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Ribokinase family evolution and the role of conserved residues at the active site of the PfkB subfamily representative, Pfk-2 from *Escherichia coli*

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ABSTRACT

Phosphofructokinase-2 (Pfk-2) belongs to the ribokinase family and catalyzes the ATP-dependent phosphorylation of fructose-6-phosphate, showing allosteric inhibition by a second ATP molecule. Several structures have been deposited on the PDB for this family of enzymes. A structure-based multiple sequence alignment of a non-redundant set of these proteins was used to infer phylogenetic relationships between family members with different specificities and to dissect between globally conserved positions and those common to phosphosugar kinases. We propose that phosphosugar kinases appeared early in the evolution of the ribokinase family. Also, we identified two conserved sequence motifs: the TR motif, not described previously, present in phosphosugar kinases but not in other members of the ribokinase family, and the globally conserved GXGD motif. Site-directed mutagenesis of R90 and D256 present in these motifs, indicate that R90 participates in the binding of the phosphorylated substrate and that D256 is involved in the phosphoryl transfer mechanism.

Introduction

The ATP-dependent phosphorylation of fructose-6-P or fructose-1-P to produce fructose-1,6 bisphosphate, and tagatose-6-phospahte to produce tagatose-1,6-bisphosphate, are important reactions in sugar catabolic pathways of bacteria. These enzymatic activities, namely 6-phosphofructokinases, 1-phosphofructokinases and 6-phosphotagatokinases, could be found within the ribokinase family of sugar kinases. The first two structures of phosphosugar kinases from the ribokinase family have been recently reported. Our group determined the crystal structure of the 6-phosphofructokinase isozyme 2 (Pfk-2, E.C. 2.7.1.11) from *Escherichia coli* in its tetrameric form in complex with two ATP molecules per monomer [1]. This enzyme is active as a dimer but is inhibited and form tetramers in the presence of high concentrations of the substrate MgATP [2]. This regulatory mechanism is modulated by the fructose-6-P concentration and has been demonstrated to be important for avoiding futile cycle occurrence under gluconeogenic conditions [3]. Previously, Miallau et al. (2007) reported the crystal structures of *Staphylococcus aureus* (*S. aureus*) tagatose-6-P kinase representing the apo, binary and ternary complexes of the enzyme with substrate and analogues, illustrating aspects of substrate recognition [4]. Interestingly, they identified a conserved motif, SGSLPXG, specific for phosphosugar kinases of the ribokinase family. However, up-to-date, no site-directed mutagenesis studies have been reported to evaluate the role of conserved residues at the active site of phosphosugar kinases from the ribokinase family.

Enzymes with a variety of specificities have been grouped within the ribokinase family. The evolutionary relatedness between Pfk-2 (from *E. coli*), tagatose-6-P kinase (LacC from *S. aureus*), 1-phosphofructokinases (from *E. coli* and *Rhodobacter capsulatus*) and ribokinase (from *E. coli*) was first recognized by Orchard and Kornberg in 1990 [5] and Wu et al. in 1991 [6]. Bork et al. (1993) identified sequence patterns common to phosphosugar kinases, ribokinases, fructokinases and inosine-guanosine kinases [7]. More recently, new members were further identified in the UniProtKB Swiss-Prot database (http://www.expasy.ch/prosite/) using the two highly conserved motifs assigned to the ribokinase family (accession numbers PS00583 and PS00584) [8].

After the crystal structure determination of *E. coli* ribokinase in 1998 by Sigrell et al. [9], structures of several other enzymes were documented to share the two-domain fold and characteristic topology of ribokinase. These enzymes are: the adenosine kinases from *Homo sapiens* [10] and *Toxoplasma gondii* [11,12], Aminoimidazole riboside kinase (AIR kinase)[1] from *Salmonella enterica* [13], 2-keto-3-deoxy gluconate kinase from *Thermus thermophilus* [14], nucleoside kinase from *Metanocaldococcus* [15], adenosine kinase from *Mycobacterium tuberculosis* [16], tagatose-6-P kinase from

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[1] *Abbreviations used:* AIR kinase, aminoimidazole riboside kinase; Pfk-2, phosphofructokinase-2; SEC, Size-exclusion chromatography; PdxK, pyridoxal kinase

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Staphylococcus aureus [4], phosphofructokinase-2 from *E. coli* [1], the 2-keto-3-deoxy gluconate kinases from *Termothoga maritima* [17] and *Sulfolobus solfataricus* [18], and ketohexokinase from *Homo sapiens* [19]. Furthermore, several unpublished structures have been deposited in the PDB and classified as homologous to ribokinase at the SCOP [20] and CATH [21] databases. The tertiary structure in these enzymes consists of a major domain, with a central beta sheet with the arrangement 5↑4↑1↑8↑9↑10↑11↑12↓13↑ (in which strands 11 to 13 form a β-meander) surrounded by α-helices, and a minor domain containing a four stranded beta sheet with the arrangement 3↓2↑6↑7↓. The minor domain covers the active site as a lid and also contributes hydrogen bonding and Van der Waals interactions that result, for most of the members, in a dimeric association. These dimers can form higher oligomeric states (like tetramers, in the case of Pfk-2 in the presence of MgATP [1]) or hexamers, in the case of 2-keto-3-deoxygluconate kinase [14]). A monomeric state is observed in the case of eukaryotic adenosine kinases due to the insertion of complete secondary structure elements that occlude the interfacial surface [10].

