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On the Specialization History of the ADP-Dependent Sugar Kinase Family

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1. Introduction

Sugars are one of the most common carbon sources used by heterotrophic organisms. Indeed, sugar phosphorylation is thought to be a key step in the cellular metabolism since, just after transport into the cell, these molecules are phosphorylated to trap them for further metabolic processing. There are several known pathways used to produce pyruvate from the incoming sugar (like glucose or galactose) which is accompanied by the synthesis of ATP and the production of reductive power. Amongst them, the Embden-Meyerhof pathway, or glycolysis, seems to be the most commonly used. Some microorganisms can also use the Entner-Doudoroff pathway. Also, although the pentose phosphate pathway is generally associated with nucleotide synthesis and reductive power in the form of NADPH it also can be linked to the flux from glucose to pyruvate as this pathway has fructose-6-phosphate and glyceraldehyde-3-phosphate as intermediates. Some microorganisms, such as *Lactococcus lactis*, use a pathway very similar to glycolysis, but instead of start from glucose they use galactose as main carbon source. In this fashion glucokinases are replaced by galactokinases and phosphosfructokinases by tagatose-6-phosphate kinases (van Rooijen et al., 1991).
Interestingly, all the above mentioned pathways ultimately converge through glyceraldehyde-3-phosphate. In this way, the main difference between them is what happens with the hexoses. Here, one of the most important reactions are the initial phosphorylations, e.g. phosphorylation of glucose, fructose-6-phosphate, galactose, tagatose-6-phosphate.
Early on the 90s it was already recognized that the transfer of the γ-phosphate of ATP to several sugars was catalyzed by at least three different non-homologous protein families: the hexokinase family, the ribokinase family, and the galactokinase family (Bork et al., 1993). The hexokinase family contains enzymes with wide specificities including glucokinases, ribulokinases, gluconokinases, xylulokinases, glycerokinases, fructokinases, rhamnokinases, and fucokinases (Bork et al., 1993). The galactokinase family contains enzymes that catalyze the phosphorylation of galactose, mevalonate, P-mevalonate, and homoserine (Bork et al., 1993). The ribokinase family on the other hand is very interesting since its members catalyze the transfer of the terminal phosphate of ATP to sugars like ribose, fructose, sugar containing molecules such as nucleosides, and sugar phosphate molecules like fructose-6-phosphate, fructose-1-phosphate, and tagatose-6-phosphate (Bork et al., 1993). This makes the ribokinase family the group with the broadest specificity amongst the above mentioned. It is clear that while the three groups share some similar substrates and hence are a great example of

convergent evolution the ribokinase family is the only one that contains enzymes able to phosphorylate sugar phosphates.
In particular, glucokinases have been extensively studied since they are in the top on many metabolic pathways, and hence some sort of metabolic hub, and also they are responsible for most of the flux control in glycolysis (Torres et al., 1988). On the other hand, while in normal conditions the phosphofructokinase from rat liver shows almost no control over the glycolytic flux, in starving conditions it becomes almost as important as glucokinase (Torres et al., 1988) which suggests that they become key in gluconeogenic conditions. Moreover, phosphofructokinases are extensively studied because they are highly regulated enzymes. In this light, phosphofructokinases have also been recognized as one of the key enzymes of glycolysis.
From the ribokinase family, one of the most studied enzyme is the phosphofructokinase-2 from *Escherichia coli* which is often referred to as a member of the PfkB subfamily (Cabrera et al., 2010). It is possible to find a second phosphofructokinase, called phosphofructokinase-1[1], in the genome of *E. coli* which belongs to another family called PfkA. In this family, the most extensively studied members are the phosphofructokinase-1 from *E. coli* and the phosphofructokinase from *Bacillus stearothermophilus* (Evans et al., 1981; Schirmer & Evans, 1990). Initially it was thought that both PfkB and PfkA groups had a common origin (Wu et al., 1991), but now we know that they are two non-homologous families. Interestingly, while not phylogenetically related, both phosphofructokinase-1 and phosphofructokinase-2 from *E. coli* show strong inhibition at high concentrations of their substrate MgATP (Atkinson & Walton, 1965; Kotlarz & Buc, 1981), which suggests that this is a key requirement of this metabolic step. This reinforces the idea that these enzymes are strongly related to the balance between glycolysis and gluconeogenesis.
Indeed, it has been already demonstrated that the substrate inhibition is needed for the avoidance of a futile cycle of phosphorylation/dephosphorylation of fructose-6-phosphate/fructose-1,6-bisP which will ultimately lead to a net hydrolysis of ATP (Torres et al., 1997). Interestingly, some microorganisms present phosphofructokinases (also members of the PfkA family) which use polyphosphates as a source of phosphate and hence they do not appear to be regulated (Peng & Mansour, 1992).

2. Glucose degradation in the members of the Archaea domain

Nowadays, organisms can be classified in three principal domains of life: Bacteria, Eukarya, and Archaea (Woese & Fox, 1977; Woese et al., 1990). Interestingly, although there are some known archaea that growth in mesophilic conditions, most of them are extremophiles. Two main phylogenetic groups can be found inside Archaea: *Euryarchaeota* and *Crenarchaeota* (Allers & Mevarech, 2005). Also, recently based in environmental samples, two more groups called *Korarchaeota* and *Nanoarchaeota* has been proposed (Allers & Mevarech, 2005).
Considering the potential for technological applications, most of the attention has been directed to study those archaea able to grow in extreme temperature conditions (known as thermophiles or hyperthermophiles), extremely high salinities (known as halophiles), extremely low pH (known as acidophiles), and most commonly a combination of them. From the *Crenarchaeota*, the *Sulfolubus* and *Aeropyrum* genera receive lots of attention since both are aerobic thermophilic organisms. In the *Euryarchaeota*, the methanogenic organisms are

[1] Sometimes phosphofructokinase-1 and phosphofructokinase-2 from *E. coli* are called the major and minor enzyme respectively.

intensively studied. One of the most studied organism here is *Methanocaldococcus jannaschii*[2] (Jones et al., 1983) since it is one of the few organisms known to produce methane at extreme temperatures. Besides it, the *Halobacterium* and *Haloferax* genera are used as models for halophilic organisms while organisms from the *Thermococcus* and *Pyrococcus* genera are used as models of hyperthermophilic organisms. Here, by far, the most studied organism is *Pyrococcus furiosus*.

