Photosynthetic Phosphoribulokinase Structures: Enzymatic Mechanisms and the Redox Regulation of the Calvin-Benson-Bassham Cycle

Ailing Yu,1,2 Yuan Xie,1,2 Xiaowei Pan, Hongmei Zhang, Peng Cao, Xiaodong Su, Wennui Chang, and Mei Li

INTRODUCTION

Oxyphototrophic organisms such as cyanobacteria, algae, and land plants convert carbon dioxide and water into carbohydrates and release the by-product oxygen, a process that can be divided into the light reactions and the Calvin-Benson-Bassham (CBB) cycle (light-independent reactions). ATP and NADPH are produced in the light reaction process before being utilized by the enzymes of the CBB cycle responsible for CO₂ assimilation and carbohydrate production in oxyphototrophs. Phosphoribulokinase (PRK) is an essential enzyme of the CBB cycle in photosynthesis, catalyzing ATP-dependent conversion of ribulose-5-phosphate (Ru5P) to ribulose-1,5-bisphosphate. The oxyphototrophic PRK is redox-regulated and can be further regulated by reversible association with both glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and oxidized chloroplast protein CP12. The resulting GAPDH/CP12/PRK complex is central in the regulation of the CBB cycle; however, the PRK-CP12 interface in the recently reported cyanobacterial GAPDH/CP12/PRK structure was not well resolved, and the detailed binding mode of PRK with ATP and Ru5P remains undetermined, as only apo-form structures of PRK are currently available. Here, we report the crystal structures of cyanobacterial (Synechococcus elongatus) PRK in complex with ADP and glucose-6-phosphate and of the Arabidopsis (Arabidopsis thaliana) GAPDH/CP12/PRK complex, providing detailed information regarding the active site of PRK and the key elements essential for PRK-CP12 interaction. Our structural and biochemical results together reveal that the ATP binding site is disrupted in the oxidized PRK, whereas the Ru5P binding site is occupied by oxidized CP12 in the GAPDH/CP12/PRK complex. This structure-function study greatly advances the understanding of the reaction mechanism of PRK and the subtle regulations of redox signaling for the CBB cycle.

The Calvin-Benson-Bassham (CBB) cycle is responsible for CO₂ assimilation and carbohydrate production in oxyphototrophs.

The CBB cycle is regulated by light/dark transitions through the redox states of the chloroplast stroma (Buchanan, 1980; Scheibe, 1991; Geiger and Servaites, 1994). Chloroplast thioredoxins (TRXs), including TRX f and TRX m (Maeda et al., 1986; Kamo et al., 1989; Pohlmeyer et al., 1996), which is central for the regulation of the CBB cycle and usually possesses two Cys pairs at both N- and C-terminal regions, is redox-regulated by TRXs (Wedel et al., 1997; Harrison et al., 1998; Kung et al., 1999). However, PRK from a cyanobacterium, Rhodobacter sphaeroides (RsPRK; Buchanan, 1974), as a result of which the CBB cycle is initiated. The PRK structure and catalytic mechanism were previously studied in an anoxygenic bacterium, Rhodobacter sphaeroides (RsPRK; Harrison et al., 1998; Kung et al., 1999). However, PRK from oxyphototrophic organisms differs significantly from RsPRK in primary sequence, oligomeric state, and regulatory mechanism (Buchanan, 1980; Tabita, 1980). In contrast to the octameric RsPRK, PRKs from oxyphototrophic organisms are commonly presented as homodimers, and each monomer contains a pair of Cys residues at the N-terminal region. PRKs from plants and eukaryotic algae are redox-regulated through reversible reduction and oxidation of this Cys pair (Latzko et al., 1970; Wirtz et al., 1982; Milanez et al., 1991; Brandes et al., 1996), which is absent in RsPRK. Reduced PRK represents its active state, while the oxidized form is inactive, although a previous report suggested that the latter form exhibits basal activity (Marri et al., 2005). Cyanobacterial PRKs also contain a similar Cys pair at the N-terminal
region; however, earlier studies suggested that in vivo, cyanobacterial PRK is more resistant than plant PRK to oxidative inactivation (Tamoi et al., 1998; Kobayashi et al., 2003). Sequence alignment results revealed that cyanobacterial PRKs lack the loop between the two Cys residues in plant-type PRKs (termed the clamp loop), which is considered to participate in TRX binding (Gurrieri et al., 2019). The architecture of the dimeric PRK had not been determined until the crystal structure of PRK from the archaean Methanospirillum hungatei (MhPRK) was reported (Kono et al., 2017). However, MhPRK lacks the Cys pair and is not subject to redox regulation. Recently, the structures of oxidized PRK from the cyanobacterium Synechococcus sp strain PCC6301 and of reduced PRK from Arabidopsis (Arabidopsis thaliana) and Chlamydomonas reinhardtii were solved (Gurrieri et al., 2019; Wilson et al., 2019). These structural data revealed the dimerization pattern of photosynthetic PRK and confirmed that the two Cys residues involved in redox regulation are positioned apart in the reduced PRK while forming a disulfide bond in the oxidized form. As one of the Cys residues is located in the P-loop region, which usually functions in ATP binding, these findings suggested that disruption of its ATP binding site results in the inactivation of oxidized PRK (Wilson et al., 2019).

GAPDH catalyzes the conversion of α-glycerate 1,3-bisphosphate to glyceraldehyde 3-phosphate in the presence of NAPDH (Melandri et al., 1968). Two types of GAPDH, namely GapA and GapB, are present in plant chloroplasts, and they give rise to A2- and A2B2-type enzymes (Brinkmann et al., 1989; Scaglariari et al., 1998; Petersen et al., 2006). Most algae do not contain GapB, and the A2 type is the major form of GAPDH in eukaryotic algae and cyanobacteria (Petersen et al., 2006). GapB is almost identical to GapA but has an additional C-terminal extension (CTE) that bears a pair of Cys residues targeted by TRXs (Baalmann et al., 1996; Petersen et al., 2006). The A2B2 heterotetramer is subjected to redox regulation via TRX through this Cys pair (Baalmann et al., 1996; Sparla et al., 2002). Previously reported structural data showed that the CTE of oxidized GapB binds at the interface of two GAPDH monomers within one tetramer, occupying their active sites and exhibiting an autoinhibition state of A2B2-GAPDH two GAPDH monomers within one tetramer, occupying their active sites and exhibiting an autoinhibition state of A2B2-GAPDH (Brinkmann et al., 1989; Scaglariari et al., 1998; Petersen et al., 2006). Most algae do not contain GapB, and the A2 type is the major form of GAPDH in eukaryotic algae and cyanobacteria (Petersen et al., 2006). GapB is almost identical to GapA but has an additional C-terminal extension (CTE) that bears a pair of Cys residues targeted by TRXs (Baalmann et al., 1996; Petersen et al., 2006). The A2B2 heterotetramer is subjected to redox regulation via TRX through this Cys pair (Baalmann et al., 1996; Sparla et al., 2002). Previously reported structural data showed that the CTE of oxidized GapB binds at the interface of two GAPDH monomers within one tetramer, occupying their active sites and exhibiting an autoinhibition state of A2B2-GAPDH (Fermani et al., 2007). The A2B2-GAPDH is a stable and constitutively active tetramer (Wedel and Soll, 1998; Graciet et al., 2004; Tamoi et al., 2005; Oesterheit et al., 2007); it is inactivated upon forming a complex with the oxidized CP12 (Clasper et al., 1991; Pohlmeier et al., 1996; Wedel et al., 1997). The C-terminal fragment of CP12 is homologous to the CTE of GapB (Baalmann et al., 1996; Petersen et al., 2006). Several crystal structures of A2-GAPDH in complex with CP12, either the short C-terminal tail or the full length, were previously determined (Matsumura et al., 2011; Fermani et al., 2012; McFarlane et al., 2019). These studies showed that the C-terminal region of CP12 occupies the similar position at the interface of two GAPDH monomers as CTE of oxidized GapB, and therefore inhibits GAPDH activity. Earlier reports showed that A2B2-GAPDH in several land plants can also be deactivated by associating with CP12 (Wedel and Soll, 1998; Scheibe et al., 2002; Howard et al., 2011).

