The Structural and Functional Characterization of Mammalian ADP-dependent Glucokinase*

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Jan P. Richter‡, Alexander K. Goroncy‡, Ron S. Ronimus§, and Andrew J. Sutherland-Smith‡

From the ‡Institute of Fundamental Sciences, Massey University, Palmerston North 4410, New Zealand and §AgResearch Limited, Palmerston North 4442, New Zealand

The enzyme-catalyzed phosphorylation of glucose to glucose-6-phosphate is a reaction central to the metabolism of all life. ADP-dependent glucokinase (ADPGK) catalyzes glucose-6-phosphate production, utilizing ADP as a phosphoryl donor in contrast to the more well characterized ATP-requiring hexokinases. ADPGK is found in Archaea and metazoa; in Archaea, ADPGK participates in a glycolytic role, but a function in most eukaryotic cell types remains unknown. We have determined structures of the eukaryotic ADPGK revealing a ribokinase-like tertiary fold similar to archaeal orthologues but with significant differences in some secondary structural elements. Both the unliganded and the AMP-bound ADPGK structures are in the “open” conformation. The structures reveal the presence of a disulfide bond between conserved cysteines that is positioned at the nucleotide-binding loop of eukaryotic ADPGK. The AMP-bound ADPGK structure defines the nucleotide-binding site with one of the disulfide bond cysteines coordinating the AMP with its main chain atoms, a nucleotide-binding motif that appears unique to eukaryotic ADPGKs. Key amino acids at the active site are structurally conserved between mammalian and archaeal ADPGK, and site-directed mutagenesis has confirmed residues essential for enzymatic activity. ADPGK is substrate inhibited by high glucose concentration and shows high specificity for glucose, with no activity for other sugars, as determined by NMR spectroscopy, including 2-deoxyglucose, the glucose analogue used for tumor detection by positron emission tomography.

Glucose metabolism is central to the biochemistry of all living systems with the enzymatic phosphorylation of glucose playing a key role in cellular energy metabolism by ensuring that this energy-rich substrate is available to the cell. A relatively recently discovered ADP-dependent glucokinase (ADPGK)³ (EC 2.7.1.147) catalyzes the phosphorylation of D-glucose to glucose-6-phosphate using MgADP as phosphoryl donor in contrast to the more typical ATP-utilizing hexokinases and glucokinases. ADPGK was first identified in Archaea, being involved in a modified Embden-Meyerhof glycolytic pathway (1), in which Archaea can also use an ADP-dependent phosphofructokinase (ADPPFK; EC 2.7.1.146). Bioinformatic analysis led to the identification of ADPGK in metazoa and the subsequent cloning and initial characterization of mammalian ADPGKs (2, 3). Mammalian ADPGKs show modest sequence similarity to archaeal orthologues (~20% amino acid identity). ADPGK is highly expressed in a wide variety of both normal and tumor mammalian tissues (3) and has been found to be localized to the endoplasmic reticulum in T cells (4) consistent with the sequence-based annotation of an N-terminal signal peptide. Furthermore, ADPGK has been identified as a cholesterol binding protein in a proteomics screen (5). Despite the role of archaeal ADPGK in glycolysis, overexpression of ADPGK in H460 and HC116 human tumor cells showed no cell proliferative or glycolytic effects (3). ADPGK knock-out in these cell lines showed no detectable effect on glycolysis or extracellular acidification as assay end points, although O₂ consumption was generally lower for the ADPGK knock-out cells (6). Knock-out of ADPGK had no effect on cell survival under normal growth conditions, but ADPGK is protective for H460 cells under anoxia and reduced glycolysis (hexokinase 2 silencing) stress conditions, but not for HCT116 cells (6). ADPGK activity is associated with T cell receptor signaling, resulting in mitochondrial reactive oxygen species production as activated T cells switch from mitochondrial respiration to a dependence on glycolysis (4). Apart from this role in T cells, a function for ADPGK remains to be defined for most eukaryotic cell types.