In these organisms, sugar degradation proceeds either through the Entner-Doudoroff or the Embden-Meyerhof pathway (Verhees et al., 2003). For instance, members of the *Thermoproteus*, *Thermoplasma*, and *Sulfolobus* genera degrade glucose through a modified version of the Entner-Doudoroff pathway where sugars are phosphorylated only at the 2-keto-3-deoxygluconate or glycerate level. While the former version is still able to produce one ATP molecule per glucose the later does not produce any ATP (for a review see Verhees et al. (2003)). On the other hand, up until the early 90s it was thought that some archaea of the *Euryarchaeota* used a modified unphosphorylated version of the Entner-Doudoroff pathway to degrade glucose (Mukund & Adams, 1991) which was called pyroglycolysis. However, in 1994 it was possible to demonstrate that, in fact, the flux to pyruvate proceeds through a highly modified version of the Embden-Meyerhof pathway (Kengen et al., 1994).Here, although all the intermediates are present, only four of the ten textbook enzymes are conserved (Verhees et al., 2003). In this pathway, the redox reactions are carried out by ferredoxin containing enzymes which latter use the electrons to reduce protons (producing hydrogen) to couple the proton motive force to ATP synthesis by means of a membrane bound hydrogenase enzyme (Sapra et al., 2003). Between the oxido-reductases present in these organisms, perhaps the most interesting is the glyceraldehyde-3-phosphate oxido-reductase. This enzyme is responsible for the single-step conversion of glyceraldehyde-3-phosphate to 3-phosphoglycerate in a phosphate independent manner (Mukund & Adams, 1995). Besides redox reactions, one of the most striking modifications seen in this version of the Embden-Meyerhof pathway is that the phosphorylation of glucose and fructose-6-phosphate is carried out by enzymes that use ADP and not ATP or polyphosphates as the phosphoryl donor (Kengen et al., 1994). These ADP-dependent enzymes are, in fact, homologous to each other and they show no sequence identity over the noise level with any of the hitherto known ATP, or polyphosphate dependent kinases (Tuininga et al., 1999). For this reason it was initially proposed that they belong to a new protein family called PfkC.

Given that these ADP-dependent enzymes were initially discovered in the hyperthermophilic archaeon *P. furiosus* (Kengen et al., 1994), it has been argued in the literature that the main reason for this "ADP-dependence" is the fact that ADP has a higher thermostability than ATP and also that both nucleotides are essentially equivalent since both have a similar standard ΔG of hydrolysis. However, these arguments are highly misleading since, (i) as metabolism is a non-equilibrium process the free energy change upon phosphoryl transfers depends on the concentration of the metabolites, (ii) several ATP-dependent enzymes can be found in hyperthermophilic organisms, (iii) the ADP-dependent enzymes are also present in mesophilic organisms (see below), and (iv) the half life of ATP at high temperatures is higher than some other metabolic intermediates present in the Embden-Meyerhof pathway (Dörr et al., 2003).

The adaptive value of the appearance of the ADP-dependent enzymes has been a matter of great debate. As we have argued before (Guixé & Merino, 2009), it is most likely unrelated

[2] This organism was initially named *Methanococcus jannaschii* and was later renamed as *Methanocaldococcus jannaschii* to aknowledge the fact that those organisms from the *Methanococcus* genus are not thermophilic.

to the temperature at which most of the *thermococcales* grow. The most intriguing question arising here is what happens with the adenylate charge inside these archaea. As they present a glyceraldehyde-3-phosphate ferredoxin oxidoreductase (Mukund & Adams, 1995) which produces 3-phosphoglycerate in a single step that does not produce ATP and also considering both glucose and fructose-6-phosphate are phosphorylated using ADP as phosphoryl donor, it was though that this modified glycolysis had a net ATP production of zero. However, it has been demonstrated by Sakuraba et al. (2004) that the pyruvate kinase from *P. furiosus* catalyze the synthesis of ATP from AMP, phosphoenolpyruvate, and Pi. In this way, the pathway from glucose to pyruvate produces two ATP molecules from every glucose molecule degraded.

Up until now, we have three protein families that contain phosphofructokinases: PfkA, the ribokinase family (which contains the PfkB-like kinases), and PfkC. While the first PfkA crystal structure (The phosphofructokinase from *B. stearothermophilus*) was solved in the 80s (Evans et al., 1981), the first PfkB-like crystal structure (the ribokinase from *E. coli*) (Sigrell et al., 1998) was solved in the late 90s, and the first PfkC crystal structure (The ADP-dependent glucokinase from *Thermococcus litoralis*) just in 2001 (Ito et al., 2001). As all of them were discovered before the middle 90s most of the phylogenetic analysis were performed only on the basis of sequence data. Quite surprisingly, despite the extremely low sequence identity, the PfkC family can be structurally classified as another member of the ribokinase group (Ito et al., 2001) which is now known as the ribokinase superfamily.

3. The ribokinase superfamily

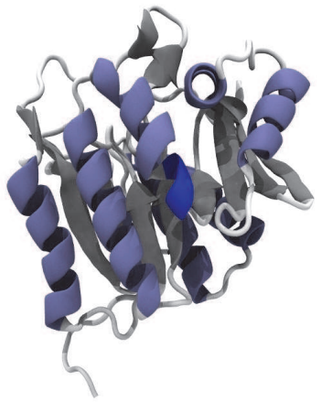
Structurally, the PfkC and PfkB-like groups contain enzymes that present two domains. The large domain, which contains the core ribokinase-like fold, is an $\alpha\beta\alpha$ structure where a central β-sheet mainly composed of parallel strands is flanked by α-helices on both sides. Also, they present a smaller β domain which in general is used as a scaffold for dimerization (Sigrell et al., 1998). However, some of the enzymes are monomers. In this case, the hydrophobic core of the small domain is formed by the insertion of some α-helices (Ito et al., 2001; Mathews et al., 1998). While not all the known PfkC enzymes are monomers (Jeong et al., 2003; Koga et al., 2000; Tuininga et al., 1999) all of them present those α-helices in the small domain. Interestingly, the way in which many of them form multimers is not known, but seems to be highly enzyme specific.

The active site of these enzymes is located in a cleft between both domains (Ito et al., 2001; Sigrell et al., 1998). For some members of the ribokinase family, it has been shown by means of x-ray crystallography that the relative orientation of the domains can be modified by the binding of the phosphoryl acceptor ligand (Schumacher et al., 2000; Sigrell et al., 1999) which has been suggested as a key step in the catalytic mechanism of these enzymes. In the PfkC case, a similar scenario has been suggested (Ito et al., 2003; Tsuge et al., 2002). Here, although the evidence is also crystallographic, it is indirect because the only enzyme crystallized in the apo form and complexed with a substrate is the ADP-dependent phosphofructokinase from *Pyrococcus horikoshii* (Currie et al., 2009) which does not show any domain movement. However, it was not possible to obtain a crystalline form of the enzyme in the presence of fructose-6-phosphate which could be the key component to induce the domain closing. In fact, it has been previously shown by us based on molecular modeling that the open conformation of these enzymes is most likely inactive (Merino & Guixé, 2008).