The protein CP12 has been identified in most oxygenic phototrophic organisms (Wedel and Soll, 1998; Groben et al., 2010). Classical CP12 proteins possess a highly conserved motif, AWD_VEE, at the central part of its sequence in addition to the N- and C-terminal Cys pairs (Wedel and Soll, 1998; Graciet et al., 2003; Stanley et al., 2013). Reduced CP12 is a fully intrinsically disordered protein (Grob et al., 2010; Launay et al., 2016), while the conformation of oxidized CP12 is stabilized by the two intramolecular disulfide bridges (Matsumura et al., 2011; Fermani et al., 2012). Oxidized CP12 is able to interact with the GAPDH tetramer and further assemblies with oxidized PRK dimer, forming the GAPDH/CP12/PRK complex (Wedel and Soll, 1998; Graciet et al., 2003; Marri et al., 2005, 2008). The enzymatic activities of both GAPDH and PRK were previously shown to be suppressed upon forming a complex, and the activity of PRK in the complex is further decreased compared with the free oxidized PRK dimer (Marri et al., 2009). The activation of PRK in the complex occurs quickly, while the transition of free oxidized PRK to its active state is slower (Marri et al., 2005), although the mechanism for this difference remained unclear. A subsequent study suggested that the GAPDH/CP12/PRK complex provides a pool of both enzymes, ready to be released with full activity (Marri et al., 2009). In addition to regulating the CBB cycle, CP12 was suggested to play wider roles, including to protect both GAPDH and PRK from oxidative stress (Marri et al., 2014) and to facilitate the stabilization of PRK during or after its synthesis in vivo (López-Calcagno et al., 2017). Several models of the GAPDH/CP12/PRK complex were previously proposed based on small-angle x-ray scattering analysis (Del Giudice et al., 2015). Recently, a cryo-electron microscopy (cryo-EM) structure of the cyanobacterial GAPDH/CP12/PRK complex from Thermosynechococcus elongatus BP-1 (TeGAPDH/CP12/PRK) was solved at an overall resolution of 4 Å (McFarlane et al., 2019). This work provided important information regarding the ternary complex assembly and the CP12 structure; however, the CP12-PRK interaction interfaces, with a local resolution of 6.2 Å, were not well resolved. The detailed intermolecular interactions of the ternary complex GAPDH/CP12/PRK requires high-resolution structures for further elucidation. In addition, although the structures of the PRK dimer from oxygenic phototrophs were recently solved (Gurrieri et al., 2019; Wilson et al., 2019), the binding mode of ATP and Ru5P with PRK remains unknown. Here, we solved the crystal structures of ligand-bound PRK as well as the GAPDH/CP12/PRK complex, enabling us to identify the active site of PRK together with its regulatory mechanisms for the redox signaling responsible for activating the CBB cycle.

RESULTS

Overall Structure of PRK

Here, we determined the crystal structures of PRK from Synechococcus elongatus PCC7942 (SePRK) in complex with ADP and glucose-6-phosphate (G6P), and from Arabidopsis in both their reduced (AtPRKred) and oxidized (AtPRKox) forms, at resolutions ranging from 2.3 to 2.5 Å. Furthermore, we solved the crystal structure of the AtGAPDH/CP12/PRK complex at 3.5 Å resolution (Table 1). In the four crystal structures, protein molecules were built almost entirely, with only several terminal residues missing (Table 1).

The PRK molecules in the four structures, either in the free form or in the complex, are highly similar, adopting an αβ6α fold (Figure 1A; Supplemental Figure 1). Four small β-strands (β1’ to
Following α3 flank at one side of the PRK molecule, while β7 is located at the other side of the molecular surface. Consistent with previously reported structures of dimeric PRK (Gurrieri et al., 2019; Wilson et al., 2019), β7 appears to be primarily responsible for the monomer-monomer interactions (Figures 1A and 1B). We also found that the structure of the α5–α6 region varied considerably between the four PRK structures (Figure 1C). The fragment between α5 and α6 was previously suggested to be a mobile “lid” in RsPRK, and it may cover the active site from the aqueous environment after substrate binding (Harrison et al., 1998; Runquist et al., 1998). The lid region cannot be traced in the SePRK structure, while it forms a flexible loop in PRK in the AtGAPDH/CP12/PRK complex (Figure 1C). By contrast, this part is stabilized by the neighboring symmetrical PRK molecule packed in the crystals, thus forming a short helix in both AtPRK structures (Figure 1C; Supplemental Figure 2). Comparison with other oxygenic phototrophic PRK structures showed that the α5–α6 region adopts the least conserved conformation in the whole PRK.

### Table 1. Data Collection and Refinement Statistics

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PDB, Protein Data Bank. Blank cells indicate no data.

[^a]: No PDB code for AtPRK-Hg.
[^b]: Highest resolution shell is shown in parentheses.
[^c]: RMSD, root mean square deviation.
[^d]: Ramachandran plot was calculated using the program Molprobity (Chen et al., 2010).
molecule (Supplemental Figure 3), indicating that the lid is internally mobile, a property that might be critical for the proper functioning of PRK.

