Conformational Changes in Inositol 1,3,4,5,6-Pentakisphosphate 2-Kinase upon Substrate Binding

ROLE OF N-TERMINAL LOBE AND ENANTIOMIC SUBSTRATE PREFERENCE

José Ignacio Baños-Sanz, Julia Sanz-Aparicio, Hayley Whitfield, Chris Hamilton, Charles A. Brearley, and Beatriz González

From the Departamento de Cristalografía y Biología Estructural, Instituto de Química-Física “Rocasolano,” CSIC, Serrano 119, 28006-Madrid, Spain and the School of Biological Sciences and School of Pharmacy, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, United Kingdom

Background: IP₅₂-K is essential for higher inositide metabolism and signaling. IP₅₂-K undergoes conformational changes upon nucleotide and inositide binding, with the N-lobe being essential for substrate recognition and IP₅ enantiomer selection. Understanding the determinants of enzyme function and substrate specificity will enable rational design of inhibitors.

Inositol 1,3,4,5,6-pentakisphosphate 2-kinase (IP₅₂-K) catalyzes the synthesis of inositol 1,2,3,4,5,6-hexakisphosphate from ATP and IP₅. Inositol 1,2,3,4,5,6-hexakisphosphate is implicated in crucial processes such as mRNA export, DNA editing, and phosphorus storage in plants. We previously solved the first crystallographic findings. Our work suggests that the clasp of the axial 2-OH of IP₅. IP₅₂-K has been cloned and characterized from many organisms, from yeast to humans. This work is focused on the study of one of those kinases, the inositol 1,3,4,5,6-pentakisphosphate 2-kinase (IP₅₂-K, IPK1), an enzyme that catalyzes the synthesis of a crucial compound, inositol 1,2,3,4,5,6-hexakisphosphate (IP₆), by phosphorylation of the axial 2-OH of IP₅. IP₅₂-K has been cloned and characterized from many organisms, from yeast to humans (3–8). Ipk1 gene disruption in mice results in early lethality of the embryos (9). IP₆ participates in mRNA export (10), regulation of chromatin state (11), developmental processes (12), and apo-
**IP₅ 2-K Conformational Changes upon Substrate Binding**

In addition, IPₚ serves as substrate for the synthesis of diphosphoinositol polyphosphates (inositol pyrophosphates), emergent molecules with multiple functions (14). In plants, IPₚ has been shown to be involved in the maintenance of basal resistance to plant pathogens (15) and represents a reserve of phosphorus in storage tissues. The high phosphorus content of seeds is a cause of major problems for both human health and the environment (16). In developing countries, grain-based diets provide an excess of IPₚ, which exacerbate iron and zinc malnutrition due to the potent metal-chelating properties of IPₚ. In addition, monogastric animals are unable to digest IPₚ, thus excreting it which leads to water eutrophication (17). Consequently, there is a high demand for the development of low phytate seeds (18–20).

IP₅ 2-K belongs to the inositol polyphosphate kinases (IPKs) family, which comprises three other subfamilies as follows: IP₃ 3-kinases, IP multikinas (IPmKs), and IP₆ kinases (2). The first structure of an IPK, the IP₃ 3-K isofrom A (21, 22), became available in 2004 and was followed by the structure of yeast IPmK (23). More recently, we reported the first structure of an IP₅ 2-kinase (24) from Arabidopsis thaliana, which showed, unexpectedly, that this enzyme belongs to the IPK family, albeit the most distant member. IP₅ 2-Ks, as well as other IPKs, conserve key features with protein kinases (PKs); they fold in two lobes (N- and C-lobes), connected by a hinge, and the N-terminal lobe core conserves an α+β fold. In addition, the essential features of ATP recognition between both lobes are conserved. However, the C-lobe of IPKs is not conserved with PKs, and the inositol-binding site, located in a separated portion of this C-lobe, is characteristic for each enzyme within the IPK family. In particular, IP₅ 2-K shows the most divergent region for inositol binding (referred to as CIP-lobe) that is formed by multiple α-helixes folded into a unique large domain that encompasses almost half of the protein (24). There is a different family of inositol phosphate kinases, exemplified by the Entamoeba histolytica enzyme (25), that show the ATP-grasp fold.

The most recent addition to this family is the diphosphoinositol pentakisphosphate kinase subfamily, the structure of which was recently solved (26). Although these enzymes display a different fold from IPKs, both share some essential features of catalysis (26). Representatives of all the inositol phosphate kinases mentioned, except IP₅ Ks, have been crystallized and many of them in presence of substrates or products, yielding complete or partial clues about their catalytic mechanism.

The structural analysis of IP₅ 2-K offers a better understanding of its function at the molecular level. The reported complexes of IP₅ 2-K with inositol, both substrate (IP₄) and product (IP₆), and the ternary complexes with substrates (IP₄/AMPPNP) and products (IP₆/ADP) allowed us to identify key residues defining inositolite- and nucleotide-binding sites, as well as crucial elements in the enzymatic mechanism (24). However, there are several aspects that remain to be understood. In particular, all the complexes display a common conformation of the active site that does not correlate with different changes observed in the fluorescence spectra upon substrate binding. We attributed these changes to putative conformational changes that could not be fully characterized because the inositol presence was a requirement for the crystallization of AtIP₅ 2-K samples (24, 27); consequently, its apo-form remained elusive. In this work, we have followed a strategy for growing crystals from IP₅ 2-K in its free state that was also applied to obtain complexes with nucleotide, always in absence of inositol. The results provide a complete picture of the conformational changes produced upon inositide and/or nucleotide binding that allowed full identification of the molecular landmarks responsible for IP₅ 2-K function. In agreement with the structural studies, fluorescence analysis shows significant IP₅ 2-K changes upon substrate binding. In addition, the exact role of the IP₅ 2-K N-lobe in protein conformational changes and substrate specificity has been also investigated. For this purpose, we have explored discrimination between enantiomers of two IP₄s, Ins(1,4,5,6)P₄ and Ins(3,4,5,6)P₄, that have been reported to be substrates of plant IP₅ 2-K in *vivo*, and we solved the structure of IP₅ 2-K in ternary complex with ATP analog and Ins(3,4,5,6)P₄. Intriguingly, despite the wealth of literature on Ins(1,4,5,6)P₄ metabolism in yeasts and Ins(1,4,5,6)P₄ metabolism in yeasts and Ins(1,4,5,6)P₄ metabolism in animals, neither of these two isomers is considered physiological substrates of IP₅ 2-K enzymes in these kingdoms. Our experiments provide a structural basis for such consideration.

The results presented here, together with our previous work, represent the first structural description of a complete catalytic cycle of IP₅ 2-K enzymes and gives insights into the discrimination of enantiomeric substrates in IP₅ 2-K.