Table 1 shows most of the members of the ribokinase superfamily with known crystallographic structures. Based on this structural data it is possible to add other specificities

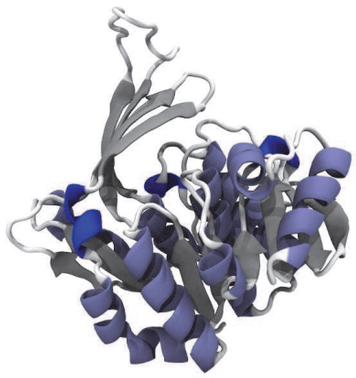
	PDB Code	Organism	Function
PfkC like	1UA4	*Pyrococcus furiosus*	Glucokinase
	1GC5	*Thermococcus litoralis*	Glucokinase
	1L2L	*Pyrococcus horikoshii*	Glucokinase
	1U2X	*Pyrococcus horikoshii*	Fructose-6-phosphate kinase
Vitamin kinase like	1JXH	*Salmonella typhimurium*	4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate kinase
	1EKQ	*Bacillus subtilis*	Hydroxyethylthiazole kinase
	1V8A	*Pyrococcus horikoshii*	Hydroxyethylthiazole kinase
	1UB0	*Thermus thermophilus*	Phosphomethylpyrimidine kinase
	1LHP	*Ovis Aries*	Pyridoxal kinase
	1TD2	*Escherichia coli*	Pyridoxal kinase (PdxY)
	2DDM	*Escherichia coli*	Pyridoxal kinase (PdxK)
	2F7K	*Homo sapiens*	Pyridoxal kinase
	2I5B	*Bacillus subtilis*	Pyridoxal kinase
	1KYH	*Bacillus subtilis*	Unknown function
	2AX3	*Thermotoga maritima*	Unknown function
	2R3B	*Enterococcus faecalis*	Unknown function
PfkB like	2AFB	*Thermotoga maritima*	2-keto-3-deoxygluconate kinase
	2VAR	*Sulfolobus solfataricus*	2-keto-3-deoxygluconate kinase
	2DCN	*Sulfolobus tokodaii*	2-keto-3-deoxygluconate kinase
	1V1A	*Thermus thermophilus*	2-keto-3-deoxygluconate kinase
	2QCV	*Bacillus halodurans*	5-dehydro-2-deoxygluconate kinase
	1TZ6	*Salmonella enterica*	Aminoimidazol riboside kinase
	1BX4	*Homo sapiens*	Adenosine kinase
	1LII	*Toxoplasma gondii*	Adenosine kinase
	2PKN	*Mycobacterium tuberculosis*	Adenosine kinase
	2C49	*Methanocaldococcus jannaschii*	Nucleoside kinase
	1RKD	*Escherichia coli*	Ribokinase
	1VM7	*Thermotoga maritima*	Ribokinase
	2FV7	*Homo sapiens*	Ribokinase
	2QHP	*Bacteroides thetaiotaomicron*	Fructokinase
	2HW1	*Homo sapiens*	Ketohexokinase
	2ABQ	*Bacillus halodurans*	Fructose-1-phosphate kinase
	2F02	*Enterococcus Faecalis*	Tagatose-6-phosphate kinase
	2JG1	*Staphylococcus aureus*	Tagatose-6-phosphate kinase
	3CQD	*Escherichia coli*	Fructose-6-phosphate kinase
	3BF5	*Thermoplasma acidophilum*	Unknown function
	1VK4	*Thermotoga maritima*	Unknown function
	2NWH	*Agrobacterium tumefaciens*	Unknown function
	2RBC	*Agrobacterium tumefaciens*	Unknown function
	2AJR	*Thermotoga maritima*	Unknown function
	2JG5	*Staphylococcus aureus*	Unknown function

Table 1. Crystal structures of the ribokinase superfamily found in the PDB database.



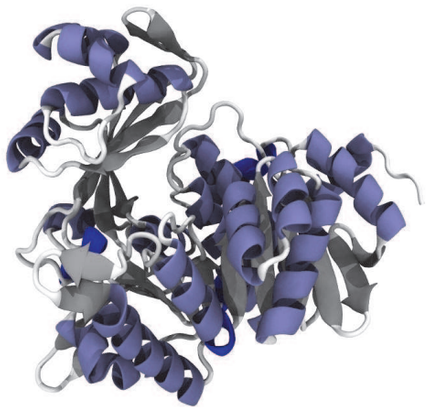
Vitamin kinase like branch

Pyridoxal kinase
Hydroxyethylthiazole kinase
Phosphomethylpyrimidine kinase



PfkB like branch

6-phosphofructokinase
6-phosphotagatose-kinase
1-phosphofructokinase
Nucleoside kinase
Adenosine kinase
Ribokinase
2-keto-3-deoxygluconate kinase
Aminoimidazol riboside kinase



ADP-dependent branch (PfkC)

Glucokinase
6-phosphofructokinase

Fig. 1. Schematic representation of the three branches of the ribokinase superfamily. For the vitamin kinase like branch the pyridoxal kinase (pdxK) from *E. coli* (PDBID 2DDM) is used as example, for the PfkB like branch the ribokinase from *E. coli* (PDBID 1RKD) is used, and for the ADP-dependent branch the glucokinase from *T. litoralis* (PDBID 1GC5) is shown.

to the superfamily, such as adenosine kinase[3] (Mathews et al., 1998), 2-keto-3-deoxygluconate kinase (Ohshima et al., 2004), and aminoimidazole riboside kinase (Zhang et al., 2004). Beyond the sugar containing molecules, three dimensional structure comparison showed that kinases like 4-methyl-5-β-hydroxyethylthiazole kinase (Campobasso et al., 2000), pyridoxal kinase (Li et al., 2002), and 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate kinase (Cheng et al., 2002) are also members of the ribokinase superfamily. Interestingly, these enzymes lack the small domain.

Already based on substrate specificities three major branches can be recognized (Figure 1). One of them contains those enzymes that catalyze the transfer of the γ-phosphate of ATP to molecules such as pyridoxal, or pyrimidine derivatives which we know as vitamin kinase like branch. The second contains all the enzymes that catalyze the transfer of the γ-phosphate of ATP to sugar containing molecules, such as fructose-6-phosphate, adenosine, aminoimidazole riboside, etc. We know this as the PfkB like branch. The last of them contains the enzymes that catalyze the transfer of the β-phosphate of ADP to glucose and fructose-6-phosphate which, as was mentioned before is known as PfkC family or ADP-dependent sugar kinase family.

Based mainly on the presence of the small domain and the monomer complexity Zhang et al. (2004) proposed that the most ancient activity of the superfamily should be that catalyzed by the simplest enzyme which is 4-methyl-5-β-hydroxyethylthiazole kinase. In that way, they propose that the increase of complexity in the monomers fold indicates a newer enzyme. By this hypothesis, the ADP-dependent enzymes and the monomeric adenosine kinases should be the newest acquisitions of the superfamily. However, this hypothesis was never tested. Nevertheless, although it could capture the essence of the evolutionary history of this group, considering the linearity of the hypothesis, it is rather unlikely that the true history of the group is entirely represented by it.