The crystal structures of three archaeal ADPGKs have been determined in varying liganded states revealing an overall similarity in topology to the ribokinase superfamily and no homology to hexokinases. Archaeal ADPGK crystal structures have been determined for apo-ADPGK from Pyrococcus horikoshii OT3 (PDB code 1L2l) (7) and glucose- and AMP-bound Pyrococcus furiosus ADPGK (PDB code 1UA4) (8). Thermococcus litoralis ADPGK structures have been determined in the ADP-bound form (PDB code 1GCS) (9) and apo (PDB code 4BBR) and AMP/glucose ternary complexes (PDB code 4BB8) (10). The ADPGK structure contains two α/β domains; the large domain is a Rossmann-type fold of an eight-stranded β-sheet enclosed by eight α-helices, with five helices on one side and three on the other. The substrates glucose and ADP bind in a shallow groove in the large domain that is covered over by the small domain acting as a “lid.” The different archaeal ADPGK
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Materials and Methods

Cloning of Recombinant Mammalian ADPGK—Sequence analysis predicted a possible N-terminal signal sequence for mammalian ADPGK (e.g. SignalP4.1 (12), mouse ADPGK (mADPGK) 1–18, and human ADPGK (hADPGK) 1–22, so a number of N-terminally truncated ADPGK cDNA sequences were designed, as well as full-length sequences. The cDNA for mADPGKΔ51 amino acids 52–495 and hADPGK (Δ50, Δ151, or full-length) codon-optimized for expression in Escherichia coli were PCR-subcloned into vector pBAD-TOPO, and the resulting vectors pBAD-mADPGKΔ51 and pBAD-hADPGK were sequence-verified. Mutant hADPGK constructs (D84A, R228A, H264A, H382A, H382V/H387V, H387A, D481A, D481E, and D481E) were prepared using whole plasmid PCR with primers containing the required nucleotide substitution with pBAD-hADPGK as template. The hADPGK numbering is +1 relative to mADPGK after amino acid 313. The presence of the mutated nucleotide substitution(s) was confirmed by DNA sequencing.

Purification of Mammalian ADPGK—For ADPGK expression E. coli LMG194 were transformed with the appropriate pBAD construct encoding hADPGK variants or mADPGKΔ51 and grown in 2× YT medium containing 100 µg/ml ampicillin. Cultures were grown at 37 °C to an A600nm of 0.8 and induced with 0.1% (w/v) L-arabinose. The cells were harvested after 12 h by centrifugation for 20 min washed in cold PBS buffer and frozen at −80 °C. The cell pellets were resuspended in lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 4 mM DTT, Roche complete protease inhibitor tablet EDTA-free) and lysed by two passes through a French press. The soluble fraction was separated from cell debris by centrifugation at 46,000 × g for 90 min at 4 °C. Imidazole was added to the supernatant to a final concentration of 20 mM. The solution was loaded onto a 5-ml Ni2+-NTA HiTrapFF column. ADPGK was eluted with a linear gradient from 20 to 500 mM imidazole in phosphate buffer (25 mM Na2HPO4, pH 8.0, 500 mM NaCl, 500 µM DTT, or 2 mM β-mercaptoethanol). The elution fractions containing ADPGK were pooled, dialyzed against low ionic strength binding buffer (40 mM Tris, pH 8.0, 20 mM NaCl), loaded onto a Uno Q6 column (Bio-Rad) pre-equilibrated with binding buffer, and eluted with a linear gradient to 1 m NaCl in Tris buffer, pH 8.0. The elution fractions containing ADPGK were pooled and concentrated, and glycerol was added to 20% (w/v) before storage at −20 °C (hADPGK) or directly frozen (mADPGK).

Analytical Gel Filtration Chromatography—Analytical gel filtration was used to estimate the oligomerization state of hADPGK on a Superdex 200 10/300 GL column (GE Healthcare) in 40 mM Tris buffer (pH 8.0), 200 mM NaCl. For column calibration blue dextran, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, and cytochrome c were used as molecular weight standards in addition to previously characterized recombinant mADPGK. The purified human ADPGK for kinetic studies was shown to be in monomeric state in solution by analytical gel filtration, comparable to the mADPGK (2).