**EXPERIMENTAL PROCEDURES**

**Purification, Crystallization, and Data Collection—Wild type and W129A IP₅ 2-K mutant were produced in *Escherichia coli* Rosetta (DE3) pLysS strain and purified as reported previously (28). The crystallization of W129A–IP₅ 2-K mutant, in its apo- and AMPPNP-bound forms is detailed in Baños-Sanz et al. (28). Briefly, to obtain the W129A IP₅-2K crystals, equal amounts of protein solution (10 mg/ml) and precipitant (29% w/v PEG4000, 0.2 M LiSO₄, and 0.1 M Tris/HCl, pH 8.5) were mixed and equilibrated against a reservoir containing 0.5 ml of precipitant using the sitting drop technique. Seeding was used to obtain high quality crystals. Crystals appeared in 5 days at 18 °C. For data collection, crystals were cryoprotected using a gradual increase in the PEG content from 29 to 35% of the crystal mother liquor. For AMPPNP-bound IP₅ 2-K, the mutated protein was mixed with 2 mM AMPPNP, and crystals were obtained in 0.5 M (NH₄)₂SO₄, 0.1 M sodium citrate tribasic, pH 5.6, and 0.8 M Li₂SO₄ and cryo-protected with this condition plus 20% glycerol. Seeding was again crucial to facilitate crystal growth. The ADP-bound IP₅ 2-K crystals were obtained in the same condition, except the protein was incubated previously to crystallization trials with 2 mM ADP. To obtain wild type crystals in complex with Ins(3,4,5,6)P₄ and AMPPNP, we have followed our protocol previously reported (27). In this case, protein was incubated with 1 mM IP₄, plus 2 mM AMPPNP, crystallized, and cryoprotected under the same condition.

---

*The following nomenclature is used: stereoisomers and enantiomers of inositol phosphates are numbered according to the δ-assignment, thus δ- and l-Ins(1,4,5,6)P₄ are identified as Ins(1,4,5,6)P₄ and Ins(3,4,5,6)P₄, respectively.*
conditions as for the other wild type crystal complexes (24). Diffraction data for all the crystals was collected using synchrotron radiation from European Synchrotron Radiation Facility (Grenoble, France). All the data were processed using iMosflm (29) and merged with Scala (30) from the CCP4 package (31). A summary of data collection statistics is presented in Table 1.

**TABLE 1**
Crystal data collection, structure determination, and refinement

| Crystal | W129A | W129A/AMPPNP | W129A/ADP | IP5 2-K/AMPPNP/IP5 |
|---------|-------|-------------|-----------|------------------|
| Space group | P2₁,2,2 | P2₁,2,2 | P2₁,2,2 | P2₁,2,2 |
| Cell | 66.00 | 63.06 | 63.72 | 61.62 |
| Wavelength | 1.05 Å | 1.05 Å | 1.05 Å | 1.05 Å |
| Resolution range | 47.44 Å to 2.25 Å | 53.37 Å to 2.05 Å | 39.49 Å to 2.50 Å | 57.47 Å to 2.93 Å |
| Refinement | 1.0053 Å | 0.9334 Å | 0.9334 Å | 0.9334 Å |
| Mean R(ref) | 99.6% (100%) | 100.00% (100%) | 100.00% (100%) | 100.00% (100%) |
| Missing residues | 1-2, 47-60, 152-161, 334-341, 47-58, 155-158 | | |
| Ramachandran data | Most favored 92.5% | 91.2% | 92.4% | 92.3% |
| Disallowed 0.3% | 0.5% | 0.3% | 0.3% | 0.3% |

The mutants were grown in *Lentinus sulphureus* was used as template (24). The incorporation of the mutations was assessed by DNA sequencing. Mutants were grown in *E. coli*
**IP₅ 2-K Conformational Changes upon Substrate Binding**

Rosetta (DE3) pLysS strain as wild type protein. Cell pellets were resuspended in buffer A (20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM DTT plus a Complete EDTA free protease inhibitor mixture (Roche Applied Science)) and disrupted with a French press. The filtrated cell lysate was applied onto a Sepharose CL-4B column equilibrated previously with buffer A, and the protein was eluted with buffer A plus 200 mM lactose. Protein-containing fractions were pooled and diluted 3-fold with 20 mM Tris, pH 8, to reduce the salt concentration, loaded onto a heparin column, washed with buffer B (20 mM Tris, pH 8, 50 mM NaCl, 2 mM DTT), and eluted with a gradient between buffer B and C (20 mM Tris, pH 8, 1 mM NaCl, 2 mM DTT). The eluted fused protein was cleaved by tobacco etch virus protease (protease/protein mass ratio 1:80), shaking the sample gently at 4 °C overnight. To remove the lectin of L. sulphureus tag, we used another Sepharose CL-4B column. Mutants were concentrated between 0.5 and 1 mg/ml and stored at −80 °C until use.

**Enzyme Assays**—Kinase assays were performed in 0.2-ml volumes on a Molecular Devices SpectraMax M5 Plate reader using an assay similar to that described previously (38, 39). The assay mixture contained 20 mM Hepes, pH 7.5, 1 mM MgCl₂, 0.1 mM ATP, 2 mM P-enolpyruvate, 0.15 mM NADH and 7.5 units lactate dehydrogenase, 15 units ml⁻¹ PK, 5 μM inositol phosphate. Reactions were started by addition of the above assay mixture to enzyme and 100 or 500 ng of native protein or mutant as specified in the text. Assays were run for 15 min at 25 °C and initial linear rate measurements (ΔOD min⁻¹) were determined using the SoftMax Pro software of the plate reader: initial rates were linear for 3–9 min. Assays were typically performed with 4–5 replicate wells and were repeated no less than three times.

**Kinetic Analysis of Native and W129A Mutant**—Kinase assays were performed with 100 ng of native or W129A protein and 0.3–10 μM Ins(1,3,4,5,6)P₅. Assays were performed with 4–5 replicate wells and repeated three times. Kᵡ app and Vₘₐₙₐₐₐ max app values were determined by nonlinear least squares regression of a plot of V app versus substrate concentration fitted to the Michaelis-Menten equation in GraFit Version 5 (Eirithacus Software Ltd.).

**Fluorescence Assays**—Substrate binding to native and W129A protein was followed at 25 °C by monitoring changes in intrinsic fluorescence recorded with an excitation wavelength of 295 nm, bandwidth 5 nm, and emission from 310 to 380 nm, bandwidth 10 nm, on a Cary Eclipse fluorescence spectrometer. The photomultiplier voltage was set to 800 V. Protein (14.5 μg) was suspended in 1 ml of 20 mM HEPES, pH 7.5, 1 mM MgCl₂ in a 10 × 3-mm cuvette. Inositol 1,3,4,5,6-pentakisphosphate or AMPPNP was added from 0.1 and 5 mM stocks, respectively. The total volume of ligand added was less than 1% of the sample volume. Data were exported in GraFit Version 5 (Eirithacus Software Ltd.). Plots of fractional change in fluorescence (F o − F)/F o versus ligand concentration, determined at 335 nm) were analyzed by nonlinear least squares regression to a single site binding model in GraFit Version 5. Experiments were repeated three times.