The two Cys residues essential for the redox regulation are located at the N-terminal region, within the P-loop (C17) and at the C-terminal end of β2 (C56; Supplemental Figure 1). Previous studies reported structures of both reduced and oxidized forms of PRK; however, the reduced form was obtained from Arabidopsis and C. reinhardtii (Gurrieri et al., 2019), while the oxidized form was obtained from a cyanobacterium (Wilson et al., 2019). Here, we solved the structures of AtPRK in both reduced and oxidized forms, thus allowing accurate structural comparison within the same species. Furthermore, we identified the conformational changes induced exclusively by the different redox states of PRK.
AtPRK. As shown in Figure 2A, the folding observed for the AtPRK\textsubscript{red} structure is largely identical to the folding of AtPRK\textsubscript{ox}. The only visible difference between the two structures is the orientation of the P-loop (residues DSGCGKST\textsubscript{P-loop}), which is flipped by \( \pm 180^\circ \) owing to the formation of the C17-C56 disulfide bond in the AtPRK\textsubscript{ox} structure (Figures 2A and 2B). The structural features for the P-loop region were also observed in the recently reported structures of the reduced and oxidized PRKs (Supplemental Figure 3; Gurrieri et al., 2019; Wilson et al., 2019). The two Cys residues are 14 Å from each other in the AtPRK\textsubscript{red} structure, whereas the flipped P-loop places C17 closer to C56 in the oxidized AtPRK (Figure 2B), allowing the formation of a disulfide bond without affecting other parts of the PRK molecule, even the position of C56. The clamp loop, which precedes \( \beta \text{2} \) and was previously suggested to provide the binding site for TRX (Gurrieri et al., 2019), forms a long coil loop (Figure 2A). The C-terminal residues of the clamp loop (T46 to I48) shape as an additional \( \beta \)-strand, which is oriented in an antiparallel manner relative to \( \beta \text{2} \) (Figure 2C). In comparison, the N-terminal half of the clamp loop, although interacting with the \( \beta \text{1} \) to \( \beta \text{4} \) region, is more flexible, as characterized by their higher B-factors (Figure 2C).

**Figure 2.** The P-Loop and Clamp Loop Regions of the AtPRK.

(A) Superposition of reduced (cyan) and oxidized (yellow) AtPRK. The clamp loop of reduced and oxidized AtPRK are highlighted in blue and orange, respectively.
(B) Comparison of the P-loop region between reduced (cyan) and oxidized (yellow) AtPRK structures. The P-loop is flipped \( \pm 180^\circ \) between the two AtPRK structures. Residues C17 and C56 are shown in ball-and-stick mode.
(C) B-factor representation of the AtPRK\textsubscript{red} structure. The thicker ribbon indicates the higher B-factor. In addition to the terminal loops and P-loop region, the N-terminal part of the clamp loop (clamp loop-N, colored marine) is characterized by higher B-factors. By contrast, the C-terminal part of the clamp loop (clamp loop-C, colored gray) is stabilized by the second \( \beta \)-strand (\( \beta \text{2} \), colored pink) and shows lower B-factors. The zoom-in view of the detailed interactions between clamp loop-C and \( \beta \text{2} \) is shown in the right box. Residues are shown as sticks and labeled. The hydrogen bond interactions are represented as black dashed lines.

Overall Structures of AtGAPDH/CP12/PRK and AtCP12 within the Complex

The AtGAPDH/CP12/PRK complex was earlier reported to possess a molecular mass of 500 kD (Marri et al., 2009), allowing analysis by cryo-EM. Therefore, we used both x-ray crystallography and cryo-EM methods to solve the complex structure. We obtained 3.5 Å resolution x-ray diffraction data and a 4.9 Å cryo-EM reconstruction map of the GAPDH/CP12/PRK complex from Arabidopsis (Table 1; Supplemental Figure 4). We further refined the crystal structure (Figure 3A), as its higher resolution would provide more details. We next fitted the refined crystal structure into the cryo-EM density map, confirming that the crystal structure corresponds to the cryo-EM reconstruction map (Figure 3B). However, the peripheral region of the complex, especially the PRK-CP12 interface, displayed a flexible nature, with a weaker density (around 7 Å resolution) compared with the other parts of the complex in the cryo-EM map (Supplemental Figure 4D). The flexible PRK-CP12 interface was also observed in the recently reported cryo-EM structure of the TeGAPDH/CP12/PRK complex (McFarlane et al., 2019), indicating that this region is highly flexible.
Since the cryo-EM reconstruction was of low resolution and revealed features similar to that of the crystal structure, we did not further refine the structure based on the cryo-EM map and used the crystal structure of the complex for further analysis.

The structure of the AtGAPDH/CP12/PRK complex can be described as spindle-like and is composed of two A4-GAPDH tetramers, two oxidized PRK dimers, and four oxidized CP12 monomers (Figure 3A). The two A4-GAPDHs are located at the two end points, while the two PRK dimers form the side edges of the spindle. CP12 functions as a linker and associates with both enzymes. In the complex structure, the oxidized CP12, in a hook-like shape, is mainly composed of three \( \alpha \)-helices (Figure 3C). The N-terminal helix (N-helix; residues G6 to T21), together with a long central helix (residues E30 to G56), forms a hairpin structure, constituting the N-terminal domain (NTD), which is stabilized by a disulfide bond between C22 and C31. The conserved region of CP12 (\( \alpha_4\text{AWDEVEE}_{\alpha_4} \); López-Calcagno et al., 2014) is located at the N-terminal half of the central helix (Figure 3C). The C-terminal domain (CTD; residues P59 to N78) is more globular and composed of a short C-helix followed by a loop, which are connected by the C-terminal disulfide bond (C64 to C73).

Comparison of the four AtCP12 molecules within the complex showed that, when aligned independently, their NTD and CTD can both be superposed well (Figure 4A). However, the overall structures are considerably different because of the different conformations adopted by the loop connecting the two domains. The flexible loop between the NTD and CTD of CP12 was also observed in cyanobacterial GAPDH-CP12 complex structures (McFarlane et al., 2019). In addition, the two PRK dimers within the complex exhibit slightly different conformations, and both have a twist of \( \approx 10^\circ \) of one monomer with respect to the other monomer when compared with the free PRK dimer (Figure 4B). By contrast, the GAPDH tetramers in complex adopt an identical conformation to its free form (Figure 4C). Comparison of our AtGAPDH/CP12/PRK structure with the recently reported cryo-EM structure of TeGAPDH/CP12/PRK showed that although their overall folds are
similar (Supplemental Figure 5A), CP12 and PRK in the two complexes adopt different conformations (Supplemental Figures 5B and 5C). In addition, our crystal structure of the AtGAPDH/CP12/PRK complex is slightly asymmetrical, presumably a result of the molecular packing of the crystals (Supplemental Figure 5D). Together, these structural findings clearly demonstrated that CP12 monomers and PRK dimers adjust their conformations to assemble with the rigid GAPDH tetramers, allowing the formation of the ternary complexes with slightly altered conformations. This flexible feature of the complex might facilitate its formation in the crowded chloroplast environment, thus ensuring the effective regulation of the CBB cycle.