As it can be inferred from Figure 1 and Table 1 the ribokinase superfamily is an excellent example of how gene duplication has been used several times by nature to produce new specificities. This process has been recognized before as one of the most important steps in the creation of new protein functions (Chothia et al., 2003). Indeed, most of the proteins present inside a genome belong to a few protein families or a combination of them (see for example Chothia et al. (2003)). This degeneracy causes that the number of protein families represented in a genome are much smaller that the number of genes there.

Just as an example, a simple PSI-BLAST search on the genome of *E. coli* using the phosphofructokinase-2 as query finds 28 non-redundant proteins including: 6-phosphofructokinase, 1-phosphofructokinase, ribokinase, 2-keto-3-deoxygluconate kinase, and several proteins of unknown function. All of them present the PfkB-like fold (see Figure 1) which shows that this family is a very interesting example of gene duplications. However, the study of this feature is complicated by the lack of information on the function of several of the PfkB-like proteins.

4. Structural evolution of the substrate specificity in the ADP-dependent sugar kinase family

The ADP-dependent sugar kinases have been found in several members of the *Pyrococcus*, *Thermococcus*, *Methanosarcina*, *Methanosaeta*, *Methanococcoides*, *Methanococcus*, *Methanocaldococcus*, and *Archaeoglobus* genera (Hansen & Schönheit, 2004; Kengen et al., 1994; Koga et al., 2000; Tuininga et al., 1999; Verhees et al., 2001). Also, it has been possible to

[3] These enzymes have a slightly different fold compared with the other nucleoside kinases (such as inosine-guanosine kinases) from the superfamily mentioned by Bork et al. (1993)

identify a distant homolog of these enzymes in the genome of higher eukaryotes, which has been proven to be an ADP-dependent glucokinase (Ronimus & Morgan, 2004). The metabolic role of the eukaryotic ADP-dependent glucokinases is unclear, but they have been suggested to be used in ischemic conditions (Ronimus & Morgan, 2004).

To date, the crystallographic structures of the ADP-dependent glucokinases from *Thermococcus litoralis* (Ito et al., 2001), *Pyrococcus horikoshii* (Tsuge et al., 2002), *Pyrococcus furiosus* (Ito et al., 2003), and the ADP-dependent phosphofructokinase from *Pyrococcus horikoshii* (Currie et al., 2009) have been solved. As opposed to the vitamin kinase or the PfkB-like branches of the ribokinase superfamily, to date just two specificities have been observed in the ADP-dependent branch (see Table 1). Considering that the ribokinase superfamily contains enzymes that catalyze the transfer the terminal phosphate of a nucleotide phosphate to the methyl alcohol end of a big number of small molecules which includes pyridoxal, pyrimidine derivatives, nucleosides, and several sugars, the PfkC family seems to be the one with the smallest substrate specificity in this group.

While there are many phosphoryl acceptor substrates in this superfamily, just two nucleotides, ADP and ATP, are described as the primary phosphoryl donors. Given the metabolic importance of the phosphoryl donor this specificity problem has received more attention than the acceptor problem in the literature. Of course, the specificity is not strict, and some other nucleotides can replace them. For instance, it has been shown that several ADP-dependent enzymes can use other purines (such as GDP) or even pyrimidines (such as UDP, (Currie et al., 2009)) as phosphoryl donors (Guixé & Merino, 2009). Also, GTP can be used by the phosphofructokinase-2 from *E. coli* and even produce substrate inhibition (unpublished results). Yet, it is important to remember that only those nucleotides with the right number of phosphates (either two for the ADP dependent enzymes or three for the ATP dependent) can be used, as it has been reviewed by us elsewhere (Guixé & Merino, 2009). This shows that any hint for the transition between nucleotide specificities has been obscured by evolution and specialization.

From an evolutionary perspective, while Zhang's hypothesis (Zhang et al., 2004) can be oversimplifying the problem, it captures the most common trend in the evolution of protein families: newer versions within the group tend to increase their structural complexity (Fong et al., 2007). Through their reasoning, ADP-dependent kinases should be closely related to the monomeric adenosine kinases. What has not been properly mentioned in the literature before is the fact that while the tertiary structure of the PfkC enzymes is almost equivalent to that of the PfkB enzymes, the topology of the C-terminal region is completely different (Figure 2). Indeed, this is the reason why it was not possible to group the ADP-dependent enzymes with the other members of the ribokinase superfamily just based on sequence comparison.

A BLAST search on the genome sequence of the archaeon *P. furiosus* reveals three PfkB-like enzymes of unknown function. As it is also possible to find vitamin kinase like enzymes in the genome of the *thermococcales* (see for instance Table 1), then it is possible to deduce that all three modern branches of the ribokinase superfamily have been originated by ancient gene duplication events followed by extensive topological modifications. While the addition of the small domain can be viewed as a trivial modification since it only involves the insertion of sequence, the C-terminal topological reordering involves a non-cyclic permutation. Now, considering that the ADP-dependent enzymes should be the modern ones, in order to be compatible with the topological reordering an ATP-dependent enzyme should present an extra strand in the C-terminal end of the protein extending the central β-sheet. Figure 2 shows that indeed, this requirement is fulfilled by some PfkB-like enzymes. Quite surprisingly, the sugar-phosphate kinases and not adenosine kinases are those who show the extra strand. This

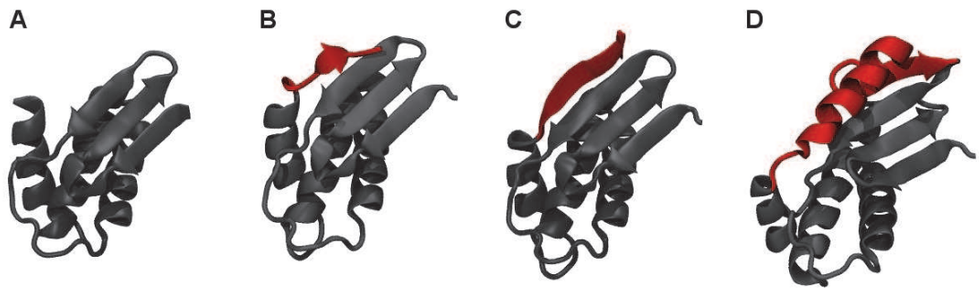


Fig. 2. β-meander region of several members of the ribokinase superfamily such as Pfk-2 from *E. coli* (**A**), a fructose-1-phosphate kinase from *B. halodurans* (**B**), a putative phosphofructokinase from *S. aureus* (**C**), and the ADP-dependent glucokinase from *P. furiosus* (**D**). In red is shown the C-terminal extension thought to be needed for the circular permutation.

suggests that the ADP-dependent enzymes are most closely related to the other glycolytic enzymes present in the superfamily, which seems to be reasonable given the similarity of their substrates.