**RESULTS**

**Structure of IP₅ 2-K Apo-form Represents the Open Conformation**—Previously reported IP₅ 2-K inositide-bound structures, with or without bound nucleotide, show a common conformation that leaves a hollow for ATP binding formed between the N-lobe and C-lobe. The inositide is mainly bound to the C-lobe (at the unique region named φ₁₋₃-lobe), but it also binds to a few N-lobe residues bringing together both lobes (Fig. 1A). We will refer to this state as “closed” or “inositide-bound” form. In this form, the β- and γ-phosphates of the ATP are linked to the G-loop (Glu18–Asn21), a feature common to PKs, and the active site is enclosed by residues from the structural elements α6 (Leu128–Ala133) and L3 (Gly251–Ser261) that interact forming a kind of clasp (Fig. 1A, zoom). Because the clasp seems to preclude the nucleotide from entering/leaving the active site, we suspected that the clasp elements might be unconcected in a putative open conformation, and therefore, we expected that L3 would present high mobility. Accordingly, the IP₅ 2-K-IP₅ crystal complex showed some disorder at L3 (Protein Data Bank code 2xao). Moreover, AtIP₅ 2-K exhibits changes in gel filtration elution profile (data not shown) and in tryptophan intrinsic fluorescence (see below) upon substrate binding, indicative of structural changes, which in turn could arise from changes in the environment of Trp129. We have therefore undertaken an in-depth study of the clasp role through characterization of several IP₅ 2-K mutants (W129A, W129V/R130A, R130A, and ΔS253/ΔE255) (Table 2). One of these fully active mutants, W129A, allowed us to obtain crystals from the enzyme in its apo-form, i.e. in its unbound state. The crystals obtained present a different unit cell, space group, and crystal packing than the inositide-bound structures (Table 1) (28).

The W129A IP₅ 2-K structure shows a similar overall fold to that reported previously for the enzyme, but the N-lobe and C-lobe are farther apart from each other forming a more open cleft (Fig. 1B). We will refer to this form as “open form” or “apo-form.” Superposition of Cα from open and closed (24) conformations gives an r.m.s.d. of 2.50 Å; however, a close inspection reveals that there is a significant motion of two independent portions of the protein (Fig. 1C) as follows: one is formed by most of the N-lobe, which includes residues 6–42 and 103–148 (r.m.s.d. of 1.27 Å superposing 81 residues from each state); and the other is composed of a segment of the N-lobe previously referred to as N-I (24) that includes residues 63–102, and the whole C-lobe (r.m.s.d. of 0.87 Å for 302 residues). This motion upon substrate binding can be described as an 18.4° rotation of one portion of the enzyme, which pivots about three regions of the polypeptidic chain acting as hinges; two of them are the two segments that connect N-I to the remaining part of the N-lobe (hinge 1, 42–63; hinge 2, 102–103) and the third one is the linker between N- and C-lobe (hinge 3: 148–159), a typical hinge common to the family of PKs (Fig. 1C). As expected, these hinges are the most disordered parts in the electron density reflecting its intrinsic flexibility. Apart from this general rigid-body movement, local changes occur particularly at α6 and L3 segments (Fig. 1C, and bottom
The conformation is dependent on the clasp formation. A detailed comparison of open and closed IP₅ 2-K forms reveals that the CIP-lobe pocket remains unaltered upon inositolide binding (Fig. 1D). Therefore, we can assume that the major inositolide-binding site is preformed in the apo-form. However, this site is not fully created in the open form because the N-lobe is too far away to accomplish inositolide recognition. This recognition involves interactions with the side chain of residues Arg₄₅ and Arg₁₃₀ and the main chain of the G-loop (Fig. 1D).

A last interesting feature is the presence of three sulfate ions in the crystal at positions similar to those occupied by Pγ(A TP).
and by P1 and P6 (inositide), as found in the IP$_{5}$-2-K ternary complex structure (Fig 1, B and D). These sulfate moieties, present in the crystal buffer, mimic the binding positions of the substrate in the open form of IP$_{5}$-2-K.

IP$_{5}$-2-K Nucleotide-bound Structure Presents a Half-closed Conformation—In our previous work, we have reported the structure of IP$_{5}$-2-K ternary complexes with either substrates (IP$_{6}$ + AMPPNP) or products (IP$_{6}$ + ADP) bound at the active site (21, 24). The structure of these ternary (nucleotide + inositide) complexes revealed a common conformation with inositide-IP$_{5}$-2-K binary complexes. However, the permanent presence of the inositide in our crystals precluded the assessment of specific conformational changes produced upon ATP binding. A new full crystal screening allowed us to crystallize W129A IP$_{5}$-2-K with the nucleotide (ATP or ADP) (28). Although these crystals appeared in very different conditions and showed different cell parameters with respect to the apo-form, they present the same space group and crystal packing. Superposition of the nucleotide-bound coordinates to those corresponding to the open and closed conformations of IP$_{5}$-2-K shows that the enzyme is in an intermediate state somewhere between the apo-form (r.m.s.d. = 1.47 Å) and the closed form adopted by the ternary complex (r.m.s.d. = 1.71 Å) (Fig. 2A). Therefore, IP$_{5}$-2-K reaches a half-closed conformation upon ATP binding that corresponds to a 10° rotation of the same portion of the N-lobe described above. We refer to this form as the "nucleotide-bound" or "half-closed" form. The ATP-binding site is essentially the same as that found in the ternary complexes, with only two significant differences as follows. First, in the absence of inositide there is no evidence of Mg$^{2+}$-binding in the electron density map. This could be due to the different lobes of orientation in this form, which conforms a Mg$^{2+}$-binding site slightly distorted as compared with that observed in the closed form. Second, a small tilt in ATP position is observed upon inositide binding with a better fit of the nucleotide into a slightly more closed pocket in the ternary complex (Fig. 2B). Despite this, the nucleotide pocket formed conserves all the essential features of the nucleotide recognition by the protein kinase superfamily, revealing that this conformation is physiologically relevant. In contrast, the C-lobe remains unaltered upon nucleotide binding, and the N-lobe, although closer, is still too far away to complete the inositide-binding site; consequently, the clasp observed in the closed-form is not formed.

In the half-closed form that we obtained with nucleotide, sulfate ions were found bound in similar positions to that occupied in the closed form by inositide phosphates P1, P6, and P5 (Fig. 2A). These sulfates putatively mimic the binding of inositide in this half-closed form. Finally, unlike what was observed in the open and closed conformations, hinge 1 and hinge 3 are ordered in this form.

Snapshots of the Conformational Changes—The crystal structures of the two IP$_{5}$-2-K states here reported, together with our previous work (24), provide more detail of the enzymatic mechanism. Our experimental results reveal essential structural motions associated with nucleotide and inositide binding that not only involve a large movement of two protein portions as rigid bodies but also local changes on the clasp region (Fig. 3). As predicted before, the clasp is fully formed only in the closed state by interaction of L3 (from the C-lobe) and α6 (from the N-lobe), through Trp$^{129}$–Glu$^{255}$ and Arg$^{130}$–Gly$^{254}$ links (Fig. 3A, right). The formation of this clasp requires the rupture of the Gln$^{255}$–Lys$^{300}$ ion pair that links L3 to the C-lobe, in both the open and nucleotide-bound conformations (Fig. 3A, left and middle panels). This breakage releases L3 to act as the "lid" that closes the pocket upon inositide binding. The conformation of both α6 and L3 changes significantly among the different structural states of IP$_{5}$-2-K (Fig. 3A). In fact, the segment α6 is not structured in the open conformation; the helix is fully formed only in the closed state. In addition, L3 is rearranged in the closed state, taking an extended conformation formed by a new pattern of interactions with IP$_{5}$-2-K residues of loop Thr$^{235}$–Asn$^{239}$ (L2), where the conserved $^{237}$QNN motif is located (Fig. 3B). Thus, the interaction Asn$^{238}$–Arg$^{241}$ observed in the open and half-closed conformation is broken leaving L2 able to interact with L3 through Gln$^{257}$–Thr$^{257}$ and Asn$^{238}$–Ser$^{256}$ links. Consequently, most of the structural changes observed in IP$_{5}$-2-K are due to inositide binding, but it is worth mentioning that in the nucleotide-bound form the clasp seems partially created through interaction of Arg$^{130}$ (α6) and Gly$^{352}$ (L3).