**Interactions of CP12 with GAPDH and PRK within the AtGAPDH/CP12/PRK Complex**

Each GAPDH tetramer of the GAPDH/CP12/PRK complex simultaneously links with two monomers of both PRK dimers through CP12 (Figure 3A). The C-terminal loop of the CP12-CTD is inserted into the interfacial region of two GapA monomers (Figure 5A), resembling the previously reported structure of GAPDH in complex with CP12 (Fermati et al., 2012). The NTD of CP12 contacts PRK, with its central helix located in a long groove on the surface of one PRK molecule (Figure 5B). A recent report showed that an adenylate kinase (ADK3) from C. reinhardtii contains a C-terminal extension similar to the CTD of CP12. The oxidized ADK3 is able to interact with GAPDH through its C-terminal extension but is unable to recruit PRK (Zhang et al., 2018). The C-terminal extension of ADK3 probably interacts with GAPDH in the similar way to the CTD of CP12. The long groove of PRK accommodating CP12 is formed by the P-loop (D14 to T21), $\alpha_2$ (D64 to Q71), and lid (R160 to L169) regions, which shape the bottom and both sides of the groove, respectively (Figure 5B). Remarkably, these regions are also involved in establishing the active site of PRK (see below). All three fragments together form a positively charged surface of the groove and surround the conserved $\lambda_{\text{AWD_\text{VEE}}}$ region of CP12 (Figure 5C). The conserved acidic residues E40 and D36 of CP12 face the groove of PRK and form hydrogen bonds with the alkaline residues R65 and R68 from the $\alpha_2$ region of PRK (Figure 5D). Mutation of either R65 or R68 to Ala in PRK prevents the formation of the ternary complex (Figure 5E), indicating that both play critical roles in stabilizing the PRK-CP12 interaction.

**Figure 4.** Structural Comparison of Proteins within the AtGAPDH/CP12/PRK Complex or with Their Free Forms.

(A) Structural comparison of four CP12 monomers within the AtGAPDH/CP12/PRK ternary complex superposed on their NTD (top) and CTD (bottom). The four CP12 molecules are colored differently.

(B) Structural comparison of two PRK dimers within the AtGAPDH/CP12/PRK ternary complex superposed on the left monomer (top) and comparison between one PRK dimer within the ternary complex structure (colored marine) and the AtPRKox structure (colored yellow) superposed on the left monomer (bottom).

(C) Superposition of the GAPDH part in the AtGAPDH/CP12/PRK structure (blue-white), the previously reported GAPDH-CP12(CTD) structure (PDB code 3RVD; magenta), and the GAPDH tetramer structure (PDB code 1RM4; pale yellow).
Figure 5. The Intermolecular Interactions in the AtGAPDH/CP12/PRK Ternary Complex.

(A) The CTD of CP12 is inserted into the interfacial region of two GAPDH monomers, blocking their active sites. The CP12 is shown as a cartoon in salmon; the two GAPDH monomers are shown in surface mode and colored in pale green and gray, respectively. The NAD$^+$ ligands bound to the GAPDH monomers are shown as sticks.

(B) The central helix of CP12 is located in a long groove on the surface of PRK. The CP12 is shown as a cartoon in salmon, and the conserved regions are highlighted in hot pink. The PRK molecule is shown in surface mode, and the groove is composed of the second helix ($\alpha_2$, magenta), the lid region (yellow), and the P-loop (cyan).

(C) The conserved region ($\omega_{\text{AWD, VEE}}$) within the central helix of CP12 is located in the positively charged region of the groove in PRK. PRK is shown in electrostatic potential surface mode; red represents negative charge, and blue represents positive charge. CP12 is shown as a cartoon in salmon, with the conserved region ($\omega_{\text{AWD, VEE}}$) highlighted in yellow-orange.

(D) The detailed interaction between CP12 and PRK in the AtGAPDH/CP12/PRK ternary complex. CP12 (salmon) and PRK (teal) are shown as a cartoon. Residues crucial for the interaction are shown as sticks and labeled. The hydrogen bonds are represented as black dashes. The conserved region ($\omega_{\text{AWD, VEE}}$) within the central helix of CP12 is highlighted in yellow-orange.

(E) Characterization of the ternary complex through size-exclusion chromatography and SDS-PAGE. The size-exclusion chromatography profiles (left) of the wild type as well as three mutants (R65A, R68A, and C17S) of AtPRK incubated with NAD$^+$-bound GAPDH and oxidized CP12 are shown in black, blue, green, and magenta lines, respectively. Peak 1 is the GAPDH/CP12/PRK ternary complex, and the peak 1 fraction in the black line (wild-type PRK incubated with GAPDH and CP12) is identified through SDS-PAGE (right). The wild type and the C17S mutant of AtPRK are able to form the GAPDH/CP12/PRK ternary complex.

(F) Structural superposition of AtPRK$_\text{red}$ (lime) and the oxidized PRK (light blue) within the AtGAPDH/CP12/PRK ternary complex. The P-loop region in reduced AtPRK does not interfere with its binding to CP12 (shown in salmon cartoon). The P-loop regions are circled by the black dashed line and indicated.
Moreover, we found that the conformation adopted by the P-loop in the reduced form of PRK does not hinder CP12 binding (Figure 5F), suggesting that the reduced AtPRK is able to form a similar complex to that of the oxidized form. To test our hypothesis, we incubated the PRK mutant C17S with GAPDH and oxidized CP12. As the mutant C17S is not able to form a disulfide bond with C56, it represents the constitutively reduced form of PRK. Our size-exclusion chromatographic result showed that the mutant C17S forms a similar GAPDH/CP12/PRK complex to the oxidized wild-type PRK (Figure 5E). Our results are consistent with previous observations in pea (Pisum sativum) and C. reinhardtii showing that reduced PRK also exists in the GAPDH/CP12/PRK complex in vivo (Lebreton et al., 2003; Howard et al., 2008).

Active Site of PRK and Recognition of ATP and Ru5P

All previously reported structures of PRK are present in their apo-forms and thus lack the details of their binding to either ATP or Ru5P. To identify the cofactor binding pattern, we cocryrstallized PRK from Arabidopsis and S. elongatus (AtPRK and SePRK) with various ligands, including ATP/G6P and AMPPNP (a non-hydrolyzed ATP analog)/Ru5P. Ultimately, only the structure of SePRK in complex with ADP and G6P was obtained (Figure 6A). In this structure, although we cocryrstallized SePRK with ATP, an ADP molecule is clearly visible in the density map, likely because of the hydrolysis of its γ-phosphate group during the crystallization process.

The SePRK structure harbors two deep narrow grooves consisting of primarily positively charged residues forming an L shape (Figure 6B). Each groove is occupied by one ligand; the ADP binding groove (named the ATP site) is mainly formed by residues from the P-loop (residues 59-DSGCGKSTG52) and the α5 region. The α-phosphate of ADP interacts with T22, while the β-phosphate group is hydrogen bonded with S21. In addition, the adenosine ring is positioned parallel to the purine ring of W140 and is hydrogen bonded with T304 (Figure 6A). In the second pocket, the G6P molecule is stabilized by residues from the α2, α6, and β2 - β3′ regions. Its phosphate group is hydrogen bonded with R52 and R163, and the hydroxyl groups are stabilized by R49, Y88, and H90 (Figure 6A). G6P and Ru5P differ in their stereochemical structures. G6P is characterized by a cyclic glucose skeleton with six carbons, while Ru5P comprises a linear ribulose with five carbon atoms. However, both contain a phosphate group at their terminal hydroxyl group, implying that they may interact with an identical region that also accommodates the phosphate group. Hence, we hypothesized that the binding pocket occupied by G6P in our structure represents the Ru5P binding site (named the Ru5P site).

