Crystal Structure of Inositol Phosphate Multikinase 2 and Implications for Substrate Specificity*

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Inositol polyphosphates perform essential functions as second messengers in eukaryotic cells, and their cellular levels are regulated by inositol phosphate kinases. Most of these enzymes belong to the inositol phosphate kinase superfamily, which consists of three subgroups, inositol 3-kinases, inositol phosphate multikinases, and inositol hexakisphosphate kinases. Family members share several strictly conserved signature motifs and are expected to have the same backbone fold, despite very limited overall amino acid sequence identity. Sequence differences are expected to play important roles in defining the different substrate selectivity of these enzymes. To investigate the structural basis for substrate specificity, we have determined the crystal structure of the yeast inositol phosphate multikinase Ipk2 in the apo-form and in a complex with ADP and Mn$^{2+}$ at up to 2.0 Å resolution. The overall structure of Ipk2 is related to inositol trisphosphate 3-kinase. The ATP binding site is similar in both enzymes; however, the inositol binding domain is significantly smaller in Ipk2. Replacement of critical side chains in the inositol-binding site suggests how modification of substrate recognition motifs determines enzymatic substrate preference and catalysis.

Soluble inositol polyphosphates (InsPs)² and inositol pyrophosphates are involved in a multitude of essential cellular functions, such as apoptosis (1–3), mRNA export (4), DNA repair (5), embryogenesis (6), regulation of endocytosis (7), stress response (8–10), regulation of telomere length (11, 12), and chemotaxis (13). Among the 30 different InsPs that have been described in eukaryotic cells (reviewed in Ref. 14), inositol 1,4,5-trisphosphate (InsP₃) is best known as second messenger in insulin signaling-mediated Ca²⁺ release from the endoplasmic reticulum (15). InsP₃ also serves as the precursor for synthesis of higher phosphorylated InsPs by the sequential action of inositol phosphate kinase (IP) kinases.

Based on sequence homology, IP kinases are classified into three families, inositol 5/6-kinases, inositol 2-kinases, and the IPK superfamily, which includes three subgroups: inositol 3-kinases (IP3Ks), inositol multikinases (IPMKs), and inositol hexakisphosphate kinases. Members of this superfamily share several strictly conserved signature motifs with each other and are predicted to assume the same overall fold (16), although their sequence conservation is very low (Fig. 1).

Despite their overall similarity, the three groups display distinct enzymatic activities and substrate specificities. IP3Ks and inositol hexakisphosphate kinases catalyze a single reaction, 3-kinase and diphosphoinositol synthase activity, respectively. IPMKs, on the other hand, display catalytic activities toward more substrates, generally acting as 6/3-5-kinases. Human and plant IPMK act primarily as a 5-kinase and are more active as a 6-kinase than a 3-kinase (17–21). The Drosophila IPMK is a 6/3-kinase with additional 5-kinase activity for inositol 1,3,4,6-tetrakisphosphate (22). In contrast, the yeast IPMK, Ipk2, acts as a 6- and 3-kinase, producing inositol 1,4,5,6-tetrakisphosphate and inositol 1,3,4,5,6-pentakisphosphate, but no 5-kinase activity has been reported for this enzyme (16, 23). Furthermore, Ipk2 has been found to function as a diphosphoinositol polyphosphate synthase, producing PP-inositol 1,3,4,5-tetakisphosphate from inositol 1,3,4,5,6-pentakisphosphate (24). Although all IP kinases act on soluble inositol phosphates, the human IPMK has recently been reported to function as a nuclear inositol lipid kinase that initiates a nuclear inositol lipid signal transduction pathway (25).