Clasp Role—As shown in Table 2, we have measured the activity of several IP$_{5}$-2-K mutants of clasp-contributing residues to assess their contribution to the enzymatic mechanism. Assays were performed by a coupled enzyme assay allowing steady-state measurements. In particular, we have analyzed the effect of single and double substitutions in α6 key residues (W129A, R130A, and W129V/R130A) and deletions in L3 (ΔSer$^{253}$/ΔGlu$^{255}$). First, the activity of the W129A mutant is uncompromised leading to the conclusion that Trp$^{129}$ participation in clasp formation is not critical for enzyme activity. It is worth noting that the bond formed by Trp$^{129}$ in the clasp is not the unique determinant for the clasp formation. Second, mutation of Arg$^{130}$ results in more than 6-fold decreased activity toward Ins(1,3,4,5,6)P$_{5}$ and toward other substrates (see below), and the double mutation W129V/R130A results also in a 5-fold reduced activity, a fact that could be ascribed mainly to the Arg$^{130}$ mutation. Arg$^{130}$ is involved in both clasp formation and inositide binding through P1 (Figs. 1D and 5); therefore, its contribution may be more critical for enzyme function. Finally, shortening of L3 by two residues (ΔSer$^{253}$/ΔGlu$^{255}$) results in an IP$_{5}$-2-K mutant 3-fold more active that the native form. Modeling this shorter loop in the IP$_{5}$-2-K structure suggests that deletion of these two residues precludes clasp formation. We suggest that the increased activity may be attributed to an improved kinetics of the enzyme, perhaps through removal of
IP$_5$ 2-K Conformational Changes upon Substrate Binding

Figure 2. Conformational changes upon nucleotide binding. 
A. Comparison of the three IP$_5$ 2-K obtained conformations (open, half-closed, and closed forms). The superposition has been made on the C-lobes and segment N-I as before. The IP$_5$ 2-K nucleotide-bound form is shown as orange (N-lobe) and white (C-lobe) schematics. The nucleotide and the sulfate moieties found in this form are shown as transparent violet surface and green surface, respectively. As the C-lobes superpose very well in the three cases, only the rotated N-lobe region and L3 have been shown for the open (red) and closed (blue) forms. This picture highlights the sequential structural changes upon nucleotide and inositide binding, as well as the clasp elements variation.

B. Comparison between the nucleotide-binding site as shown in the half-closed (orange) and closed (blue) forms. The superposition has been made by matching the ATP molecules. The ATP-binding site is very similar in both structures, although it is more constrained in the closed form. Magnesium found in the closed form is shown as red sphere.

the lid, that would decrease the energetic barrier of the process, for instance through a more favorable release of products.

As mentioned above, we have shown that W129A-IP$_5$ 2-K retains as much activity as the native enzyme (Table 2). We have performed kinetic measurements for native and W129A IP$_5$ 2-K (Fig. 4 and Table 3). Our results show that, although there are no dramatic changes, the native IP$_5$ 2-K presents ~3-fold greater affinity for the inositide substrate ($K_m$ app, 3.7 ± 1.0 μM) than the mutated enzyme (13.7 ± 3.0 μM). However, $V_{max, app}$ of W129A mutant (0.95 ± 0.16 μmol min$^{-1}$ mg$^{-1}$) is 2-fold higher than that of the native enzyme (0.54 ± 0.07 μmol min$^{-1}$ mg$^{-1}$), telling us that the mutant is more efficient. The removal of the Trp129 side chain probably affects the inositide binding pocket formation, as it is next to Arg130. In turn, Trp129 removal likely weakens clasp formation producing a more efficient enzyme, which correlates with our previous suggestion that lid removal could be favoring the product release.

Role of the N-lobe in Inositide Binding and Specificity—Inositol 1,3,4,5,6-pentakisphosphate is bound to the N-lobe through phosphates P1 (Arg$^{130}$) and P3 (Arg$^{45}$ and G-loop main chain) (Fig. 1D). To assess the importance of P1 and P3 to inositide binding and ensuing conformational change, we made several attempts to crystallize IP$_5$ 2-K in presence of the enantiomers Ins(3,4,5,6)P$_4$ and Ins(1,4,5,6)P$_4$, which are expected to bind in the same orientation as Ins(1,3,4,5,6)P$_5$. The presence of a plane of symmetry along the C2–C5 axis of Ins(1,3,4,5,6)P$_5$ allows, in comparison with the two IP$_5$s, an assessment of the contribution of residues coordinating the enantiotopic 1- and 3-phosphates.

We obtained crystals of IP$_5$ 2-K in ternary complex with Ins(3,4,5,6)P$_4$ and AMPPNP. These crystals turned out to be similar to the previously solved ternary complexes and binary complexes of IP$_5$ 2-K and inositide (r.m.s.d., 0.425 Å) (24) and confirm that Ins(3,4,5,6)P$_4$ binds in the same orientation as Ins(1,3,4,5,6)P$_5$ (Fig. 5). The most interesting feature of the new ternary complex is that Arg$^{130}$ is perfectly oriented to potentially bind the absent P1, and it maintains interaction with L3 forming the clasp as in IP$_5$-bound structures. This result suggests that Arg$^{130}$ participation in P1 binding and clasp formation represents two separate facets of IP$_5$ 2-K function. As mentioned before, the R130A mutant presents reduced but significant IP$_5$ 2-K activity, suggesting that although Arg$^{130}$ is an important residue, it is not essential for catalysis. In parallel, as we were unsuccessful in attempts to grow crystals in the presence of inositol phosphate (5 M) and ATP (100 μM) (Table 4), Ins(3,4,5,6)P$_4$ and Ins(1,4,5,6)P$_4$ are bound to the protein G-loop, it seems likely that the G-loop plays a main role in inositide binding and in subsequent conformational changes.

Because there remains conjecture concerning the relative contribution of different pathways to IP$_6$ synthesis in plants (19), and because Ins(1,4,5,6)P$_4$ and Ins(3,4,5,6)P$_4$ have been reported to be substrates in vitro of plant enzymes (6, 18), we have compared the activity of IP$_5$ 2-K toward Ins(1,4,5,6)P$_4$ and Ins(3,4,5,6)P$_4$ for native protein and for a range of mutants in N-lobe residues. Our assays were performed at a fixed concentration of inositol phosphate (5 μM) and ATP (100 μM) (Table 4). Ins(3,4,5,6)P$_4$ and Ins(1,4,5,6)P$_4$ are equally active substrates for IP$_5$ 2-K. The ratio of activity against Ins(1,4,5,6)P$_4$/Ins(3,4,5,6)P$_4$ is increased significantly by the R45A mutation and reduced slightly by W129A mutation. However, and remarkably, substitution involving Arg$^{130}$ (R130A) increase.
markedly the discrimination of the enzyme in favor of \( \text{Ins}(3,4,5,6)P_4 \). These results highlight the importance of Arg\(^{130} \) in \( P_1 \) binding. We are not aware of other structure-based studies of discrimination between enantiomeric substrates in inositol phosphate kinases.