To test our hypothesis about the position of the Ru5P site and to locate the γ-phosphate group of ATP, we docked Ru5P and ATP into our SePRK structure through the AutoDock Vina program (Figure 6C; Trott and Olson, 2010). In addition, we docked a G6P molecule into the SePRK structure to assess the docking program. We found that the docked G6P is located in a position identical to that we observed in the crystal structure (Supplemental Figure 6), indicating that it is feasible to apply the docking method to explore the ligand binding site in our PRK structures. In the docked model of SePRK in complex with Ru5P and ATP, the phosphate group of Ru5P locates at a similar position to that of G6P, while the C1 hydroxyl group of Ru5P locates at a distance 5.8 Å away from that of G6P (Figure 6D). This docking result confirms the hypothesized position of the Ru5P site and suggests that the Ru5P site is composed of residues of the α2 (D42, R49, and R52) and β2′ to β3′ (Y88 and H90) regions as well as R163 from α6 (Figure 7A). Moreover, docking of an ATP molecule suggests that its γ-phosphate is fixed by S16, K20, and S21 from the P-loop (Figure 7A). These residues are completely conserved among the oxygenic phototrophic PRKs (Supplemental Figure 1), indicating that they play an essential role during catalysis.

Key Residues in Ligand Binding and Catalysis of AtPRK

Despite extensive screening for crystallization conditions, we failed to obtain the ligand-bound AtPRK structure. However, our structural superposition analysis showed that AtPRKapo exhibits an overall structure nearly identical to that of SePRK (Figure 1B), suggesting that both enzymes bind ATP and Ru5P in the same pockets in a similar manner. On the basis of these findings, we generated several AtPRK mutants (Supplemental Figure 7) altered in the conserved residues potentially involved in catalysis and ligand binding according to our findings on SePRK structure and tested their activity. Our enzymatic assays showed that the mutant forms D58A, Y104F, and H106A almost completely lost catalytic ability, while the mutant forms S15A, K19A, S20A, R65A, and W156A showed dramatically reduced catalytic activity when compared with the wild type (Figure 7B).

Residues S15, K19, S20, and W156 in AtPRK correspond to residues S16, K20, S21, and W140 in SePRK, which participate in ATP binding in SePRK. In agreement with the structural observation on SePRK, the binding analysis between AtPRK and ATP through the surface plasmon resonance (SPR) method revealed that the two mutations K19A and W156A drastically decreased the binding affinity of PRK to ATP (Figure 7B; Supplemental Figure 8). However, mutation of S15A and S20A from the P-loop failed to show such an effect on ATP binding of AtPRK. Our docking results suggested that S15, S20, and K19 (S16, S21, and K20 in SePRK) are all involved in interacting with the γ-phosphate of ATP (Figure 7A). These data suggest that K19 is essential for interacting with ATP, as its positively charged side chain favors the binding and stabilization of the negatively charged phosphate group of ATP. While the two Ser residues S15 and S20 play minor roles in binding ATP, they are critical for catalysis, presumably by ensuring the proper orientation of γ-phosphate of ATP.

In addition, our structural analysis suggested that residues D58, R65, Y104, and H106 (corresponding to D42, R49, Y88, and H90 in SePRK) are involved in Ru5P binding. We therefore measured the binding affinity of wild-type and mutant (D58A, R65A, Y104F, and H106A) PRK with Ru5P using an isothermal titration calorimetry (ITC) method. Our ITC results indicated that these four residues are indeed involved in Ru5P binding (Figure 7B; Supplemental Figure 9), which is in agreement with our docking results as well as a previous report showing that R64 in C. reinhardtii PRK (corresponding to R65 in AtPRK) is important for binding the substrate (Roesler et al., 1992). However, considering the relatively high concentration of Ru5P in our enzymatic assay (1.2 mM), the reduced activities of these mutants are likely to represent their different kcat values compared with that of the wild-type PRK.
Figure 6. The Active Site of PRK.

(A) The structure of SePRK bound with ADP and G6P. Fragments involved in ADP and G6P binding are labeled in blue. The ligands ADP and G6P and the coordinated residues are shown as sticks and labeled in black. The 2\(\Delta F\) electron density maps of ADP and G6P contoured at 1.0 \(\sigma\) are shown as blue mesh.

(B) The electrostatic potential surface of SePRK. Red represents negative charge, and blue represents positive charge. The ADP and G6P molecules, occupying two positively charged grooves on the PRK surface, are shown as sticks and circled with green lines.

(C) The electrostatic potential surface of SePRK with docked ligands ATP and Ru5P. Red represents negative charge, and blue represents positive charge. The ATP and Ru5P are shown as sticks and circled with orange lines.

(D) Superposition of bound ADP and G6P molecules (green for carbon atoms) with docked ATP and Ru5P molecules (yellow for carbon atoms) in SePRK. The ligands are shown as sticks. The distance between the C1 hydroxyl groups of Ru5P and G6P is 5.8 Å, shown by the black dashed line in the left panel. The distance between the C1 hydroxyl group of Ru5P and the \(\gamma\)-phosphate group of ATP is 5.2 Å, shown by the black dashed line in the right panel.
Furthermore, the results obtained with the two loss-of-function mutants D58A and H106A strongly suggest that these two residues directly participate in the catalysis in addition to binding Ru5P. This finding is in agreement with a previous analysis demonstrating that D42 in RsPRK (equivalent to D58 in AtPRK) is essential for its catalytic activity (Harrison et al., 1998). Based on our docking results obtained from the SePRK structure, D58 and H106 in AtPRK are possibly located at the crossing point of the ATP site and the Ru5P site, in close proximity with the potential positions of ATP $\gamma$-phosphate and the C1 hydroxyl oxygen of Ru5P (Figure 7A). Together, these findings provide strong evidence that D58 and H106 function as the catalytic base that activates the nucleophilic attack.

Insights into the Catalysis and Regulation of PRK

Superposition of our PRK structures shows that the P-loop is the only region that adopts different conformations between the AtPRK$_{red}$ and AtPRK$_{ox}$ structures (Figures 2A and 2B). Further comparison with the SePRK structure clearly demonstrates that the ATP site is disrupted in the AtPRK$_{ox}$ structure (Figure 8A), which was also suggested previously for the oxidized cyanobacterial PRK (Wilson et al., 2019). However, the Ru5P site is located somewhat distantly from the P-loop and stays unaffected in the oxidized form (Figure 8A). These structural observations demonstrate that the inactivation of oxidized PRK is because it is unable to bind ATP, and they suggest that the switch between the active (reduced) form and the inactive (oxidized) form of PRK only requires the conformational change of its P-loop, without affecting the Ru5P site in PRK.