Yeast inositol multikinase Ipk2 was cloned and characterized by two groups (16, 23, 26). Knock-out experiments showed that Ipk2Δ cells are viable when grown at 30 °C but exhibit a drastic reduction of cellular inositol 1,3,4,5-tetakisphosphate, inositol 1,3,4,5,6-pentakisphosphate, and inositol hexakisphosphate levels, together with a 170-fold increase of InsP₃ concentration, demonstrating that Ipk2 function is essential for production of higher phosphorylated InsPs in yeast (26). Yeast Ipk2 seems to perform a unique second function among the IPMKs in that it had previously been characterized under the name ArgRIII (or ArgK²) as part of a transcriptional regulatory complex in arginine metabolism (27). The main function of Ipk2 in this transcriptional complex appears to be the stabilization of the transcription factors ArgRI and Mcm1 from degradation (28). Protein-protein interaction is mediated by a unique inserted region containing a stretch of 15 aspartates in 16 residues (polar-D loop) that is required for the interaction with ArgRI and Mcm1 (28). The polar-D loop insertion is not conserved in the mammalian IPMKs, and this second function might be restricted to the Saccharomyces cerevisiae enzyme.
The available structural information for inositol kinases is currently limited to the structure of InsP₃ 5/6-kinase from Entamoeba histolytica (29), which is not a member of the IPK protein family, and the structure of the catalytic core domain of human inositol 1,4,5-trisphosphate 3-kinase from the IP3K subgroup (30, 31). No structural information is currently available for inositol kinases.
able for the IPMK subgroup. To further investigate the structural determinants for catalytic activity and substrate specificity of inositol phosphate multikinases, we have determined the crystal structure of Ipk2 in the native form and in a complex with ADP and Mn²⁺. This is the first structure of an IPMK family member. The overall structure is related to IP3K, despite the low sequence identity of 17.7% between both enzymes. Structural comparison with the highly specific inositol 3-kinase highlights critical binding interactions in the active site, which are consistent with substrate binding in several possible orientations as required for the Ipk2 multikinase catalytic activity.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Crystallization**—Full-length Ipk2 from *S. cerevisiae* was cloned from genomic DNA into the pET26b vector (Novagen). The expression construct contained a C-terminal hexahistidine tag. Protein was overexpressed in *Escherichia coli* strain BL21Star (Invitrogen) at 20 °C. Bacterial cells were lysed by ultrasonification on ice. The soluble protein was bound to nickel-agarose affinity resin (Qiagen), washed with a buffer containing 20 mM Tris (pH 8.5), 250 mM NaCl, and 10 mM imidazole. His-tagged protein was then eluted with a buffer containing 20 mM Tris (pH 8.5), 250 mM NaCl, and 150 mM imidazole. The protein was further purified with anion exchange chromatography at pH 8.5, using a linear gradient of 0 mM to 1 M NaCl concentration, and size exclusion chromatography at pH 8.5 and 200 mM NaCl. The purified protein was concentrated to 25 mg/ml in a buffer containing 10 mM Tris (pH 8.5), 20 mM NaCl, and 7% glycerol. The sample was flash-frozen in liquid nitrogen and stored at −80 °C. The C-terminal His tag was not removed for crystallization.

For the production of selenomethionyl proteins, the expression construct was transformed into B834(DE3) cells (Novagen). The bacterial growth was carried out in defined LeMaster medium (32), and the protein was purified using the same protocol as for the wild-type protein.

Crystals of Ipk2 were obtained at 20 °C with the sitting drop vapor diffusion method. The reservoir solution contained 100 mM HEPES, pH 7.7, 200 mM CaCl₂, and 28% polyethylene glycol 400 (w/v). The crystals were cryoprotected by rapid soaking in a solution containing mother liquor with the addition of 20% (v/v) ethylene glycol and flash-frozen in liquid nitrogen. Crystals were grown to maximum dimensions of 0.45 × 0.12 × 0.12 mm.