**Intrinsic Fluorescence Decreases upon Substrate Binding** — The new structures that we have obtained provide description of conformational changes consequent upon ligand binding. We have undertaken ligand binding analysis of native and W129A mutants by fluorescence. For this purpose, we titrated \( \text{Ins}(1,3,4,5,6)P_5 \) substrate and recorded changes in intrinsic tryptophan fluorescence, exciting at 295 nm to limit fluorescence to tryptophan residues. We performed similar experiments titrating the nucleotide analog AMP-PNP. Before performing substrate/analog titrations, we undertook Stern-Volmer analysis of quenching of tryptophan fluorescence with iodide as the quencher. The linearity of the unmodified Stern-Volmer plot and the \( y \) axis intercept of 1.03 for native and 1.02 for W129A (data not shown) indicate that the tryptophan residues of native and W129A protein are all accessible to the quenching agent. We also assessed the temperature dependence of fluorescence quenching by iodide. The increased gradient and linearity of the plot at higher temperature indicate that the quenching process is collisional. These data are compatible with all tryptophans solvent-exposed in native and W129A protein. \( \text{IP}_5 \) 2-K has a total of four tryptophan residues among which two of them are on protein surface but partially buried in an hydrophobic pocket (Trp\(^{13} \) and Trp\(^{69} \)), although the other two are totally exposed (Trp\(^{129} \) and Trp\(^{381} \)). More precisely, Trp\(^{129} \) would change its conformation from a partially exposed (closed form) to a completely exposed (open form, mutated by Ala) upon binding.

Incremental addition of \( \text{Ins}(1,3,4,5,6)P_5 \) yielded a saturating decrease in fluorescence for both native and W129A \( \text{IP}_5 \) 2-K (Fig. 6, A and B). Although it is clear that the decrease in fluorescence for the W129A mutant arises from residues other than tryptophan Trp\(^{129} \), that for native protein additionally includes Trp\(^{129} \). Fluorescence decreases in both cases are moderate. Assuming that Trp\(^{281} \), permanently solvent-exposed, is not a main contributor, the moderate changes are in concordance with slight distortions produced in Trp\(^{13} \) and Trp\(^{69} \) pockets upon inositide binding observed in the crystals. Plots of fractional change in fluorescence versus ligand such as that shown for native protein (Fig. 6C) yielded \( K_a \) values for \( \text{Ins}(1,3,4,5,6)P_5 \) of 0.35 ± 0.12 \( \mu \)M for native protein and 0.42 ± 0.03 \( \mu \)M for W129A \( \text{IP}_4 \) 2-K. These data show that binding of

---

**FIGURE 3. Snapshots of the different \( \text{IP}_5 \) 2-K conformations.** A, sequential view of \( \text{IP}_5 \) 2-K active site structure in open (red), half-closed (orange), and closed (blue) forms. The N- and C-lobes are distinguished by different color shades. The key protein elements that cover the active site and that change conformations between the different forms (G-loop, L3, α6) are highlighted in green. Interactions for clasp formation in the closed form as well as the singular intramolecular ion pair (Asp\(^{255} \)-Lys\(^{709} \)) in open and half-closed forms are highlighted. Sulfate moieties are shown as green sticks. B, \( \text{IP}_5 \) 2-K active site along its different forms represented as sticks and following the code colors of A. Specific interactions formed and broken upon nucleotide and inositide binding are shown. Sulfates and substrates are shown as black sticks, highlighting phosphates and sulfate atoms as blue spheres.
Ins(1,3,4,5,6)P₅ causes conformational change in IP₅ 2-K (either wild type or mutant), a result consistent with differences in conformation found between ligand-free and IP₅ 2-K inositol-bound structures. Remarkably, the affinity for inositide is similar in both proteins.

Titrations of AMPPNP also gave a saturating decrease in fluorescence, again for both native and W129A IP₅ 2-K (Fig. 6, D and E). Plots of fractional change in fluorescence versus ligand such as that shown for native protein (Fig. 6F) yielded $K_\text{d}$ for AMPPNP of 37 ± 9 μM and 55 ± 2 μM, respectively, for native and W129A protein. These structural changes reported by fluorescence upon AMPPNP binding are in agreement with the crystallographic differences in conformation between W129A IP₅ 2-K ligand-free and in complex with the nucleotide. The affinities for the nucleotide showed by the wild type and W129A IP₅ 2-K are quite similar, although slightly decreased for the W129A protein. Because the clasp would not be properly formed in this mutant, probably a less stable nucleotide complex is formed.

**DISCUSSION**

We have obtained different snapshots of IP₅ 2-K functional states (Figs. 2A and 3) by x-ray crystallography. Each different state is unequivocally correlated with binding of nucleotide or inositide at the active site. Thus, the protein shows an open conformation in the absence of substrates, a half-closed conformation when bound to nucleotide, and as reported previously (24), a closed conformation in the inositide-bound state. In agreement with the crystal structures presented here, IP₅ 2-K intrinsic fluorescence decreases upon nucleotide and inositide binding (Fig. 6). We have predicted previously such conformational changes (24). Very recently, some experimental evidence of these changes has been reported by partial proteolysis experi-
ments that show stabilization of some regions of IP₅ 2-K upon inositolide and nucleotide binding (40). This work shows that high resolution analysis of the conformational events has been possible by a single mutation (W129A) allowing description of a complete picture of the different conformational states experienced by IP₅ 2-K throughout the substrate binding stages of catalysis. The assumption that the W129A mutant is a mimic of WT IP₅ 2-K is based on the experimental evidence presented here. In addition, the W129A mutant presents similar protection from proteolysis in the presence of nucleotide and inositide as wild type IP₅ 2-K, as well as a similar band shift on native gels in presence of IP₆ (data not shown).

Inositide Alone Can Promote Closed Conformation and Clasp Formation—An intriguing aspect from our studies is that inositide is sufficient and necessary to trigger the closed conformation and complete the clasp formation, and it can do so independently of ATP binding. We were able to crystallize IP₅ 2-K with AMPPNP/Ins(3,4,5,6)P₄ in the closed form; however, trials with AMPPNP/Ins(1,4,5,6)P₄ were unsuccessful. One potential explanation of these observations is that the inositide P3 coordination to Arg₄⁵ and/or the G-loop is essential to favor the closed form upon inositide binding (Fig. 5). In fact, because Arg₄⁵ mutation does not significantly compromise enzyme activity, we suggest that the G-loop may be the principal N-lobe element for inositide binding and subsequent conformational changes. In addition, the clasp is fully formed only in the closed conformation. Mutation of clasp elements that would preclude its formation yield active enzymes, suggesting that the clasp formation is not essential for protein activity (Table 2). On the contrary, the clasp might be relevant for modulating protein kinetics as suggested by enzymatic and kinetic measurements. In conclusion, both events, domain closure and clasp formation, are coupled to inositide binding, and the G-loop could be playing a direct role in the conformational changes. A main role for a G-loop has been largely studied in protein kinases, where it has been suggested that it is a regulatory flap above the ATP-binding site showing great flexibility (41, 42).