In addition to the redox regulation, another important regulatory mechanism of PRK activity involves controlling the reversible assembly of the GAPDH/CP12/PRK complex. In this ternary complex, both GAPDH and PRK remain inactive, as CP12 binds to their active sites. The central helix of CP12 is located exactly in the Ru5P site of PRK (Figure 8B). The two acidic residues E40 and D36, interacting with R65 and R68 of PRK (Figure 5D), insert into the Ru5P site. D36 could potentially clash with Ru5P (Figure 8B). As a result, the CP12 would interfere with the binding of PRK to Ru5P, which explains the further decrease of PRK activity within the complex.

Interestingly, we found that ATP binding is a precondition for PRK to interact with Ru5P. While we detected no available signal when titrating Ru5P into AtPRK alone using ITC (Supplemental Figure 9A), we obtained reliable data by titrating Ru5P into a mixture of AtPRK incubated with ATP (Figure 7B; Supplemental Figure 9B). While the affinity of Ru5P to PRK increased in the presence of ATP, incubating PRK with Ru5P failed to affect its ATP binding (Supplemental Figure 8C). This experimental observation suggests that binding Ru5P requires the conformational change of PRK, which is induced by ATP binding. PRK was previously proposed to follow an ordered sequential mechanism for catalysis (Lebreton and Gontero, 1999), indicating that PRK binds ATP and Ru5P simultaneously and catalyzes the direct transfer of the
phosphate group from ATP to Ru5P, without forming the enzyme-phosphate intermediate. Our results presented here suggest that during the ordered sequential catalytic reactions, PRK binds ATP first before binding Ru5P.

DISCUSSION

In this study, we solved the structure of SePRK in complex with ADP and G6P and generated a docked model of SePRK bound with ATP and Ru5P based on our crystal structure. Our results show that the ATP site is mainly formed by residues from the P-loop and α5 (Figure 6A). Two residues of PRK, K19 and W156 (numbered as in AtPRK), are crucial for ATP binding. Together, they stabilize the phosphate groups and the adenosine ring of ATP through ionic bond and π-π interaction, respectively (Figure 7). Moreover, we found that mutation of either of the two conserved Ser residues in the P-loop (S15 and S20) results in a significant decrease of PRK activity, without affecting its ATP binding ability (Figure 7B). Since the two Ser residues are located near the γ-phosphate group of ATP in our docked model, it is possible that they facilitate the proper orientation of ATP, especially its γ-phosphate group. This suggestion explains their crucial role for the catalytic activity of PRK. The oxidized PRK fails to bind ATP, as the disulfide bond formed between C17 and C56 changes the conformation of the P-loop, thus disrupting the ATP site (Figure 8A). Except for the P-loop region, other parts of the AtPRKred and AtPRKox are highly identical (Figure 2A). Together, these observations suggest that PRK undergoes a minimum conformational change to switch between the reduced and oxidized states, with the other parts remaining relatively stable, which may facilitate the rapid activation-deactivation cycle of PRK.

Our structures show that while the ATP site is disrupted in the oxidized PRK, the Ru5P site remains unaffected by the C17-C56 disulfide bond formation. The Ru5P site is shaped by residues from α2, φ6, and β2’-β3’, including D58, R65, Y104, and H106 (numbered as in AtPRK), which are pivotal for Ru5P binding (Figure 7). The binding of Ru5P is inhibited by the assembly of the GAPDH/CP12/PRK complex, as the central helix of CP12 is located in the

Figure 8. The ATP Site and the Ru5P Site in PRK.

(A) Structural superposition of AtPRKox (yellow) and SePRK (pink) with docked ATP and Ru5P molecules (shown as sticks). The ATP site is disrupted in the AtPRKox structure (yellow), as the P-loop clashes with the ATP molecule, whereas the Ru5P site is unaffected in the AtPRKox structure.

(B) Structural superposition of AtPRK in the ternary complex (slate) and SePRK (pink) with docked ATP and Ru5P molecules (shown as sticks). The Ru5P site is at the PRK-CP12 interface of the AtGAPDH/CP12/PRK complex and is disrupted upon binding CP12 (green), whereas the ATP site is unaffected. The two conserved residues D36 and E40 of CP12 are shown as sticks and labeled. Residue D36 of CP12 is potentially clashing with Ru5P.
Ru5P site and potentially clashes with Ru5P in the groove on the PRK surface. By contrast, the CP12-PRK interaction does not affect ATP binding with PRK (Figure 8B). This observation is in line with an earlier report showing that the GAPDH/CP12/PRK complex is able to bind ATP (Marri et al., 2005). These results indicate that ATP and Ru5P binding are independently regulated through the reversible formation of the C17-C56 disulfide bond and the GAPDH/CP12/PRK complex, respectively.

Our biochemical and structural data together suggest that residues D58 and H106 (numbered as in AtPRK) directly participate in the catalytic reaction of PRK, since mutation of any of the two residues leads to the completely loss of PRK activity (Figure 7B). In the docked model, the γ-phosphate group of ATP and the C1-hydroxyl group of Ru5P are positioned at the crossing point of the L-shaped groove, separated by only 5.2 Å (Figure 6D). This distance is comparable to that between substrate/product and cofactor in other kinases reported previously (Shirakihara and Evans, 1988; Sigrell et al., 1998) and allows the direct transfer of a phosphate group from ATP to Ru5P with their slight conformational changes. Both D58 and H106 are located close to the phosphate group of ATP and the hydroxyl group of Ru5P (Figure 7A); thus, they may play an essential role in the catalytic process, such as functioning as the catalytic base that activates the nucleophilic attack.

Figure 9. Potential TRX Binding Sites on AtPRK and AtCP12.

(A) Electrostatic potential surface of AtPRKred. The clamp loop is shown in cartoon mode, highlighted in blue and indicated by black arrows. The residue C56 is shown in sticks and indicated. The potential binding pocket of TRX is circled by a green dashed line.

(B) Electrostatic potential surface of TRX from spinach (Spinacia oleracea; PDB code 1FAA). The two Cys residues are shown in sticks and indicated.

(C) Electrostatic potential surface of AtCP12. The disulfide bond between C22 and C31 at the NTD is shown in sticks and indicated. The potential binding pocket of TRX is marked by a black dashed box.