**Data Collection**—X-ray diffraction data were collected on an ADSC CCD detector at the X4A beamline of the National Synchrotron Light Source in Brookhaven. For the initial structure determination, a selenomethionyl single wavelength anomalous diffraction data set to 3.1 Å resolution was collected at a wavelength of 0.9790 Å at 100 K. The diffraction images were processed and scaled with the HKL package (33). The crystals belong to the space group P6₁, with cell dimensions of \( a = b = 185.6 \) Å and \( c = 50.1 \) Å. There are two molecules in the asymmetric unit, giving a \( V_m \) of 3.1 Å³/dalton. To obtain higher resolution data, a native data set was collected on a larger crystal to 2.0 Å resolution. Cell dimensions of the native crystal were \( a = b = 186.5 \) Å and \( c = 50.1 \) Å. Diffraction data for the ATP-bound form were collected to 2.4 Å resolution from a crystal that had been soaked in a solution of 40 mM ATP and 20 mM MnCl₂ in mother liquor for 15 min. The data processing statistics are summarized in Table 1.

**Structure Determination and Refinement**—The locations of selenium atoms were determined with the program Solve (34), based on the anomalous differences in the single wavelength anomalous diffraction data set. Reflection phases to 3.1 Å were calculated with Solve, and phases were extended to 2.0 Å resolution of the native data set with DM (35) from the CCP4 program suite (36). After phase extension and noncrystallographic symmetry averaging, the resulting experimental electron density map was of excellent quality. The initial atomic model was built with the program Arp/wArp (37). Six of the eight residues of the His tag are visible in molecule A, and two residues are visible in molecule B. The initial protein model was rebuilt manually with the program O (38). The initial structure refinement was carried out with the program CNS (39). After simulated annealing refinement, the last stages of crystallographic refinement were performed with the program Refmac without noncrystallographic symmetry restraints (40). The structure refinement statistics are summarized in Table 1. All molecular figures were produced with the program PyMOL (41). Molecular surfaces were generated using Gromacs (42) and Mead (43).

**RESULTS**

**Overall Structure of Ipk2**—We have determined the structure of the full-length inositol multikinase Ipk2 from yeast in space group P6₁, at 2.0 Å resolution by single anomalous dispersion methods of selenomethionyl-substituted protein (Table 1). The crystallographic \( R/R_{free} \) factors are 20.8/25.3 and 19.5/24.6% for the native and the ADP-complex structure, respectively. The majority of residues (93.4 and 91.9%) are in the most favored region of the Ramachandran plot. There are two molecules in the asymmetric unit. The contacts among the noncrystallographically related molecules are generally weak and hydrophilic in nature. The protein migrates as monomers on a gel filtration column (data not shown). Several loop regions in the Ipk2 structure are disordered, and we have not included the corresponding residues in our current model. These loop regions comprise residues 1–26, 46–57 (between \( a_1 \) and \( a_2 \)), and 76–110 (between \( b_2 \) and \( b_3 \)) in the N-terminal domain and 287–316 in the C-terminal domain. Not surprisingly, the unique aspartate-rich poly-D loop region between residues 287 and 316 is disordered in our structure and was omitted from the final model. This loop is located on the opposite side of the active side between helix \( a_9 \) and \( b_10 \). The C-terminal His tag (-LEHHHHHHH) is partially ordered in our structure. Three histidine residues were included in molecule A, but only the Leu and Glu of the His tag were ordered in molecule B.

The structure consists of three domains, an N-terminal \( \alpha + \beta \) subdomain, a larger C-terminal \( \alpha + \beta \) subdomain, and an inositol binding domain (Fig. 2). The N-domain extends from residue 26 to 118 and consists of three antiparallel \( \beta \)-strands (\( \beta_1-\beta_3 \) and two \( \alpha \)-helices (\( \alpha_1 \) and \( \alpha_2 \)). The \( \beta \)-sheet has a strand order of 1-3-2, and helices \( \alpha_1 \) and \( \alpha_2 \) connect strands \( \beta_1 \) and \( \beta_2 \) on both ends of the \( \beta \) sheet.

The C-domain is formed by residues 127–134 and 165–355. It is an \( \alpha + \beta \) fold with a central six-stranded \( \beta \) sheet. The
strand order of this sheet is 6-5-1-2-4-3. One side of the β sheet is stabilized by three helices. Helices α7 and α8, together with the additional small helix α6, connect strands β7 and β8. The third helix is α10 at the C-terminal end of the protein. An additional small β-sheet is formed by strands β8 and β11, where β8 is inserted between the stabilizing helix α8 and strand β9 of the central β sheet, and β11 connects the last strand β10 with the last helix α10. The N- and C-domains are connected by a loop from residues 119 to 126.