Mammalian ADPGK Crystal Structure Determination—Crystals were obtained from a mADPGKΔ51 solution of initial concentration 9.0 mg/ml (20 mM HEPES, 25 mM NaCl, 1 mM TCEP) after hanging drop vapor diffusion for 2 weeks with precipitant 0.2 M NH4Cl, 20% (w/v) PEG 3350 (JCSG screen condition A9; Molecular Dimensions) at 21 °C. These crystallization conditions were refined with an additive screen. X-ray diffraction data were collected on a crystal grown in the above conditions supplemented with Silver Bullets Bio condition A1 (Hampton Research). For AMP crystal soaking experiments, larger mADPGKΔ51 single crystals were obtained by seeding. Crystallization drops were set up with 0.2 M NH4Cl and 20% (w/v) PEG 3350 mixed with mADPGKΔ51 solution of initial concentration 9.0 mg/ml and incubated at room temperature for 2 weeks. Seeding was then performed placing small needle shaped crystals into an equilibrated drop of the same experiment where no nucleation event had occurred, and the crystals were incubated for another 2 weeks. AMP was introduced to select crystals by addition to a final concentration of 5 mM, and the experiment was incubated for another 4 days at room temperature, during which minimal cracking of the crystals was observed.

X-ray diffraction data of 0.5° oscillation images over a total scan range of 120° were collected on the MX2 beam line at the Australian synchrotron at 100 K from single crystals of apo-mADPGKΔ51 frozen in a loop by rapid immersion in liquid N2 using perfluoropolyether oil as cryoprotectant. For the AMP-bound mADPGKΔ51, 0.5° oscillation images were collected on a Rigaku Micromax HF007 equipped with R-Axis IV detector at 120 K frozen in a loop with paratone N as cryoprotectant. Crystals were of orthorhombic symmetry with a solvent content of ~43% with one molecule in the asymmetric unit. Diffraction intensities were processed with XDS (13) and Pointless/Aim-

Cultures were grown at 37 °C to an protease inhibitor tablet EDTA-free) and lysed by two passes mADPGK in 2-deoxyglucose, the glucose analogue in widespread clinical use for 90 min at 4 °C. Imidazole was added to the supernatant to a final concentration of 20 mM. The solution was loaded onto a 5-ml Ni2+-NTA HiTrapFF column. ADPGK was eluted with a linear gradient from 20 to 500 mM imidazole in phosphate buffer (25 mM Na2HPO4, pH 8.0, 500 mM NaCl, 500 µM DTT, or 2 mM β-mercaptoethanol). The elution fractions containing ADPGK were pooled, dialyzed against low ionic strength binding buffer (40 mM Tris, pH 8.0, 20 mM NaCl), loaded onto a Uno Q6 column (Bio-Rad) pre-equilibrated with binding buffer, and eluted with a linear gradient to 1 m NaCl in Tris buffer, pH 8.0. The elution fractions containing ADPGK were pooled and concentrated, and glycerol was added to 20% (w/v) before storage at −20 °C (hADPGK) or directly frozen (mADPGK).
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TABLE 1