In addition, the results of our binding assays indicated that PRK binds ATP prior to Ru5P. However, since the ATP site is solvent-accessible, it is possible that the bound ATP is hydrolyzed before Ru5P binding, resulting in the waste of ATP. Nevertheless, a number of kinases have been reported to bind ATP before the substrate. The ATP binding induces the conformational change of fragments adjacent to the ATP site in these kinases, thus shielding the ATP molecule and protecting it from hydrolysis (Schlauderer et al., 1996; Sigrell et al., 1998; Li et al., 2004). It is possible that PRK uses a similar mechanism, undergoing conformational change upon ATP binding and protecting ATP. Previous observation showed that ATP binding leads to the dissociation of the GAPDH/CP12/PRK complex (Marri et al., 2005), consistent with our suggestion that binding ATP induces the conformational change of PRK. In addition, the changed conformation of PRK may further facilitate the Ru5P site formation, explaining our experimental observation that Ru5P only binds PRK after ATP association. Furthermore, our binding assay demonstrated that PRK has strong affinity for Ru5P (Figure 7B; Supplemental Figure 9B), considering the relatively high concentration of Ru5P in the chloroplast (around 25 to 75 μM; Küken et al., 2018). This strong affinity may further ensure that Ru5P binds PRK immediately after ATP binding, which may also help to avoid the nonspecific hydrolysis of ATP. Possible candidates for these mobile fragments in PRK include the lid region, which is highly flexible, as shown by our comparison results of different PRK structures (Figure 1C; Supplemental Figure 3). This mobility is a pivotal characteristic of the lid, which allows this region to change conformation and perform various functions, such as covering the active site during catalysis, a commonly suggested role for the lid region in many kinases, including RsPRK (Schlauderer et al., 1996; Runquist et al., 1998).

Previous studies suggested that only the oxidized AtPRK forms the ternary complex in vitro (Marri et al., 2005). However, our biochemical data showed that mutant C17S of PRK is still able to form a complex with CP12 and GAPDH under oxidized conditions (Figure 5E). This result suggests that reduced PRK also associates with oxidized CP12 and forms the GAPDH/CP12/PRK complex. Analysis of our complex structure revealed that the reduced PRK is able to form the complex in a manner similar to the oxidized form of PRK, as the different conformation of the P-loop does not interrupt PRK association with CP12 (Figure 5F). In agreement with these observations, reduced PRK in pea and C. reinhardtii were previously reported to form a GAPDH/CP12/PRK complex in vivo (Lebréton et al., 2003; Howard et al., 2008). The finding that reduced PRK can form the GAPDH/CP12/PRK ternary complex may explain the quick recovery of PRK activity when it dissociates from the complex, as it is better prepared for binding ATP than the free oxidized PRK form.

Plant-type PRK is redox-regulated mainly by chloroplast TRX f and TRX m as previously suggested, which target C56 of PRK (Brandes et al., 1996). The clamp loop between two Cys residues (C17 and C56) in PRK was suggested to provide the binding site for TRX (Gurrieri et al., 2019); however, the AtPRK structures solved in our study and in an earlier report (PDB code 6H7H; Gurrieri et al., 2019) together showed that the clamp loop adopts the same conformation in both oxidized and reduced forms (Supplemental Figure 3), indicating this is a thermodynamically stable status.
of AtPRK. Moreover, in our PRK structures, the pocket above C56 shaped by the clamp loop appears too narrow to accommodate the TRX protein (Figures 9A and 9B). In addition, the positively charged N-terminal part of the clamp loop may be unfavorable for TRX binding, for the catalytic Cys residues of TRX are also located in a region containing primarily positively charged residues (Capitanì et al., 2000). These structural features imply that the conformational change of the clamp loop is required for TRX binding. Structural analysis showed that the N-terminal part of the clamp loop is flexible (Figure 2C); hence, it may swing away to make room for TRX and stabilize TRX binding. In addition, the swing of the clamp loop also exposes the residue C56 that is located at the C-terminal end of β2 (Figure 9A), which is otherwise buried under the clamp loop.

CP12 is also redox-regulated by TRXs (Marri et al., 2009). Under oxidizing conditions, CP12 first forms the C-terminal disulfide bond to enable GAPDH binding, then links the N-terminal disulfide bridge and binds PRK (Marri et al., 2005; Ferrmani et al., 2012). Previous redox titration analysis suggested that the dissociation of the GAPDH/CP12/PRK complex mainly depends on the reduction of CP12, which is less demanding in terms of the reducing potential than GAPDH alone (Marri et al., 2009). The N-terminal part of CP12 is located at the complex surface and negatively charged (Figure 9C); thus, it is likely to be the most accessible target for TRX. We propose that the disassembly of the ternary complex is initiated through the dissociation of CP12 with PRK, which is in agreement with previous reports showing that CP12 and GAPDH form a stable binary complex (Ferrmani et al., 2012; McFarlane et al., 2019). Upon reduction of the disulfide bond in CP12-NTD, the major part of CP12 adopts a disordered conformation to release PRK, and further reduction of the C-terminal disulfide bond of CP12 disassembles the entire complex. The released GAPDH and PRK expose their active sites, allowing the reduction of oxidized PRK. The complex dissociation upon CP12 reduction was suggested to be crucial for ensuring the tight coupling of GAPDH and PRK (Marri et al., 2009). PRK and GAPDH represent two key enzymes in the CBB cycle, utilizing ATP and NADPH produced through light reactions for catalysis, respectively. By controlling the activity of two key enzymes simultaneously, the GAPDH/CP12/PRK complex formation allows the regulation of the whole CBB cycle. This process represents an economic way to avoid accumulating or wasting of intermediate products. Together, the redox signal is fine-tuning the CBB cycle through regulating the activation-deactivation of PRK and the reversible formation of the ternary complex.

METHODS

Cloning

The genes encoding AtPRK and SePRK were PCR-amplified using primer pairs 1 and 2 from cDNA libraries of Arabidopsis (Arabidopsis thaliana) and Synechococcus elongatus PCC7942, respectively. The resulting amplified products were gel-extracted and ligated into the linearized vector pET28a (Novagen) between Ncol and Xhol restriction sites for expression with a C-terminal His tag. The mutants of AtPRK were prepared by site-directed mutagenesis using overlap-PCR (Gibson, 2011). All primers for mutations were designed on the website https://www.bioinformatics.org/primerx/. The PCR products were all analyzed by agarose gel electrophoresis and extracted for plasmid reconstruction. The details of all primers are listed in Supplemental Table 1.

To obtain the His tag-free proteins for the formation of the AtGAPDH/CP12/PRK complex, the genes encoding AtPRK, AtGAPDH, and AtCP12 were PCR-amplified with primer pairs 3, 4, and 5 from the cDNA library of Arabidopsis. The resulting amplified product was gel-extracted and ligated into the linearized vector pMCSG7 through ligation-independent cloning. The expression vector pMCSG7 encodes an upstream His tag followed by a tobacco etch virus protease cleavage site, which allows removing of the His tag from the mature proteins.

Escherichia coli strain Top10 was used for plasmid cloning, and the insertion of the genes was confirmed by DNA sequencing. The recombinant plasmids were transformed into E. coli BL21 (DE3) for protein expression.