The inositol-binding domain is inserted between strand 4 and strand 5 of the C-terminal domain. In the case of Ipk2, the inositol-binding domain consists of only two helices, and we have designated this part of the structure as a separate domain based on the homology with the larger inositol binding domain of IP3K.

A data base search with the program Dali (44) confirmed that the structure of IP3K (30, 31) is the closest related protein structure. IP3K has been classified as a member of the Saicar-synthase like superfamily in the SCOP data base (45), which also contains the phosphatidylinositol kinase PIPIIKβ (46) and shares structural and functional similarity with ATP-grasp fold proteins and protein kinases.

The ATP Binding Site—We have obtained the ADP-bound form of Ipk2 by soaking native crystals with 40 mM ATP and 20 mM MnCl2. In a 2.4 Å resolution data set, we observed electron density for a molecule of ADP and a Mn2+ ion in the active site of one of the two Ipk2 molecules in the asymmetric unit (Fig. 3A). The active site region of the unoccupied molecule is partially blocked by crystal lattice contacts and it might therefore not be accessible to the cofactor. Despite the short soaking
time, we observed only very weak density for the γ-phosphate group, indicating that either the majority of bound ATP was hydrolyzed or the position of the γ-phosphate is disordered in the absence of the substrate. Only minor structural differences are observed between the apo-form and the ADP-bound form. The overall root mean square deviation between both structures is 0.3 Å for 255 Ca atoms.

The ATP binding site is located between the N- and C-domains of Ipk2 (Fig. 3B). The adenosine moiety of ADP is positioned in a hydrophobic pocket that is lined by residues Ile29 and Leu117 from the N-domain and residues Leu121, Leu260, and Ile324 from the C-terminal domain. Leu260 is part of the SLL signature motif, and Ile124 is part of the IDF signature motif, both strictly conserved in the IPK family. The N1 and N6 atoms of adenine are recognized specifically by the kinase through hydrogen bonds to the amide nitrogen and carbonyl oxygen of Leu120 and Glu118, respectively, which anchors the adenosine in the hydrophobic pocket. The ADP-ribose group is hydrogen-bonded to Asp131, which is part of the PXXXDKXXG signature motif. The ADP phosphate groups form hydrogen bonds with Lys31 from the N-terminal domain and Asp325 from the C-terminal domain. Asp325 and Lys131 coordinate the binding of Mn²⁺ to the ADP phosphates. Residues in the ATP binding site, with the exception of Asp325, assume a very similar side chain conformation in the apoenzyme form and the ADP-bound form.

**DISCUSSION**

**Structural Homology with Inositol 1,4,5-Trisphosphate 3-Kinase**—As predicted from previous sequence alignments, Ipk2 and IP3K (Protein Data Bank code 1W2C) show a high structural similarity despite their overall lower sequence identity of 17.7% (Fig. 4A). A schematic alignment of the domain organization and the location of signature motifs for the three IPK subgroups is shown in Fig. 1A, and a structure-based sequence alignment is given in Fig. 1B. Ipk2 is 355 residues long, whereas the 461-residue-long IP3K contains an additional N-terminal Ca²⁺/calmodulin activation domain that is
not present in Ipk2. 191 Ca atoms can be aligned between both structures to a root mean square deviation of 1.3 Å.

Functionally important secondary structure elements are well conserved between both enzymes, but most of the loop regions assume different conformations, and there are two regions with significant structural differences. First, the central β-sheet of the Ipk2 C-terminal domain contains an additional short strand (β6) and two helices, α5 and α9. These inserted structural elements are located remote from the active site and could have a functional role to mediate protein-protein interactions in the Ipk2 transcriptional complex.