Indeed, this C-terminal reordering is quite interesting since this same region constitutes almost all the nucleotide binding site. However, while this permutation almost certainly alters the dynamics of the binding pocket, we are not sure if it will alter the specificity of the enzyme. Nevertheless, it requires empirical testing which is now being performed in our laboratory.

Interestingly, the α and β phosphates of ADP are accommodated in the binding site of the PfkC enzymes almost in the same way as the β and γ phosphates of ATP in the remaining members of the superfamily. This led Ito et al. (2001) to suggest that the bulky side chain of Y357 in the ADP-dependent glucokinase from *T. litoralis* which is located below the ribose moiety of ADP was pushing the nucleotide forward and then rendering an enzyme unable to use ATP. However, we indirectly demonstrated that this is not the case since for the ADP-dependent phosphofructokinase from *P. horikoshii* the presence of a significantly less bulky side chain (I340) does not produce an enzyme with ATP-dependent activity (Currie et al., 2009).

While in most of the members of the *Euryarchaeota* there are two ADP-dependent enzymes coded in their genomes, the archaeon *M. jannaschii* presents just one copy of these genes. Surprisingly, the enzyme is able to catalyze the transfer of the β-phosphate of ADP to either glucose or fructose-6-phosphate (Sakuraba et al., 2002). Based on this feature, it was proposed that this enzyme represent an ancestral state of the family, which later gave rise to the separate specificities through a gene duplication event (Sakuraba et al., 2002). However, this hypothesis had to wait six years to be tested (Merino & Guixé, 2008).

To test this hypothesis we used the Bayesian method of phylogenetic inference implemented in the MrBayes 3.1 software (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Initially, a structural based sequence profile was built by means of a structural alignment of the ADP-dependent glucokinases from *T. litoralis*, *P. horikoshii*, and *P. furiosus* and the ADP-dependent phosphofructokinase from *P. horikoshii*. Later all the ADP-dependent kinases from archaeal source were aligned to this profile. After several rounds of alignment refinement the eukaryotic ADP-dependent enzymes were added. As they share only about 15 to 20% sequence identity with the archaeal versions the alignment was guided by secondary structure predictions.

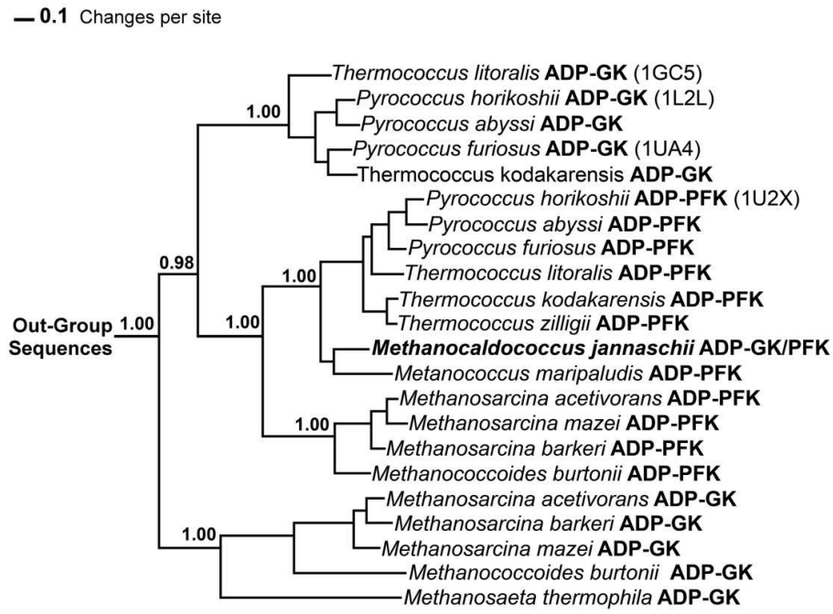


Fig. 3. Phylogenetic tree of the archaeal part of the ADP-dependent sugar kinase family. The eukaryotic sequences were used as outgroup. For displaying clarity just the posterior probabilities of the most important nodes are shown in the figure. The node shown in bold letters correspond to the bifunctional enzyme from *M. jannaschii*. Modified from (Merino & Guixé, 2008).

Considering that in the Archaea most organisms present ADP-dependent glucokinases and phosphofructokinases while in the Eukarya just glucokinases can be found we thought that the divergence between both domains happened before the gene duplication event. In this light, the eukaryotic enzymes seem to be a reasonable choice for the outgroup. Nevertheless, for consistency, other members of the ribokinase superfamily were tested as outgroups as well.

Figure 3 shows the resulting evolutionary tree. It clearly shows four main clades: a group containing the glucokinases from the *methanosarcinales*, a group containing the glucokinases from the *thermococcales*, a group containing the phosphofructokinases from the *thermococcales*, and a group containing the phosphofructokinases from *methanococcales* and *methanosarcinales*. Surprisingly, irrespective of the outgroup used, the root of the tree appears between both glucokinases groups and not dividing both specificities as should be expected from the bifunctional ancestor hypothesis. This demonstrates that the bifunctional enzyme from *M. jannaschii* does not represent an ancestral state of the family. However, given its basal position inside the phosphofructokinases it still could represent a transitional form between both specificities.

Given the tree topology, it is possible to infer that the first separation between *thermococcales* and *methanosarcinales*, i.e. the glucokinases separation close to the root, was produced by a speciation event. Most likely, the gene duplication event is located close to the last common ancestor between both specificities. As this node is located after the speciation event, it is necessary to have an extra horizontal gene transfer event in order to explain the presence of ADP-dependent glucokinases and phosphofructokinases in the genomes of *thermococcales* and

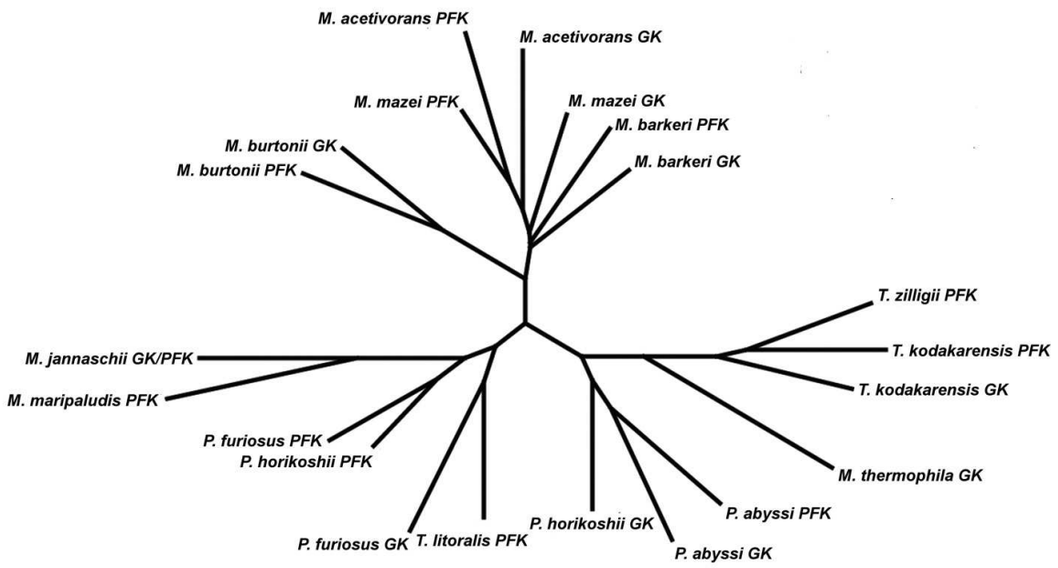


Fig. 4. Dendrogram grouping the archaeal ADP-dependent genes according to their average difference in relative synonymous codon usage. Modified from (Merino & Guixé, 2008).