Expression and Purification of Recombinant Proteins

The pET28a-transformed cells were cultured at 37°C for 4 h in Luria-Bertani medium supplied with kanamycin (50 μg/mL). The pMCSG7-transformed cells were cultured at 37°C for 4 h in Luria-Bertani medium supplied with ampicillin (100 μg/mL). The expression and purification procedure for all recombinant proteins is similar and described below.

When the optical absorption density at 600 nm of the E. coli BL21 cells reached 0.8, the protein expression was induced by the addition of 1 mM IPTG. The culture was further incubated for 16 h at 30°C. The cells were then harvested by centrifugation at 8000g for 10 min. The harvested cells were resuspended in buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 20 mM imidazole) and disrupted by sonication. The cell lysate was centrifuged at 18,000g for 40 min at 4°C. The supernatant containing the target protein was loaded onto an Ni2+-affinity column (GE Healthcare) pre-equilibrated with buffer A and then washed with buffer B (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 50 mM imidazole). The target protein was then eluted with buffer C (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 250 mM imidazole). The recombinant proteins with C-terminal His tag were further purified by gel filtration chromatography using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with buffer D (20 mM HEPES, pH 7.5, and 100 mM NaCl).

AtGAPDH/CP12/PRK Complex Formation

The recombinant AtPRK, AtGAPDH, and AtCP12 proteins with N-terminal His tag were first digested with tobacco etch virus protease at 4°C overnight. Then, the digested target protein was applied to an Ni2+-affinity column before gel filtration chromatography. To obtain the AtGAPDH/CP12/PRK complex, AtGAPDH was first treated with 2 mM NADH for 5 h at 4°C, then AtGAPDH, AtPRK, and AtCP12 were mixed and incubated at a molar ratio (subunit basis) of 8:4:4 with 0.01 mM CuSO4 for 12 h at 4°C. Finally, the in vitro-assembled complex was isolated from free proteins by gel filtration chromatography with a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with a buffer of 20 mM HEPES, pH 7.5, and 100 mM NaCl.

Each purified protein was checked by SDS-PAGE and identified by peptide mass fingerprinting on matrix-assisted laser desorption/ionization time of flight mass spectrometry.

Crystallization

To obtain the reduced and oxidized forms of AtPRK, we treated AtPRK protein with 5 mM DTT and 0.01 mM CuSO4, respectively. Before crystallization, SePRK and reduced and oxidized AtPRK were concentrated to 10 mg/mL using centrifugal filter units (Millipore) with a molecular weight cutoff of 50-kD and GAPDH/CP12/PRK complex was concentrated to 15 mg/mL using 100-kD molecular weight cutoff centrifugal filter units.
Data Collection and Structure Determination

Five diffraction data sets were collected for crystals of native AtPRKred, native AtPRKox, AtPRK-Hg, SePRK, and the AtGAPDH/CP12/PRK complex. The x-ray data of AtPRK-Hg were collected with increasing concentration, from 15% (w/v) to 40% (w/v), gradually with increasing concentration, from 15% (w/v) to 40% (w/v), gradually before data collection. The postcrystal treatment followed the method previously reported by Heras and Martín (2005).

Enzymatic Assay and Binding Affinity Analysis

The enzyme kinetic assay was performed according to a procedure reported previously with slight modification utilizing the ADP formation coupled to the NADH oxidation (Racker, 1957; Kobayashi et al., 2003). The enzymatic activity was quantified by measuring the reduction of NADH over time through monitoring the decrease in A\textsubscript{340}. The activity of the wild types and mutants of PRK was measured at 298K in the reaction solution of 100 mM Tris-HCl, pH 8.0, 4 mM MgCl\textsubscript{2}, 10 mM KCl, 0.3 mM DTT, 0.5 mM NADH, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase, 0.5 mM phosphoenolpyruvate, and 1 mM ATP. PRK was added to the reaction solution at a final concentration of 40 ng/mL and incubated for 10 min at 298K. RuSP was added to a final concentration of 1.2 mM to initialize the reaction, and then the A\textsubscript{340} was measured for 10 min.

The interaction between ATP and PRK was investigated by an SPR instrument (Biacore 8K, GE Healthcare) at 298K with running buffer containing 20 mM HEPES, pH 7.5, and 100 mM NaCl. A sensor chip SA (Series S, GE Healthcare) flow cell was activated with 1 M NaCl and 5 mM NaOH for 4 min. PRK proteins were incubated with biotin in a molar ratio of 1:1 for 30 min. Spare biotin was removed by repeated concentrating and diluting three times. Then, proteins were diluted to 40 μg/mL and immobilized to the sensor chip.

Cryogenic experiments were conducted utilizing a MicroCal iTC200 instrument (Malvern) at 298K. To analyze the affinity between PRK and RuSP, 1 mM ATP and 2 mg/mL PRK in the buffer containing 100 mM Tris-HCl, pH 8.0, 4 mM MgCl\textsubscript{2}, 10 mM KCl, and 2 mM DTT were incubated for 10 min in the cell and then titrated with 0.5 mM RuSP in the same buffer. All measurements of enzymatic activity of PRKs and the binding affinity with ATP and RuSP were repeated two to three times, with similar results obtained.

Sequence Alignment

Multiple sequence alignment (Supplemental Figure 1) was performed with ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/) and displayed
Supplemental Data

Supplemental Figure 1. Multiple sequence alignment of phosphoribulokinase.

Supplemental Figure 2. The crystal packing of AtPRKred and AtPRKox.

Supplemental Figure 3. Structural comparison of PRK structures from different species.

Supplemental Figure 4. Single particle cryo-EM analysis of AtGAPDH/CP12/PRK ternary complex.

Supplemental Figure 5. Structure and comparison of GAPDH/CP12/PRK complexes.

Supplemental Figure 6. Superposition of the docked G6P with the G6P molecule bound in SePRK structure.

Supplemental Figure 7. Characterization of the wild type and mutants of PRK.

Supplemental Figure 8. Measurements of ATP binding affinity of wild type and mutants of AtPRK as well as wild-type SePRK through the SPR method.

Supplemental Figure 9. Measurements of Ru5P binding affinity of wild type and mutants of AtPRK as well as wild-type SePRK through the ITC method.

Supplemental Table 1. The primers and vectors used for the constructs of GAPDH, CP12, and PRK (wild type and mutants).

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AUTHOR CONTRIBUTIONS

M.L. and W.C. conceived the project; A.Y. and Y.X. did the expression, purification, crystallization, data processing, and structure determination of PRK proteins; A.Y. prepared the GAPDH/CP12/PRK complex and performed the crystallization, data processing, and structure determination of the complex; A.Y. performed the activity and affinity assays; X.P., H.Z., and P.C. helped in data collection; X.S. assisted in crystal-EM data collection; M.L., A.Y., and Y.X. analyzed the structures and wrote the article; all authors discussed and commented on the results and the article.

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