Second, the inositol-binding domain of Ipk2 is significantly smaller than the IP-binding domain in IP3K. Only two of the five α-helices present in IP3K are conserved in Ipk2. However, these two helices form the core region of the inositol-binding site, and their orientation remains similar in both enzymes (Fig. 4A). Thus, the overall conformation of the inositol-binding site is comparable in both enzymes, but significant differences regarding nature and orientation of protein side chains are observed between both enzymes (discussed below). It should also be noted that the larger IP-binding domain in IP3K is not conserved in other IPK family members and could conceivably be required as an interdomain surface to coordinate the additional regulatory domain in the IP3Ks.

A comparison of the ATP binding site of both enzymes shows that the position of the cofactor is similar and that the functionally important residues are conserved (Fig. 4B). Surprisingly, Lys171 in Ipk2 remains in a conformation similar to the apoenzyme and does not form hydrogen bonds with the ribose hydroxyl groups. Instead, a solvent water molecule (W255) is observed in a position equivalent to the amino group of the IP3K Lys536 side chain. In Ipk2, Phe197 (not conserved in IP3K) disrupts the formation of a solvent water network similar to IP3K, and this might contribute to the different conformation of Lys171. However, the equivalent Lys536 in IP3K assumes a similar side chain conformation in the ADP-bound structure determined by Miller and Hurley (Protein Data Bank code 1TZD) (31), suggesting that this residue is not strictly required for coordinating ATP in Ipk2.

Although the coordination of the ADP α- and β-phosphate groups is similar in both enzymes, the position of the metal ion and the Asp325 side chain are slightly shifted by 1.5 Å. Interestingly, Lys133, which is crucial for positioning the substrate and the ATP γ-phosphate during catalysis, is rotated toward the ADP molecule. It coordinates to Asp325 and Ser258 and is in close proximity to the Mn2⁺ ion in the absence of inositol. A solvent water molecule is located in the position of the lysine amine in IP3K so that the overall geometry of cofactor-binding interactions remains similar in both enzymes.

In IP3K, an additional N-terminal β-strand and α-helix were observed that partially obstruct the ATP-binding site in the absence of the cofactor. This autoinhibitory interaction is probably part of the N-terminal Ca2⁺/calmodulin-regulated activation mechanism of this enzyme. Ipk2 is not regulated by Ca2⁺/calmodulin, but it contains an N-terminal segment of similar length. However, the first 26 residues are disordered in both Ipk2 structures, and we did not observe a similar order-disorder transition between the apoenzyme and the ADP-bound form.

**Inositol Binding Site**—Despite considerable efforts, we have not been able to produce inositol-bound crystals of Ipk2. The current crystallization conditions contain 200 mM CaCl2, which
is required for crystal formation, since a Ca\(^{2+}\) ion forms a bridging crystal contact between two crystallographically related molecules. The formation of a calcium complex with InsP\(_5\) in the soaking solution might either limit substrate solubility or interfere with substrate binding. Given the close structural homology with IP3K, we have modeled the position of InsP\(_5\) in Ipk2 from the least squares alignment between both enzymes to evaluate substrate binding.

The substrate-binding site of Ipk2 is located between the C-terminal domain and the inositol binding domain. The overall architecture is well conserved between both enzymes (Fig. 5A). In Ipk2, helix \(\alpha3\) of the inositol-binding domain, which delineates one side of the substrate binding site, is shifted toward the ATP position when compared with the substrate-bound structure of IP3K. However, a shift of the corresponding helix and an opening movement of the inositol-binding domain upon substrate binding was observed in IP3K, suggesting a similar conformational flexibility for Ipk2. The inositol-binding pocket is a highly positively charged shallow groove on the protein surface. At the bottom of this groove, Met\(^{151}\) provides a hydrophobic patch that stacks against the inositol ring. The hydrophobic side chain of Met\(^{151}\) will also interfere with the presence of a phosphate group in the 2-position and prevent binding of 2-phosphorylated inositol phosphates. The inositol 3-position in IP3K is hydrogen-bonded to Lys\(^{133}\) in the PXXXDXKXG signature motif. The 4-phosphate interacts with Lys\(^{311}\) and Lys\(^{312}\) in IP3K. Lys\(^{311}\) is replaced with His\(^{328}\) in Ipk2, which is oriented in the same direction but will contribute less favorably to phosphate group binding. Considering the fact that IPMKs need to bind substrates in several orientations, a strong interaction in this position might interfere with enzymatic 6-kinase activity. Lys\(^{147}\), which is equivalent to Lys\(^{312}\) in IP3K, is directed toward the inositol 5-position. A conformational change of helix \(\alpha3\) could position this side chain to interact with the 4- and 5-position of the inositol ring, similar to IP3K.