How are evolutionarily related the phosphosugar kinases to the other members of the ribokinase family? The sequence identities of these proteins are in the range of 18–22% [15] making it difficult to clearly establish phylogenetic relationships by conventional multiple sequence alignment tools. The question on how the structural repertoire of ribokinase family members in the PDB may help to identify these relationships has not been explored in spite of the increasing availability of these structures.

All members of the ribokinase family phosphorylate sugars and sugar-derived compounds bearing a hydroxymethyl group. When the structures of the different protein–ligand complexes are superimposed, the sugar rings of AIRs, ribose and adenosine occupy the same location with their hydroxymethyl groups oriented in the same way [13], allowing a hydrogen bond interaction with a strictly conserved aspartic residue. This residue is conserved even at the superfamily level and it has been proposed previously that it acts as the catalytic base which deprotonizes the secondary hydroxyl group in the sugar substrate that will attack γ-phosphate of ATP [22]. The GAGD motif containing this aspartic is one of the sequence patterns originally identified in the studies by Bork et al. [7] that corresponds to the PS00584 motif in Prosite (see above). On the other hand, at the phosphosugar kinase subfamily level, conserved residues at the active site must account for the interaction with a common feature of these substrates, the phosphoryl group. For example, the serine residue described to interact with the phosphate of tagatose-6-P in *S. aureus* tagatose-6-P kinase is conserved in other phosphosugar kinases [4].

In this work, we perform a phylogenetic analysis to distinguish evolutionary relationships between ribokinase family members with known structure, which also allow us to recognized two kinds of sequence motifs; one conserved in the entire ribokinase family (GXGD) and a new motif (TR) exclusively conserved among the phosphosugar kinases. The role of these motifs was assessed by site-directed mutagenesis in Pfk-2 since it is the best characterized member among the phosphosugar kinases, biochemically and structurally speaking. Our results constitute, up to date, the most complete study of the role of conserved active site residues in substrate binding, catalysis and allosteric regulation for a phosphosugar kinase of the ribokinase family.

Materials and methods

Dataset of ribokinase family members

In the SCOP database the ribokinase-like family (SCOP id.: 53614) includes 9 different protein domains (domain is the unit of description and classification in these databases) from bacterial, eukaryal and archaeal species. In order to obtain a complete dataset, structures not present in these databases were exhaustively searched in the PDB (http://www.rcsb.org) with the "advanced search" tool, by performing a blast search using as the input query the sequences of every structure identified in SCOP. A total of 70 pdb files were found corresponding to different crystalline forms of 32 unique proteins. A non-redundant set of individual chains (Table 1) was generated by selecting for those structures with ligands present at the sugar binding site or closed orientations of their minor and major domains.

Structural alignment and phylogenetic analysis

All proteins in our non-redundant dataset (Table 1) were structurally aligned by using the STAMP tool from Multiseq [23] in VMD [24]. The loops connecting β2–β3 and β6–β7 in the minor domain were removed since their structural differences drastically reduced the performance of superposition. The multiple sequences alignment (MSA) was obtained from the equivalent residues of the 32 superposed structures. Some misaligned positions were corrected by visual inspection. Finally, all the gap-including positions were removed using GeneDoc [25] giving as result a MSA of 188 positions × 32 taxa. The remaining positions correspond to the core of the ribokinase-like fold (Fig. 1B). Considering that Pfk-2 has 309 residues, these positions correspond to 61% of its entire length. We prepared our MSA in this way in order to work with Mr. Bayes v3.1.2 [26]. This program is not able to treat the insertion–deletion process under a realistic stochastic model. Since it treats gaps as missing data, gap-containing columns will not contribute with phylogenetic information. For the analysis, we use blosum62 as the fixed rate model and gamma-shaped rate variation across sites with a proportion of invariable sites. The number of generations was set to 4×10^6, obtaining for the average standard deviation of the split frequencies a value lower than 0.01.

Site-directed mutagenesis of Pfk-2

Site-directed mutagenesis of D256 and R90 were carried out using the QuickChange (Stratagene) system using as the template the pET21d plasmid (Novagen) carrying the wild type *pfk-2* gene. The mutagenic primers were 5′-GAAGCCAAAGACTGGACC**AG**CA GAATTTACAC-3′ and 5′-ACCGTTGGCGCTGGT**AA**CAGCATGGTCGGC GCG-3′ for R90Q mutation and D256N mutation, respectively (mutated codon is underlined, the replaced nucleotide is in bold). The bases changed were verified by DNA sequencing of the mutants.

Enzyme expression and purification

The mutant enzymes were produced in *E. coli* DF1020 since this strain does not express wild type phosphofructokinases. DF1020 strain was co-transformed with plasmid pGP1-2 [27] that allows the expression of the T7 RNA polymerase after heat induction and the pET21d plasmid carrying the Pfk-2 mutated gene. Cultures were grown at 30 °C in Luria broth media supplemented with ampicillin and kanamycin to a final concentration of 100 and 75 μg/ml, respectively. Protein expression was induced when the A_{600} = 0.5 by heat treatment at 42 °C for 20 min; thereafter, the culture was incubated at 37 °C for 4 h before the cells were collected by centrifugation. The mutant enzymes were purified essentially as described in Babul [28], replacing the AMP-agarose step with a second chromatography in Cibacron blue-Sepharose. The transformed *E. coli* strains produced an average of 10–15 mg of protein per liter of culture. Protein concentration was determined using the Bio-Rad protein assay with the standard curve constructed with bovine serum albumin.