In this closed form, the inositide blocks the nucleotide entrance/exit to/from the active site. This suggests that the nucleotide might bind first, producing a half-closed conformation, and the inositide later completed the domain closure. Then the product release should proceed in an inverse order, because the nucleotide is completely occluded in the inositide-bound form. The exit of ADP could be the limiting step for IP₅ 2-K activity, as it has been reported for PKs (43), because the motion to open the domains and/or to undo the clasp is necessary for nucleotide release. These suggestions are supported by the fact that clasp-affected mutants as W129A or ΔS253/ΔE255 produce more efficient or active proteins. Nevertheless, more work has to be done to confirm these entire hypotheses about substrate binding order. We consider that the capacity of inositide alone to trigger the closed conformation could reflect its role as an enzyme inhibitor and therefore in regulation of enzymatic activity. Several examples of similar proteins being inhibited by its substrate/product can be found in the literature (44–47).

Modulation of Enantiomeric IP₄ Preference of Plant IP₅ 2-Ks—The relative contribution of different pathways to IP₆ synthesis in plants is the focus of much attention (18, 19). We
have compared the activity of IP₅ 2-K toward Ins(1,4,5,6)P₄ and Ins(3,4,5,6)P₄ for native protein and for a range of mutants, because both enantiomers represent intermediates in disparate pathways reported to proceed from inositol 1,4,5-trisphosphate and inositol, respectively (19). Ins(1,4,5,6)P₄ is a product of plant multikinase action against inositol 1,4,5-trisphosphate, certainly in vitro (48, 49). AtIPKβ, a multikinase, and AtIPK1 (IP₅ 2-K) contribute to IP₆ synthesis in Arabidopsis (18). Ins(3,4,5,6)P₄ was identified in early studies of IP₆ synthesis in duckweed (50). This isomer is a substrate of plant ATP-grasp fold inositol tris/tetraakisphosphate kinases (20, 51–53), and enzymes of this class contribute to IP₆ synthesis in maize (20).

We have shown using a coupled assay that Ins(3,4,5,6)P₄ and Ins(1,4,5,6)P₄ are equally effective in vitro substrates for IP₅ 2-K, and we have now determined the role of Arg¹³⁽⁰⁾ and Arg⁴⁵⁽⁵⁾ in the specification of substrate preference between enantiomers. We note that although Arg¹³⁽⁰⁾ is fully conserved across kingdoms, Arg⁴⁵⁽⁵⁾ is not, and within plants the equivalent residue is variable (6). Moreover, despite the elucidation of structures of a variety of inositol phosphate kinases belonging to the ATP-grasp fold and inositol polyphosphate kinase families, this is the first study in which discrimination between enantiomeric substrates has been investigated. Our choice of substrates, although dictated by consideration of what is known of inositol phosphate metabolism in plants, is of obvious relevance to animal inositol phosphate metabolism centered on the same enantiomers. In addition, manipulation or inverting the enantiomeric substrate preference and therefore enantiomeric product production is of great interest from a chemical and a biotechnological point of view.

**Singular Enzyme among the Inositide Kinase Family**—The structural features for all inositol kinase families have been extensively studied to determine the structural determinants of substrate specificity. Structures are available for different inositol phosphate kinases in the presence/absence of substrates and products, providing data about structural changes produced upon binding of nucleotide and/or inositide. With respect to the IPK family, to which IP₅ 2-K belongs, none of the structures solved up to now (IP₅ 3-K (21, 22) and IP₅mK (23)) show significant structural changes upon nucleotide binding. However, data upon inositide binding is only known for IP₅ 3-K, for which a slight conformational change is described. This change consists of an inositide site rearrangement achieved by a protein intramolecular bridge breakage between the C-lobe and IP₅ binding (Arg⁴¹⁻ Asp⁷⁴) leaving Arg⁴¹ free to interact with inositide. As seen in IP₅ 2-K, a bond is also broken upon inositide binding (Lys²⁰⁰⁻ Asp²⁵⁵) to leave Lys²⁰⁰ able to bind inositide. Nevertheless, the bridges in the two proteins are not structurally related, and IP₅ 3-K does not present structurally equivalent elements of L3 or ø6. In any case, the conformational change produced in IP₅ 3-K is local, and it does not involve large movements between domains as in IP₅ 2-K. A member from the other family of inositol phosphate kinases, those from ATP-grasp fold, also shows structural differences upon inositide binding. In the recent published structure for the pyrophosphate inositol-synthesizing enzyme PPIP5K (26), the side chain of three large basic residues changes markedly upon inositide binding, revealing a major reorganization in the inositide-binding site configuration without involving backbone changes. In conclusion, this is the first time that such a conformational change involving domain movements upon inositide as well as nucleotide binding is shown for a member of the inositol phosphate kinase family. However, we must not ignore the fact that it has not yet been possible to crystallize every inositol kinase and in all their different states. Thus, none of the IP₅ K isoforms structures are available at the moment and neither are the IPmKs in the presence of inositide nor PPIP5K in the absence of nucleotide. Consequently, the possibility of undetermined conformational changes for these families of enzymes cannot be precluded.

**Comparison with Protein Kinases**—A similar domain movement has been reported for protein kinases (42, 54). In the most studied PK (PKA), the nucleotide binding generates a closure between both N- and C-lobes (43). In our previous work (24), we reported that the distinctive bridge linking α C and the N-lobe β3 in PKAs structures representing the active conformation (Lys⁷²⁻ Glu⁹¹) is not formed in IP₅ 2-K (Arg⁴⁰⁻ Glu⁸⁵ equivalent residues); therefore, we suggested that the bridge is not a hallmark for active conformation in IP₅ 2-Ks (Fig. 7). On the contrary, interaction between α C and the C-lobe is retained in both PKAs and IP₅ 2-Ks. Thus, in concordance with our previous results, the IP₅ 2-K open and half-closed conformations also lack this bridge, and in the domain motion reported here, the N-lobe region containing α C (N-I) is coupled with the C-lobe (Fig. 7), whereas in PKA the motion from open to close conformations keeps α C in contact with both lobes (43).

**Conclusions**—From our previous inositide-bound IP₅ 2-K structure (closed form) and using a rational approach, we have designed several IP₅ 2-K mutations prone to yield an open protein conformation. From a specific protein mutant, W129A, we have conducted crystallization of IP₅ 2-K in an open, free of substrates, and a half-closed nucleotide-bound conformation. Combining this information with the known closed inositide-bound form, we have provided a schematic view of the conformations adopted by IP₅ 2-K in the course of IP₆ synthesis. This is the first time that such a conformational change is reported for an inositol kinase. Moreover, these changes explain all the experimental observations, including fluorescence measurements, that pointed to a nucleo-
IP$_5$ 2-K Conformational Changes upon Substrate Binding

tide- and inositol-induced conformational changes in IP$_5$ 2-K. The different protein forms captured can be considered as a "biological mimic state," proving mutagenesis to be a successful tool in Protein Crystallography.