methanosarcinales. Indeed a similar scenario for the generation of paralogous genes has been proposed before (Gogarten et al., 2002).

To test this hypothesis we analyzed the relative synonymous codon usage of the archaeal PfkC genes (McInerney, 1998). By this methodology, the frequency of any given codon in a gene is calculated relative to the frequency expected for an unbiased codon usage. Figure 4 shows that, in general, genes are grouped very close to their paralogous. If this is not the case, they are at least inside a group that contains closely related species. The only exception is the glucokinase from *Methanosaeta thermophila* which is located inside the *thermococcales* group (Figure 4). Indeed, when the codon usage of this gene is compared with the codon usage of the archaeal genomes, it seems to be more related to the genome of *T. litoralis* than to its own genome (not shown).

While the data present above are not enough to prove the horizontal transfer hypothesis it still strongly suggests that this process has been involved in the evolution of the ADP-dependent sugar kinase family. It is important to stress out that if the event of horizontal gene transfer is ancient enough, then the accumulation of a sufficient number of mutations should have masked it. If this is our case then, to our knowledge, there is no sequence based technique to prove the hypothesis.

Sakuraba et al. (2002) demonstrated that when the bifunctional enzyme was using fructose-6-phosphate as substrate glucose can act as a competitive inhibitor. They proposed that this was produced because both sugars bind to the same site. It is important to mention that competitive inhibition does not necessarily indicates that substrate and inhibitor have the same site, but in this case it is certainly the case. To take advantage of this fact we modeled the bifunctional enzyme and its interaction with both sugars. In this way, it is possible to gain as much information as possible about the structural determinants of the sugar specificity.

Figure 5 shows the predicted interaction geometries for both substrates. For clarity just the residues in a 5 Å radius are shown. As it was inferred by Sakuraba et al. (2002) the interaction between the protein and both substrates are very similar. Indeed, just three of the residues seems to differ significantly in the way they interact with the sugars. For instance, while

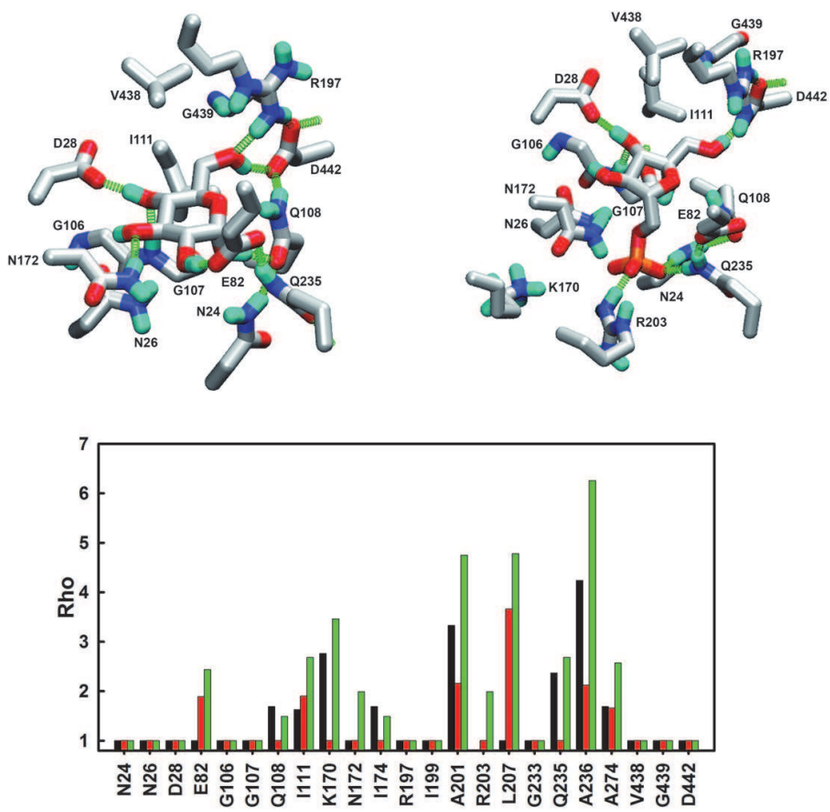


Fig. 5. **Left.** Glucose docked to the bifunctional enzyme. **Right.** Fructose-6-phosphate docked to the bifunctional enzyme. **Bottom.** Results of the real value evolutionary trace analysis for all the residues within 5 Å from the ligands. The results for the glucokinase specificity are shown in black, those for the phosphofructokinase specificity in red, and those for the whole family in green. Modified from (Merino & Guixé, 2008).

E82 makes a hydrogen bond with the hydroxyl located at C2 in glucose it does not seem to interact in any specific way with fructose-6-phosphate. Indeed, this side chain has been proposed by other authors as key for the glucokinase specificity (Ito et al., 2003; Sakuraba et al., 2002). On the other hand, R203 is making a close salt bridge with the phosphate moiety of fructose-6-phosphate while it does not interact with glucose. Although K170 is not in the 5 Å radius we had strong evidence that, as in the R203 case, this side chain was also involved in the phosphate binding (see below).