X-ray diffraction data and refined ADPGK model statistics

|                     | Apo-ADPGK | AMP-ADPGK |
|---------------------|-----------|-----------|
| PDB code            | 5CCF      | 5CK7      |
| Beam source         | Australian Synchrotron MX2 | Rigaku Micromax 007 |
| Detector            | Quantum 205 | R-Axis IV |
| Distance to detector (mm) | 315      | 200       |
| Wavelength (Å)      | 0.9184    | 1.5418    |
| Resolution range (Å) | 47.42–2.09 (2.17–2.09) | 30.00–2.99 (3.20–2.99) |
| Space group         | P2₁,2₁,2₁ | P2₁,2₁,2₁ |
| Unit cell dimensions (Å) | 45.92, 58.68, 160.94 | 45.68, 58.49, 161.71 |
| Total number of reflections | 126,165 | 53,669 |
| Number of unique reflections | 24,797 (1,318) | 9,031 (1,438) |
| Multiplicity        | 4.8 (4.9) | 5.9 (4.8) |
| Completeness (%)    | 99.5 (99.8) | 97.1 (88.3) |
| Wilson B factor (Å²) | 9.3 (2.0) | 10.1 (2.8) |
| Rmerge (%)          | 19.2      | 51.5      |
| Rpim (%)            | 0.12 (0.77) | 0.091 (0.61) |
| CC₅₀ (%)            | 0.088 (0.43) | 0.10 (0.37) |
| Rwork (%)           | 19.2 (2.93) | 22.7 (32.6) |
| Rfree (%)           | 24.2 (31.5) | 24.3 (29.8) |
| Number of non-hydrogen atoms | 3729   | 3497      |
| Number of ligand molecules | 0       | 1         |
| Number of water molecules | 282     | 27        |
| Number of protein residues | 446     | 447       |
| Root mean square deviation bonds (Å) | 0.009 | 0.015 |
| Root mean square deviation angles (°) | 1.27   | 1.69      |
| Ramachandran favored (%) | 98        | 97        |
| Ramachandran outliers (%) | 0.45    | 0.45      |
| MolProbity score    | 0.61      | 2.11      |
| MolProbity clash score | 0.29    | 8.2       |
| Average B factor (Å²) | 36.2     | 64.1      |

less (14) in the most likely space group P2₁,2₁,2₁ based on diffraction symmetry and systematic absences (Table 1). The apo-ADPGKΔ51 structure was solved by molecular replacement using Phaser (15) with a search model prepared in Phenix (16) from an ensemble of archaeal ADPGK large domain structures available from the PDB with model alteration (side chain and main chain deletions) informed by multiple sequence alignment. The molecular replacement solution was obtained with one molecule in the asymmetric unit, confirming the P2₁,2₁,2₁ space group, with positive difference density visible for the small domain and other atoms not included in the search ensemble. Extensive model rebuilding was carried out in Coot (17) in combination with auto-building and maximum-likelihood refinement conducted with Phenix (16) and Refmac (18) to build the small domain and improve the large domain structure. Solvent molecules were added to the model into positive 3σ peaks in the weighted Fₐ - Fₜ difference map where chemically sensible hydrogen bonds could be made. The refinement (including TLS refinement) at later stages converged to a final model with an R factor of 0.192 and a free R factor of 0.242 (5% of reflections excluded from refinement) for data 47–2.10 Å. The AMP-ADPGKΔ51 structure was solved by molecular replacement in Phaser using the refined structure of apo-ADPGKΔ51 as a search model. The AMP-ADPGKΔ51 structure was refined, using restraints to the apo-ADPGKΔ51 structure, converging to an R factor of 0.227 and R_free of 0.243 (5% of reflections excluded from refinement) for data 39–3.0 Å. Further x-ray data and model quality statistics are presented in Table 1. The atomic coordinates and structure factor amplitudes for the apo-ADPGKΔ51 and AMP-ADPGKΔ51 crystal structures are available in the Research Collaboratory for Structural Bioinformatics Protein Data Bank under PDB codes 5CCF and 5CK7, respectively. The structure and sequence figures were prepared with CCP4mg (19) and Esprit (20). Structural alignments were performed with Promals3D (21).
Results

Structure of Mammalian ADP-dependent Glucokinase—The apo-mADPGKΔ51 (amino acids 52–495) crystal structure has been determined using x-ray diffraction data to 2.1 Å and refined to an R factor of 0.19 and a free R factor of 0.24. The asymmetric unit of the P2₁2₁2₁ cell contains a single ADPGK molecule consistent with a monomeric structure for ADPGK with no crystallographically related intermolecular contacts greater than 739 Å² as determined with PISA (22). Size exclusion chromatography during protein purification indicated a monomeric solution state (2).