A general problem facing the protein kinase field is the design of selective inhibitors, because all protein kinases bind ATP in a very similar manner. In the case of inositol phosphate kinases, we can use the inositol-binding site for design of specific inhibitors; however, a number of crucial inositol kinases could be affected by similar molecules. To overcome this selectivity problem and avoid off-target effects, an understanding of the structural basis of substrate specificity and preference is essential. Furthermore, a common approach is to exploit protein conformational changes to design specific inhibitors. In this context, docking studies, which start from an ensemble of different states, have been proven to be successful. The main goals of this work were to provide new substrate recognition features, as elements involved in enantiotopic phosphate discrimination and structures of different protein conformations states. These are valuable tools in the search for specific and selective protein inhibitors with potential applications as biologically tools for in vivo studies. The implications of IP$_5$ 2-K in crucial cellular and developmental events have been already proven; however, elaboration of protein inhibitors could help in the determination of further roles for IP$_5$ 2-K in mammals.

Acknowledgments—We thank María Álvarez-Cao, Juana María González-Rubio, and Kendall Baker for technical assistance and the European Synchrotron Radiation Facility (Grenoble, France) for providing beam time and assistance during data collection. We thank Stephen Mills (University of Bath, UK) for the gift of Ins(1,4,5,6)P$_4$ and Ins(3,4,5,6)P$_4$ used in some of the enzyme assays described.

REFERENCES
1. Streb, H., Irvine, R. F., Berridge, M. J., and Schulz, I. (1983) Release of Ca$^{2+}$ from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5-trisphosphate. Nature 306, 67–69
2. Irvine, R. F., and Schell, M. J. (2001) Back in the water. The return of the rice. Nature 414, 687–689
3. Eves, E. B., Nichols, J., Wente, S. R., and York, J. D. (2002) Biochemical and functional characterization of inositol 1,3,4,5,6-pentakisphosphate 2-kinase. J. Biol. Chem. 275, 36575–36583
4. Philibpy, B. Q., Ullah, A. H., and Ehrlich, K. C. (1994) Purification and some properties of inositol 1,3,4,5,6-pentakisphosphate 2-kinase from immature soybean seeds. J. Biol. Chem. 269, 28393–28399
5. Stephens, L. R., Hawkins, P. T., Stanley, A. F., Moore, T., Poyner, D. R., Morris, P. J., Hanley, M. R., Kay, R. R., and Irvine, R. F. (1991) Myo-inositol pentakisphosphates. Structure, biological occurrence, and phosphorylation to myo-inositol hexakisphosphate. Biochim. Biophys. Acta 1031, 485–499
6. Sun, Y., Thompson, M., Lin, G., Butler, H., Gao, Z., Thornburgh, S., Yau, K., Smith, D. A., and Shukla, V. K. (2007) Inositol 1,3,4,5,6-pentakisphosphate 2-kinase from maize. Molecular and biochemical characterization. Plant Physiol. 144, 1278–1291
7. Sweetman, D., Johnson, S., Caddick, S. E., Hanke, D. E., and Brearley, C. A. (2006) Characterization of an Arabidopsis inositol 1,3,4,5,6-pentakisphosphate 2-kinase (AtIPPK1). Biochem. J. 394, 95–103
8. Verbsky, J. W., Wilson, M. P., Kisseleva, M. V., Majerus, P. W., and Wente, S. R. (2002) The synthesis of inositol hexakisphosphate. Characterization of human inositol 1,3,4,5,6-pentakisphosphate 2-kinase. J. Biol. Chem. 277, 31857–31862
9. Verbsky, J., Lavine, K., and Majerus, P. W. (2005) Disruption of the mouse inositol 1,3,4,5,6-pentakisphosphate 2-kinase gene, associated lethality, and tissue distribution of 2-kinase expression. Proc. Natl. Acad. Sci. U.S.A. 102, 8448–8453
10. Alcázar-Román, A. R., Borger, T. A., and Wente, S. R. (2010) Control of mRNA export and translation termination by inositol hexakisphosphate requires specific interaction with Glei. J. Biol. Chem. 285, 16683–16692
11. Shen, X., Xiao, H., Ranallo, R., Wu, W. H., and Wu, C. (2003) Modulation of ATP-dependent chromatin-remodeling complexes by inositol polyphosphates. Science 299, 112–114
12. Sarmah, B., Latimer, A. J., Appel, B., and Wente, S. R. (2005) Inositol polyphosphates regulate zebrafish left-right asymmetry. Dev. Cell 9, 133–145
13. Agarwal, R., Mumtaz, H., and Ali, N. (2009) Role of inositol polyphosphates in programmed cell death. Mol. Cell. Biochem. 328, 155–165
14. Bennett, M., Onnebo, S. M., Azevedo, C., and Saiardi, A. (2006) Inositol pyrophosphates. Metabolism and signaling. Cell. Mol. Life Sci. 63, 552–564
15. Murphy, A. M., Otto, B., Brearley, C. A., Carr, J. P., and Hanke, D. E. (2008) A role for inositol hexakisphosphate in the maintenance of basal resistance to pathogen plant pathogens. Plant J. 56, 638–652
16. Raboy, V. (2001) Seeds for a better future. “Low phytate” grains help to overcome malnutrition and reduce pollution. Trends Plant Sci. 6, 458–462
17. Raboy, V. (2003) myo-Inositol-1,2,3,4,5,6-hexakisphosphate. Physicochemistry 64, 1033–1043
18. Stevenson-Paulik, J., Bastidas, R. J., Chiuo, S. T., Frye, R. A., and York, J. D. (2005) Generation of phytate-free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. Proc. Natl. Acad. Sci. U.S.A. 102, 12612–12617
19. Raboy, V. (2007) The ABCs of low-phytate crops. Nat. Biotechnol. 25, 874–875
20. Shi, J., Wang, H., Wu, Y., Hazebroek, J., Meeley, R. B., and Ertl, D. S. (2003) The maize low phytic acid mutant ipa2 is caused by mutation in an inositol phosphate kinase gene. Plant Physiol. 131, 507–515
21. González, B., Schell, M. J., Letcher, A. J., Veprintsev, D. B., Irvine, R. F., and Williams, R. L. (2004) Structure of a human inositol 1,4,5-trisphosphate 3-kinase. Substrate binding reveals why it is not a phosphoinositide 3-kinase. Mol. Cell. 15, 689–701
22. Miller, G. J., and Hurley, J. H. (2004) Crystal structure of the catalytic core of inositol 1,4,5-trisphosphate 3-kinase. Mol. Cell 15, 703–711
23. Holmes, W., and Jogl, G. (2006) Crystal structure of inositol triphosphatase multikinase 2 and implications for substrate specificity. J. Biol. Chem. 281, 38109–38116
24. González, B., Baños-San, J. I., Villate, M., Brearley, C. A., and Sanz-Aparicio, J. (2010) Inositol 1,3,4,5,6-pentakisphosphate 2-kinase is a distant IPK member with a singular inositol-binding site for axial 2-OH recognition. Proc. Natl. Acad. Sci. U.S.A. 107, 9606–9613
25. Miller, G. J., Wilson, M. P., Majerus, P. W., and Hurley, J. H. (2005) Specificity determinants in inositol polyphosphate synthesis. Crystal structure of inositol 1,3,4,5,6-pentakisphosphate 5-kinase. Mol. Cell 18, 201–212
26. Wang, H., Falck, J. R., Hall, T. M., and Shears, S. B. (2012) Structural basis for an inositol pyrophosphate kinase surmounting phosphate crowding. Nat. Chem. Biol. 8, 111–116
27. Baños-San, J. I., Villate, M., Sanz-Aparicio, J., Brearley, C. A., and González, B. (2010) Crystalization and preliminary x-ray diffraction analysis of inositol 1,3,4,5,6-pentakisphosphate kinase from Arabidopsis thaliana. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 66, 102–106
28. Baños-San, J. I., Sanz-Aparicio, J., Brearley, C. A., and González, B. (2012) Expression, purification, crystallization, and preliminary x-ray diffraction analysis of the apo-form of IP$_5$-2K from Arabidopsis thaliana. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 68, 701–704
29. Battye, T. G., Kontogiannis, L., Johnson, O., Powell, H. R., and Leslie, A. G. (2011) iMOSFLM. A new graphical interface for diffraction-image processing with MOSFLM. Acta Crystallogr. D Biol. Crystallogr. 67, 271–281
30. Evans, P. (2006) Scaling and assessment of data quality. Acta Crystallogr. D Biol. Crystallogr. 62, 72–82
31. Collaborative Computational Project Number 4 (1994) The CCP4 suite. Programs for crystallography. Acta Crystallogr. D Biol. Crystallogr.
**IP$_2$ 2-K Conformational Changes upon Substrate Binding**