To quantify the conservation degree of the residues inside the sugar binding site we used a tree-based residue ranking system called real value evolutionary trace (Mihalek et al., 2004). Briefly, the method ranks the residues as follows:

First, let us consider a rooted evolutionary tree with N leaves (sequences). If we number the nodes in the tree starting with the root being 1 then, using as example Figure 3, the node number 2 should be the one with 0.98 posterior probability, and so on. Using this method it is possible to number $N-1$ nodes. Each node defines some groups g of sequences. The root node of course creates a group with all of them. Node number 2 creates a group that contains the ADP-dependent glucokinases from *methanosarcinales* and other with the rest of

the sequences. By this nomenclature one can define a measurement of the conservation of each position in the alignment i where

$$r_i = 1 + \sum_{n=1}^{N-1} \begin{cases} 0 & \text{if position i conserved within each group g} \\ 1 & \text{otherwise} \end{cases} \quad (1)$$

It is clear that if a residue is conserved from the root of the tree, then it will have a r_i of 1. As it gets less and less conserved r_i will be higher. To account for the sequence conservation within each group, the r_i value was weighted by sequence entropy given the expression

$$\rho_i = 1 + \sum_{n=1}^{N-1} \frac{1}{n} \sum_{g=1}^{n} \left(- \sum_{aa=1}^{20} f_{ia}^{g} \log f_{ia}^{g} \right) \quad (2)$$

where f_{ia}^{g} stands for the frequency of appearance of amino acid a inside the group g.
Figure 5 (bottom) shows the result of the ranking applied to the whole PfkC family, and both separated specificities. It is clear from the figure that most of the residues are conserved in the whole family. Interestingly, E82 is only conserved inside the glucokinase specificity, which is in good agreement with the role proposed above. Also, K170 and R203 are only conserved inside the phosphofructokinase specificity which makes them the inverse case of the E82 residue.
Interestingly, N172 is conserved inside both specificities, but it is not in the whole family. The reason for this is that within phosphofructokinases this residue is strictly an asparagine while inside the glucokinases is always a histidine. This suggests that this residue is also related with sugar specificity, but the reason is not as clear as the above examples.
Recently, we used a more elegant method known as explicit likelihood of subset covariation (ELCS) (Dekker et al., 2004) to explore the correlation between mutations to search for the structural specificity determinants.
Figure 6 shows the group of side chains with the highest ELCS score. Surprisingly, the group contain a side chain that belongs to a highly conserved motif called NXXE which has been related with metal binding to the enzymes of the superfamily (Maj et al., 2002; Parducci et al., 2006; Rivas-Pardo et al., 2011). In fact, we have demonstrated that this motif is related to the binding of the catalytic and regulatory metals in the ADP-dependent sugar kinase family (To be published). Also, the first group found by the ELCS method contains some residues that we proposed before as specificity related. The role of the R48/D65[4], R65/S76, P73/F90 mutations is not clear, but seem to be related to the dynamics of the small domain. K158/C174 (equivalent to K170 in the bifunctional enzyme), N160/H176 (equivalent to N172 in the bifunctional enzyme), and R191/D203 (equivalent to R203 in the bifunctional enzyme) are clearly interacting with the sugars. Interestingly, when the position R191/D203 presents an arginine, this positive side chain coordinates the phosphate group present in the fructose-6-phosphate molecule. On the other hand, when it presents an aspartic acid, this side chain interacts with the histidine in the N160/H176 position, allowing the histidine to be correctly positioned to make an h-bond with the O2 hydroxyl group of glucose. Curiously, the position equivalent to E82 from the bifunctional enzyme does not appear to be correlated with other positions by the ELCS method. However this could be due to the small amount of sequence information used for the analysis.

[4] We use the numbering of *Ph*PFK/*Pf*GK for the correlated mutations. See Figure 6 for clarity.

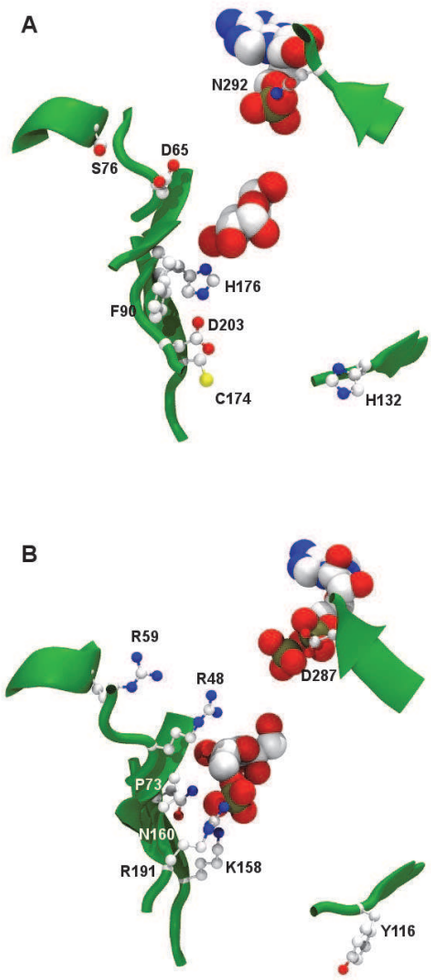


Fig. 6. First cluster of correlated mutations in the PfkC family identified by the ELCS method. **A.** Crystal structure of the glucokinase from *P. furiosus*. Glucose and AMP are shown. **B.** Structural model for the ternary complex between the phosphofructokinase from *P. horikoshii*, ADP and fructose-6-phosphate. The coordinates were derived from the molecular dynamics simulation performed in (Currie et al., 2009)

We have tested the predictions made by the evolutionary trace and the ELCS methods by means of mutagenesis using the ADP-dependent phosphofructokinase from *horikoshii* (Currie et al., 2009) as a model.

Table 2 shows the effect of each mutation on the kinetic parameters of fructose-6-phosphate and MgADP. As it can be predicted, either the mutations R191A, R191E, or K158A produce a high increase in the K_M value for fructose-6-phosphate with a little effect on k_{cat} or K_M from MgADP.

On the other hand, the N160A increase three-fold k_{cat} with a concomitant high increase in the K_M value for fructose-6-phosphate. The reason for the increase in activity is not clear, but it suggests that while this interaction increases the affinity of the protein for the sugar it

Enzyme	k_{cat}	K_M F6P	k_{cat}/K_M F6P
	s^{-1}	μM	$M^{-1}s^{-1}$
Wild Type	45.5 ± 4.0	15.2 ± 2.5	$2.98 \cdot 10^6$
A71E	39.3 ± 9.9	22.2 ± 2.6	$1.77 \cdot 10^6$
K158A	41.0 ± 6.7	6500 ± 1300	$6.30 \cdot 10^3$
N160A	151 ± 16	415 ± 13	$3.65 \cdot 10^5$
N160Q	14.7 ± 0.6	6300 ± 720	$2.33 \cdot 10^3$
R191A	27.4 ± 1.5	254.4 ± 26.1	$1.07 \cdot 10^5$
R191E	42.5 ± 1	4870 ± 170	$8.73 \cdot 10^3$

Table 2. Kinetic parameters of wild type and mutant versions of the ADP-dependent phosphofructokinase from *P. horikoshii*. Modified from (Currie et al., 2009). All experiments were performed at 50 °C.

imposes a strain in the transition state, which results in a decrease of k_{cat}. However, none of these mutations produce an enzyme with glucokinase activity.