Consistent with the Ipk2 6/3-kinase specificity, the inositol 6-OH is recognized differently between both enzymes. One of the main determinants of substrate selectivity in IP3K is Met\(^{288}\) which interferes with an inositol 6-phosphate group and thus precludes binding of inositol 1,4,5,6-tetakisphosphate. The helix with Met\(^{288}\) is not conserved in Ipk2, but Arg\(^{150}\), which replaces Tyr\(^{315}\), occupies a similar position. Assuming a conformational shift of helix \(\alpha3\) in Ipk2, Arg\(^{150}\) will create a favorable binding environment at the 6-position and should also be in a position to coordinate a phosphate group in the inositol 1-position. The inositol 1-phosphate group, finally, is recognized by Arg\(^{319}\) in IP3K. Surprisingly, this important residue is replaced by Val\(^{154}\) in Ipk2, but the equivalent hydrogen bonding position is occupied by Arg\(^{204}\) from helix \(\alpha6\) of the C-terminal domain. Helix \(\alpha6\) is one of the three additional helices in the Ipk2 C-domain not present in IP3K, and the position of Arg\(^{204}\) in the inositol-binding site underlines the importance of this helix for enzymatic function. The structural comparison with IP3K clearly shows that the Ipk2 active site provides favorable binding partners for inositol phosphate groups of inositol 1,4,5-trisphosphate that is bound in a site for either 6- or 3-phosphorylation as well as for the two different inositol 1,3,4,5-tetakisphosphates for the synthesis of inositol 1,3,4,5,6-pentakisphosphate.

Although our structural model illustrates the general nature of substrate binding interactions in Ipk2, it should be noted that inositol binding is likely to induce a shift of helices \(\alpha3\) and \(\alpha6\) concomitant with slight reorientations of substrate binding side chains to accommodate the inositol phosphate groups.

**Implications for Substrate Specificity**—Phosphorylation of the inositol 6-hydroxyl requires substrate binding in one of two possible orientations to position the 6-hydroxyl group toward the ATP. In the first orientation, the inositol ring is rotated by 180° around a vertical axis, exchanging the 5-phosphate group with the 4-phosphate group and the 1-phosphate with the 2-phosphate (Fig. 5B). In the other orientation, the inositol ring is additionally rotated around a horizontal axis, bringing the 5-phosphate group onto the 2-position and the 4-phosphate onto the 1-position (Fig. 5C). In both orientations, there is a phosphate group present in the position previously occupied by the inositol 2-hydroxyl. In comparison with IP3K, Ipk2 contains a two-residue insertion at the beginning of helix \(\alpha6\) that projects Lys\(^{300}\) toward a phosphate group in the place of the 2-position, creating a favorable binding environment. In a second replacement in this region, Glu\(^{333}\), which is oriented toward the potential phosphate group in IP3K, is replaced with the shorter and uncharged Cys\(^{168}\) in Ipk2. Both changes create a favorable binding environment for phosphate groups in the 2-position, consistent with the requirement to bind inositol substrate in various positions.

In summary, our results provide a first view of the highly versatile active site of inositol phosphate multikinases. Our structural information has significantly advanced our understanding of the molecular details governing their multiple catalytic activities. The Ipk2 active site provides favorable binding interactions at all five available binding positions for substrate phosphate groups, enabling the enzyme to interact with different phosphorylated inositol polyphosphates in different orientations as proposed by Shears (47). Modification of this binding pattern in the related IP3Ks rationalizes the increased substrate specificity of these enzymes.

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