The mammalian ADPGK structure has a ribokinase-like topology (Fig. 1) similar to that of other members of the PFKC (ADP-dependent kinase-like) group of the ribokinase superfamily. This overall fold is similar to the archaeal ADPGK and ADPPFK enzymes consisting of a large domain and a small domain connected by an intermediate hinge-containing region. Compared with archaeal ADPGK structures, the mADPGK structure is most similar to the unliganded “open” ADPGK conformation with a solvent-exposed active site cleft between the two domains (Fig. 1).

The large domain is comprised of a central curved 10-stranded mostly parallel β-sheet that packs against 5 helices on its convex face and 4 helices on its concave side. The small domain core comprises a curved 5-stranded (β-strands 2–4, 8, and 11) β-sheet with 3 α-helices (2–4) and 2 additional short β-strands (9, 10) on the top of its convex face. The concave face of the sheet is unadorned and forms the upper part of the active site located between the two domains. Loops and three short helices form the complex multielement intermediate hinge region connecting the two domains.

The region most divergent from archaeal ADPGKs is after helix α14 (i.e. residue Cys-414) where α15 is much shorter and α16 is replaced in mADPGK (430–445) with a short two-stranded antiparallel β-sheet (Figs. 2 and 3) occupying a similar position and volume on the protein structure as the helices do for archaeal ADPGK. This change requires a minor reorientation of α1 at the N terminus. The structure of the mADPGK small lid domain compared with \textit{P. furiosus} ADPGK (PDB code 3697...
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ADPGKs. Helices (α), sheets (β), and turns (TT) in the structure of mmADPGK are labeled. The mmADPGK disulfide bond Cys are marked with green triangles. hADPGK residues targeted for site-directed mutagenesis are marked with red stars. mm, M. musculus; hs, Homo sapiens; pf, P. furiosus; tl, T. luridus.
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FIGURE 3. Structural superposition of mouse and archaeal ADPGK structures. The superposition of mADPGKΔ51 and T. litoralis ADPGK (PDB code 4BBR) in ribbon representation. Regions distinctly different from the archaeal ADPGK on the small (α2) and large (α1 and β17/18) are colored (gray/blue for mADPGK and yellow/red for T. litoralis ADPGK).

1L.2L) shows a rearrangement of secondary structure elements; the structures of the α2 and α3 helices are not conserved for mADPGK with α2 shifted and α3 replaced by an extended β-strand region instead in mouse (Gly-97–Asp-105, helix α2; Fig. 3). The core of the small domain; the α4, α5 helices and β3–β4 strands are structurally conserved. Structural rigid body superposition using RAPIDO (23) allowing two domain structural alignment resulted in superposition of mADPGK with the closed P. furiosus ADPGK structure with an root mean square deviation 0.9 Å compared with 5.2 Å calculated with a single body superposition (359 Cα atoms used for superposition in both cases).