50, 760–763

32. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674

33. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D Biol. Crystallogr. 53, 240–255

34. Emsley, P., and Cowtan, K. (2004) Coot. Model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132

35. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) PROCHECK. A program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. 26, 283–291

36. DeLano, W. L. (2002) The PyMOL Molecular Graphics System. DeLano Scientific LLC, San Carlos, CA

37. Hayward, S., and Lee, R. A. (2002) Improvements in the analysis of domain motions in proteins from conformational change. DynDom Version 1.50. J. Mol. Graph. Model. 21, 181–183

38. Mayr, G. W., Windhorst, S., and Hillemeier, K. (1993) PROCHECK. A program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. 26, 283–291

39. Rowan, A. S., Nicely, N. I., Cochrane, N., Wlassoff, W. A., Claiborne, A., and Hamilton, C. J. (2009) Nucleoside triphosphate mimicry. A sugar triazolyl nucleoside as an ATP-competitive inhibitor of B. anthracis panthenate kinase. J. Biol. Chem. 284, 22458–22468

40. Gosein, V., Leung, T. F., Krajden, O., and Miller, G. J. (2012) Inositol 3-phosphate-induced stabilization of inositol 1,3,4,5,6-pentakisphosphate 2-kinase and its role in substrate specificity. Protein Sci. 21, 737–742

41. Bossemeyer, D. (1994) The glycine-rich sequence of protein kinases. A multifunctional element. Trends Biochem. Sci. 19, 201–205

42. Taylor, S. S., Radzio-Andzelm, E., Madhusudan, Cheng, X., Ten Eyck, L., and Narayana, N. (1999) Catalytic subunit of cyclic AMP-dependent protein kinase. Structure and dynamics of the active site cleft. Pharmacol. Ther. 82, 133–141

43. Cox, S., Radzio-Andzelm, E., and Taylor, S. S. (1994) Domain movements in protein kinases. Curr. Opin. Struct. Biol. 4, 893–901

44. Abdullah, M., Hughes, P. J., Craxton, A., Gigg, R., Desai, T., Marecek, J. F., Prestwich, G. D., and Shears, S. B. (1992) Purification and characterization of inositol 1,3,4-trisphosphate 5/6-kinase from rat liver using an inositol hexakisphosphate affinity column. J. Biol. Chem. 267, 22340–22345

45. Nanalskowksi, M. M., Bertsch, U., Fanick, W., Stockebrand, M. C., Schmale, H., and Mayr, G. W. (2003) Rat inositol 1,4,5-trisphosphate 3-kinase C is enzymatically specialized for basal cellular inositol trisphosphate phosphorylation and shuttles actively between nucleus and cytoplasm. J. Biol. Chem. 278, 19765–19776

46. Voglmaier, S. M., Bembeneck, M. E., Kaplin, A. I., Dormán, G., Olszewski, J. D., Prestwich, G. D., and Snyder, S. H. (1996) Purified inositol hexakisphosphate kinase is an ATP synthase. Diphosphoinositol pentakisphosphate as a high energy phosphate donor. Proc. Natl. Acad. Sci. U.S.A. 93, 4305–4310

47. Van Rooijen, L. A., Rossowska, M., and Bazan, N. G. (1985) Inhibition of phosphatidylinositol-4-phosphate kinase by its product phosphatidylinositol 4,5-bisphosphate. Biochem. Biophys. Res. Commun. 126, 150–155

48. Stevenson-Paulik, J., Odom, A. R., and York, J. D. (2002) Molecular and biochemical characterization of two plant inositol polyphosphate 6-/3-5-kinases. J. Biol. Chem. 277, 42711–42718

49. Xia, H. J., Brearley, C., Elge, S., Kaplan, B., Fromm, H., and Mueller-Roeber, B. (2003) Arabidopsis inositol polyphosphate 6-/3-kinase is a nuclear protein that complements a yeast mutant lacking a functional ArgR-Mcm1 transcription complex. Plant Cell 15, 449–463

50. Brearley, C. A., and Hanke, D. E. (1996) Inositol phosphates in the duckweed Spirodela polyrhiza. Biochem. J. 314, 215–225

51. Sweetman, D., Stavridou, I., Johnson, S., Green, P., Caddick, S. E., and Brearley, C. A. (2007) Arabidopsis inositol polyphosphate 6-/3-kinase is an outlier to a family of ATP-grasp fold proteins from Arabidopsis. FEBS Lett. 581, 4165–4171

52. Josifsen, L., Bohn, L., Sørensen, M. B., and Rasmussen, S. K. (2007) Characterization of a multifunctional inositol phosphate kinase from rice and barley belonging to the ATP-grasp superfAMILY. Gene 397, 114–125

53. Caddick, S. E., Harrison, C. J., Stavridou, I., Mitchell, J. L., Hemmings, A. M., and Brearley, C. A. (2008) Arabidopsis thaliana inositol 1,3,4-trisphosphate 5/6-kinase 4 (AtITPK4) is an outlier to a family of ATP-grasp fold proteins from Arabidopsis. FEBS Lett. 582, 1731–1737

54. Hyeon, C., Jennings, P. A., Adams, J. A., and Onuchic, J. N. (2009) Ligand-induced global transitions in the catalytic domain of protein kinase A. Proc. Natl. Acad. Sci. U.S.A. 106, 3023–3028