located in the LRR domain and with interactions with their respective interaction modulators, flg22 and BLD. This is different from how the PSK modulates heterodimerisation of PSKR1, given the fact that the island loop changes its conformation in presence of PSK, controlling the interaction of SERK1 with PSKR-LRR. The interactions which mediate the PSKR1-SERK1 binding are located in the LRR domain, specifically with residues F596, S598, T619 and S623. However, the ID loop must maintain a high internal organisation for a stable SERK1 binding event. Therefore, understanding the dynamics under which the PSK-PSKR1-SERK1 complex formation occurs may aid in the development of innovative, small molecule-based agricultural applications for crop control.

The PSKR1 simulations with a folded ID loop and without the PSK showed that the island loop is highly structurally unstable, allowing the loop to start accessing disordered states.

The binding of PSK has a negligible effect on the overall LRR domain dynamics but does affect the LRR-domain residues that interact with the ligand. LRR domains are known to be structurally stable, which correlates with the low RMSD value sampled in this area for both the apo and holo simulations. On the apo disordered loop, its elongated conformation randomly and transiently interacts with the LRR domain, but nothing
that has a significant effect on the dynamics overall. On the other hand, PSK has several features that are crucial for loop stabilisation. The sulfated tyrosine and the PSK backbone atoms are key for PSK-PSKR1 interactions, suggesting that these interactions are enthalpy-driven by Coulombic/hydrogen bond interactions. These result in a highly specific and favourable contribution to binding free energy. Interestingly, this binding site is found exclusively within the PSKR1, without any requirement for the binding partner. This contrasts with the BRI1-BLD-BAK1/FLS1-flg22-BAK1 complex formation since the ligand is bound between two proteins forming a heterodimer. Therefore, understanding the PSKR1-PSK binding mechanisms should be independent of any tertiary partner. Hence, this emerged as an interesting target for function modulation via controlling the dynamics of the loop, and subsequently, controlling the signalling cascade.

Our enhanced sampling simulations found a stable conformation which is different to the native conformation, which we denoted as state B. Interestingly, this newly identified state B was PSK-dependent, i.e. sampled only when PSK was bound. A similar albeit unstable configuration was sampled for the apo configuration of PSKR1. This metastate found in the apo simulation may help to elucidate the folding timeline for the ID-loop: the apo PSKR1-ID loop folds into a conformation similar to state B, which can partially bind PSK. In sequence, the partially bound PSK-ID loop folds into the functional configuration.

The PSK directly affects the unfolding free energy landscape for the ID loop. The unfolding barrier between the native conformation and transition conformation that leads to state B has a value of 26 kcal/mol. The relative energy of state B in comparison to the native state is 22 kcal/mol. The application of a similar sampling method without PSK results in a reduction of the barrier height to 13 kcal/mol. Several metastable states were detected between the folded holo-structure and a higher radius of gyration, with no noticeable barrier. This energy landscape is characteristic of disordered regions, which shows several local energy minima and low transition energy barriers between them.

A novel “druggable” binding site was found in state B composed of residues located on the other face of the ID-domain, opposite to the PSK binding area. This pocket has an amphiphilic characteristic. Hence, this pocket may be used for the design of small molecular modulators for an antagonist effect on the PSK signalling cascade, working as a “molecular clamp” between the loop and the LRR domain. The fragments reported by FTMAP could be used as a starting point for fragment-based ligand design campaign to design novel agrochemicals or molecular probes. Also, this pocket may be used as a point of the structure-guided studies of antagonistic effects of certain endogenous molecules on PSKR1.

As previous studies have shown, PSK signalling is crucial for plant growth in several different species. PSK is one of the key hormones on cell elongation[9], working alongside the brassinosteroid signalling pathway. Furthermore, the PSK signalling cascade has been shown to be directly related to pollen tube growth [10]. Hence,
controlling the PSKR1 function may lead to control of plant growth and reproduction. However, this may affect the immune system of the plant cell, since pathogen-associated molecular pattern (PAMP)-related genes are promoted by PSK[12,40].

Recently, Aghdam and coworkers showed that the use of exogenous PSK has a beneficial effect on fruit storage for both strawberries[41] and broccoli[42]. This opens a new window of possibilities regarding possible applications for PSK and the ID loop modulation on PSKR1. Therefore, we expect that by using molecules that could tune the ID loop between the native state and state B, we could tune the PSK signalling. This would allow for either a positive effect, i.e. prolonged cold storage of perishable crops or antagonistic effect on invasive crop growth.

Conclusions

In this work, we evaluated the stability effect of PSK on the PSK receptor 1 through molecular dynamics simulations and enhanced sampling techniques. By using equilibrium dynamics and well-tempered metadynamics, we showed a novel and stable configuration on PSKR1 which is “druggable” by small molecule modulators. We proposed a model of how PSK binds to PSKR1 via sequential folding states of the ID loop. We expect that this model would help to clarify PSK-PSKR1-SERK1 complex formation, and show how modulating the ID loop dynamics may regulate the PSK signalling pathway.

Author Contributions: J.V.S., R.C. and A.K.B. designed the study. J.V.S., M.K. and P.Z. carried out the equilibrium molecular dynamics simulations. J.V.S. performed the well-tempered metadynamics. J.V.S., M.K., and P.Z. extracted and analysed data from the MD simulations and metadynamics. A.K.B supervised the study. J.V.S produced the figures and plots and wrote the first draft of the manuscript. M.K., P.Z., R.C., and A.K.B. reviewed the manuscript.

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Supplementary data

Supporting Figure 1: ID loop radius of gyration: A) PSKR1-PSK complex; B) Apo PSKR1; C) DePSKR1
Supporting Figure 2: Root mean square deviation for all equilibrium runs: A) PSKR1-PSK complex; B) Apo PSKR1; C) DcPSKR1
Supporting Figure 3: Residue root mean square fluctuation for all equilibrium runs: A) PSKR1-PSK complex; B) Apo PSKR1; C) DcPSRKR1
Supporting Figure 4: solvent accessible surface area per residue for all equilibrium runs: A) PSKR1-PSK complex; B) Apo PSKR1; C) DcPSKR1
Supporting Figure 5: Well-tempered metadynamics convergence: A) PSKR1-PSK free energy landscape though time. B) Apo PSKR1 Free energy landscape through time. C) Gaussian height deposition for both systems through time.