ADPGK Nucleotide Binding Site—Overall, the mADPGK structure contains homologous glucose and nucleotide binding sites compared with archaeal orthologues, although there are differences in the details of key residues lining the active site. The AMP-mADPGK structure reveals clear density for AMP reaction product bound in the nucleotide binding site (Fig. 4, A and B), whereas apo-mADPGK contains ordered solvent molecules in the active site. One striking feature of the mADPGK structure is the presence of a disulfide bond between Cys-469 at the “back” (relative to the ADP binding site) of the “large nucleotide-binding loop” (residues 466–476) that wraps around the “back” (relative to the ADP binding site) of the “large nucleotide-binding loop.” For archaeal ADPGK, it has been proposed that a conserved Tyr (Tyr-357 for P. furiosus ADPGK) is responsible for the preference for ADP binding over ATP because its side chain excludes the larger triphosphate nucleotide and places the ADP into a binding mode where the β-phosphate is located in the equivalent γ-phosphate position found for ATP-dependent ribokinases (9). A homologous Tyr is not conserved for mammalian ADPGK and instead is His at the equivalent position. However, it has not been possible to convert an ADP-dependent enzyme to utilize ATP based on this hypothesis (hADPGK H382V/H387V; this study or Ref. 24). Furthermore, the mADPGK nucleotide-binding site appears more open partly because of replacement of another conserved archaeal ADPGK Tyr with Leu-383 at the C-terminal end of β14 (Fig. 2). The AMP ribose 2’ and 3’ OH contact the Thr-382 and His-381 main chain carbonyls and the His-381 side chain, respectively (Fig. 4), interactions conserved for archaeal ADPGK. The large nucleo-
tide binding loop (residues 466–476) and the strictly conserved Ile-482 (and to a lesser degree Leu-478) on either side of the catalytic Asp-480 (Fig. 2) are larger side chains compared with ribokinases (e.g. Ala-253 PDB code 1RK2) (26) that position the nucleotide along one “phosphate spacing” so that the ADPGK ADP$^-$-phosphate is in an equivalent position to the $\gamma$-phosphate of ATP in ribokinase structures.

ADPGK Glucose BindingSite—Attempts to co-crystallize or soak mADPGK with glucose have not been successful to date; in the absence of a bound structure, it is possible to infer a homologous glucose binding site at the intermediate hinge region between the large and small domains for the mADPGK structure based on archaeal ADPGK (Fig. 6). The mADPGK structure shows that the GG (157–158) ribokinase sugar-binding sequence motif is conserved where the Gly-158 backbone NH can interact with glucose 4OH (by homology to archaeal ADPGK). mADPGK also contains the conserved glucose ligands from the lid domain Asp-84 ($\beta2$) and Glu-130 ($\beta3$). If we hypothesize a lid-closing mechanism equivalent to archaeal ADPGK occurring for mADPGK, then Asp-84 and Glu-130 would be positioned so that their side chains could make hydrogen bonds with the glucose 3 and 4OH, and 2OH, respectively. His-207 contacts with 2 and 3OH, further contributing to hexose specificity (8). One difference for mADPGK is Cys-82, which is Asn for archaeal ADPGKs (e.g. P. furiosus ADPGK Asn-32 and tlADPGK Asn-40) with the mADPGK sulfhydryl potentially in contact distance of the glucose 3OH. Another difference for mADPGK is the larger amino acids His-264–Met-265 compared with archaea T. litoralis ADPGK Gln-243–Ala-244 and P. furiosus ADPGK Gln-235–Val-236. The Met-265 side chain is positioned within 4 Å of the glucose-binding site, approaching the 2OH position. The catalytic Asp-480 is positioned within 3 Å of the glucose 6OH in this binding model. RAPIDO structural superposition of mADPGK with liganded archaeal ADPGK, allowing rigid body rotation of the two domains, positions conserved residues from the lid domain.
The disulfide bond cysteines are indicated with a yellow shading. The mouse ADPGK glucose binding site (Fig. 5). The mouse ADPGK glucose binding site inferred from homology to archaeal ADPGK by structural superposition of mADPGK (gray), and glucose/AMP bound T. litoralis ADPGK (PDB code 4885, yellow). The α-glucose (green) from 4885 is displayed. All labels refer to mADPGK amino acids.

**FIGURE 5. Sequence alignment of the C-terminal region of ADPGKs from Eukaryotes and Archaea.** hs, H. sapiens; mm, M. musculus; pt, Pan troglodytes; bt, Bos taurus; ec, Equus caballus; oc, Orcytolagus cuniculus; gg, Gallus gallus; dr, Danio rerio; xt, Xenopus tropicalis; ce, Caenohabditis elegans; gg, Stronglyclentrost purpuratus; si, Solenopsis invicta; bm, Bombyx mori; dm, Drosophila melanogaster; tt, Tetrahymena thermophila; bl, L. tigris; pf, P. furiosis; ph, P. horikoshii. Cysteine residues homologous to those involved in the formation of the disulfide bond observed in M. musculus ADPGK are highlighted with yellow shading. The disulfide bond cysteines are highly conserved in Eukarya but absent in Archaea.