The A71E mutation does not affect the catalytic constants nor K_M for fructose-6-phosphate. Surprisingly, it produces an enzymes that now can catalyze the transfer of the β-phosphate of ADP to glucose with a k_{cat} of $2.7 \pm 0.05\ s^{-1}$ and a K_M of 3.95 ± 0.2 mM[5]. Also we recently have produced a N160H mutant. It dramatically increases the K_M value for fructose-6-phosphate to 6.3 ± 0.72 mM and decreases the k_{cat} almost four-fold (Table 2). As in the A71E case, it also produces a bifunctional enzyme that can use glucose as substrate. However, for this mutant no clear saturation is seen even for 25 mM glucose. Based on a Lineweaver-Burk plot, it is possible to estimate a k_{cat} of $2.42\ s^{-1}$ and a K_M value of 25.3 mM. Clearly, this mutation produces a much stronger effect than A71E.

The last two mutations are key to understanding the specialization problem since they not only enable the phosphofructokinase from *P. horikoshii* to use glucose as substrate. Competition experiments with this enzyme have shown that glucose does not bind to the wild type version, which demonstrates that the mutations somehow unblock the binding site for the binding of glucose. Curiously, both mutations points to the interaction between the protein and the hydroxyl group at C2 of glucose. This suggests that the specificity determinants are not evenly distributed amongst the binding site, but rather concentrated in hot-spots. In this light, in order to revert the specificities, just a couple of mutations are needed.

5. Are two ADP-dependent kinases better than one?

Considering that the glycolysis of *M. jannaschii* is functional with just one enzyme in charge of the phosphorylation of glucose and fructose-6-phosphate it is not clear, at a first glance, why two genes were select by nature in the other members of the *Euryarchaeota*. As it was mentioned above, glucokinases are on the top of several pathways and hence the modification of their activity affects a big part of the metabolism. Indeed, this enzyme generally have a great control of the carbon flux. On the other hand, phosphofructokinases seem to be closely related with the balance between glycolysis and gluconeogenesis. In the archaeon *P. furiosus* it has been shown that the switching between these two metabolic pathways is controlled at the expression level (Schut et al., 2003). When the ADP-dependent phosphofructokinase is expressed the fructose-1,6-bisPase is repressed and *vice versa*. Of course, just shutting the

[5] Glucokinase experiments were performed at 40 °Cgiven the instability of the auxiliar enzyme used.

bifunctional enzyme down in *M. jannaschii* will not only decrease the phosphofructokinase activity, but it will have the undesirable side effect of decreasing the glucokinase activity. In this light, the use of one enzyme for each specificity has a great impact in how the cell can regulate the carbon flux. Indeed, the fact the sugar specificity residues are correlated with some others related with regulation (such as the mutation in the NXXE motif) strongly favors our explanation.

What kind of process produce this? It is now a generally accepted hypothesis that less important genes or parts of a gene tend to change or evolve faster than less important ones, which is known as the Kimura-Ohta principle (for a review see Camps et al. (2007)). It is clear that the upon a gene duplication, the phenotypic effect of a mutation in any copy of these genes should be fairly null with the only exception of those that produces specialized genes. It can be argued that even those mutations that produce inactive proteins which should be deleterious and removed by purifying selection under normal conditions are now nearly neutral since the only extra cost is to produce a non-functional protein.

It has been shown recently that upon a change in the fitness optimum (either produced by an environmental change or an internal redistribution of fluxes) most mutations are fixed by natural selection up until the genes reach a nearly optimal sequence. Then they accumulated mutations according to a neutral model (Razeto-Barry et al., 2011). From the arguments given above, it is clear that the only way in which a duplicated gene can break the neutral regime is when a rare specializing mutation is fixed. In this case, the organism must adapt to the new distribution of internal fluxes. Ohta (1987) reached a similar conclusion based on simulations. He stated: " Positive natural selection favors those chromosomes with more beneficial mutations in redundant copies than others in the population, but accumulation of deteriorating mutations (pseudo genes) have no effect on fitness so long as there remains a functional gene. The results imply the following: Positive natural selection is needed in order to acquire gene families with new functions. Without it, too many pseudo genes accumulate before attaining a functional gene family".

As we have shown here, for our protein family, this would imply just one or two mutations since for instance, just the change of a single interaction can change the balance between both specificities. Of course, upon specialization, mutations that modulate regulation (such as those related with the NXXE motif) increases the flexibility of the metabolism.

Interestingly, although most of the *Euryarchaeota* that present the ADP-dependent kinases have two separated specificities the glucokinase from psychrophilic archaeon *Methanococcoides burtonii* have a big C-terminal deletion that should make it non-functional (Merino & Guixé, 2008). The fact that it is still possible to know that it was a glucokinase suggests that this deletion was recent. The phosphofructokinase gene in this archaeon present a glutamic acid in the position equivalent to E82 in the bifunctional enzyme, which suggests that this could be a bifunctional enzyme too. In this way, it appears that until the phosphofructokinase gene is entirely specialized it still exist the possibility of loosing the specific glucokinase gene.

6. Concluding remarks

Our studies about the evolution of the ADP-dependent sugar kinase family showed that the root of the family is located inside the glucokinase group, demonstrating that the bifunctional (glucokinase/phosphofructokinase) enzyme is not an ancestral form, but could be a transitional form from glucokinase to phosphofructokinase. Unfortunately, to date it has not been possible to obtain the crystal structure of any ADP-dependent phosphofructokinase in the presence of fructose-6-phosphate. However, based on structural modeling we have been able to understand partially the structure/specificity relation up to the point where we

can produce bifunctional enzymes from specific ones. Strikingly, the sugar discrimination is somehow concentrated in very few hot-spots in the structure. Indeed, the introduction of just one hydrogen bond or some salt bridges seems to modulate the affinity for glucose or fructose-6-phosphate respectively. Unfortunately, to date, we have been unable to absolutely switch the specificity of these enzymes.

The gene duplication event itself seems to be related with the separated regulation of the glucokinase and phosphofructokinase activity, along with the balance between glycolysis and gluconeogenesis. Indeed, with two different enzymes a finest tuning of the carbon flux inside the cell can be achieved.

7. Acknowledgements

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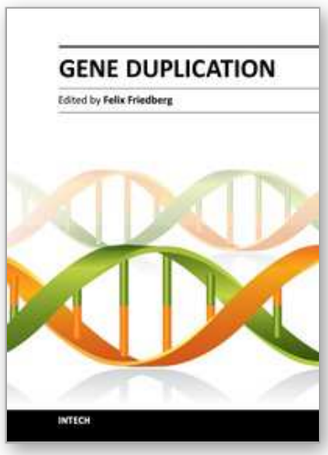
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The book Gene Duplication consists of 21 chapters divided in 3 parts: General Aspects, A Look at Some Gene Families and Examining Bundles of Genes. The importance of the study of Gene Duplication stems from the realization that the dynamic process of duplication is the "sine qua non" underlying the evolution of all living matter. Genes may be altered before or after the duplication process thereby undergoing neofunctionalization, thus creating in time new organisms which populate the Earth.

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