**FIGURE 6. Mouse ADPGK glucose binding site.** The mouse ADPGK glucose binding site inferred from homology to archaeal ADPGK by structural superposition of mADPGK (gray), and glucose/AMP bound T. litoralis ADPGK (PDB code 4885, yellow). The α-glucose (green) from 4885 is displayed. All labels refer to mADPGK amino acids.

**ADPGK Functional Characterization**—$^{31}$P NMR activity assays confirmed that D-glucose and ADP are substrates for human ADPGK. The assay monitored the disappearance of the substrate peak corresponding to ADP and appearance of signals for the products glucose-6-phosphate and AMP (Fig. 7). No activity was observed for any of the other sugars tested—2-deoxyglucose, L-galactose, D-xylose, L-arabinose, D-fructose, D-mannose, D-fructose-6-phosphate, D-methyl-D-glucopyranose, 1-O-methyl-D-glucopyranose, D-ribose, D-xylose, D-galactose, D-fructose, D-mannose, D-fructose-6-phosphate, and α-D-glucose-1-phosphate.

The kinetic analysis for human ADPGK has been extended from previous studies (3) to include enzymatic parameters for ADP and to model the inhibition observed at higher glucose and ADP substrate concentrations, as well as AMP product inhibition. The pH profile for human ADPGK was determined with a single maximum activity at pH 7.5 in the range tested (pH 6.0–9.5). Allowing for inhibition at increased substrate concentrations in the kinetic model resulted in a $V_{max}$ of 30 units/mg protein, an apparent $K_m$ for glucose of 0.48 mm (±0.09) with an apparent $K_i$ for glucose inhibition of 2.9 mm (±0.6) for hADPGK (Fig. 8). The apparent $K_m$ for ADP was 0.56 mm (±0.09) with apparent substrate inhibition $K_i$ of 9.1 mm (±1.5). The $K_i$ for AMP inhibition was 0.5 mm (±0.07) (Fig. 8). hAD-
PGK showed reduced activity with GDP (V\text{max} 1.5 units/mg), relative to ADP, with an apparent K\text{m} of 2.9 mM (R\text{H11006}0.8). Little activity was observed for CDP, V\text{max} 0.05 unit/mg. N-terminal truncations of human or mouse ADPGK (R\text{H9004}50, respectively) retained activity comparable with that of the full-length enzyme, but truncation of the N-terminal 151 amino acids (R\text{H9004}151) abolished activity. Neither full-length nor N-terminally truncated ADPGK showed any observable activity with ATP.

The hADPGK site-directed mutants showed either greatly reduced (D84A V\text{max} 0.0004 unit/mg; R228A V\text{max} 0.31 unit/mg, K\text{m} R\text{H11005}0.27 mM for ADP and 0.23 mM for glucose; H264A V\text{max} R\text{H11005}0.0051 unit/mg; D481A V\text{max} R\text{H11005}0.15 unit/mg; D481N V\text{max} R\text{H11005}0.017 unit/mg) or no detectable activity (H387A, D481E, and the double mutant H382V/H387V). Mutant hADPGK-H264A showed a greatly increased apparent K\text{m} for glucose (5 mM), and no substrate inhibition could be detected for this variant with glucose concentrations of up to 55 mM.

Discussion

The overall fold of Mus musculus ADPGK is ribokinase-like, similar to archaeal orthologues although details differ in secondary structural elements of the lid domain and on the surface around the N terminus of the large domain. For crystallization the mouse ADPGK has been N-terminally truncated at residue 52 (R\text{H9004}51) to the “core” ADPGK structure (conserved with archaea; Fig. 2). The very N terminus (residues 1–21) is predicted to be a signal sequence consistent with ER localization.

The mammalian ADPGK large nucleotide-binding loop is structured by a disulfide bond between two conserved cysteines; Cys-414 and Cys-469 for mADPGKΔ51 with the loop conformation retained in the absence of nucleotide. The only other ADPGK structure without a nucleotide bound is the P. horikoshii ADPGK (PDB code 1L2L), where the large nucleotide-binding loop is not defined in the electron density. Comparing the apo- and AMP-bound mADPGK structures shows that the large nucleotide binding loop is preformed in the absence of nucleotide. Mouse ADPGK was able to use GDP and CDP as phosphoryl donor with 55 and 12% relative (to ADP) activity remaining, respectively (2), compared with hADPGK with 5 and 0.2% activity with GDP and CDP, respectively. Human ADPGK showed no catalytic activity with UDP or TDP. In contrast, most archaeal ADPGKs, ADPPFKs, and ADPGK/PFKs can utilize nucleotide diphosphates other than ADP (27). For example, P. furiosus and T. litoralis ADPGK can use CDP at a similar rate to ADP, albeit with a much higher K\text{m} (28), and lower catalytic rates were reported for the nucleotide diphosphates GDP, UDP, and IDP. On the other hand, no alternative nucleotide substrate utilization was observed for the Archaeoglobus fulgidus ADPGK (29). No ADP-dependent kinase has been identified that can use nucleotide triphosphate as phosphoryl donor, including the mutant hADPGK-H382V/H387V.
with the apparent $K_m$ values of 0.27 mM for ADP and 0.23 mM for glucose, which is still comparable with the wild type enzyme. The R228A ADPGK is predicted to be unable to stabilize the terminal phosphate sufficiently during the reaction, whereas the substrate binding is affected to a lesser extent. A metal ion complexed with ADP is essential for ADPGK activity (32), although to date no definitive site for cation binding has been confirmed for any of the ADPGK crystal structures, as is the case for the ADPGK sequences previously reported here. An exact role for the Mg$^{2+}$ has not been elucidated, although for ribokinases, it has been hypothesized to stabilize transition state formation by increasing the electrophilicity of the phosphate for transfer (33).

Human ADPGK was found to be specific for $\alpha$-glucose. The utilization of alternative phosphoryl acceptors was determined by monitoring ADP disappearance and AMP appearance by $^{31}$P-NMR, thus eliminating possible complications of a linked enzymatic assay, where the produced phosphorylated products may not be compatible with the linker enzymes. ADPGK from *T. litoralis* and *P. furiosus* had less than 10% of their activity with the hexoses galactose, fructose, or mannose (28, 34, 35). The archaeal enzymes were also able to utilize 2-deoxyglucose as phosphoryl acceptor to a certain extent. The *A. fulgidus* ADPGK was not able to use hexoses other than $\alpha$-glucose and had low activity with 2-deoxyglucose (29). Human ADPGK, as for mADPGK shown previously (2), is not able to utilize 2-deoxyglucose as substrate. This was also the case for the mutant hADPGK-H264A, which was tested for different phosphoryl group acceptors because of its elevated $K_m$ for glucose. The fact that eukaryotic ADPGK is apparently unable to accept a hexose phosphoryl acceptor modified in the 2-position has implications for probes used in positron emission tomography, an imaging technique widely in visualization of malignant tumor tissues that exploits the elevated glucose consumption of tumor cells. 2-$^{18}$F-fluoro-2-deoxy-$\alpha$-glucose is frequently used for positron emission tomography imaging of tumors, which is metabolized after uptake into the cells by the hexokinases to 2-$^{18}$F-fluoro-2-deoxy-$\alpha$-glucose-6-phosphate, which in turn cannot be further metabolized and is visualized by the positron emission tomography scanning technique (11, 36). A lack of ADPGK activity with 2-$^{18}$F-fluoro-2-deoxy-$\alpha$-glucose could therefore lead to an underestimation of the total conversion of glucose to glucose-6-phosphate, which is an important metric in diagnosis.

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