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Critical Review

The ADP-dependent Sugar Kinase Family: Kinetic and Evolutionary Aspects

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Summary

Some archaea of the *Euryarchaeota* present a unique version of the Embden–Meyerhof pathway where glucose and fructose-6-phosphate are phosphorylated using ADP instead of ATP as the phosphoryl donor. These are the only ADP-dependent kinases known to date. Although initially they were believed to represent a new protein family, they can be classified as members of the ribokinase superfamily, which also include several ATP-dependent kinases. As they were first identified in members of the *thermococcales* it was proposed that the presence of these ADP-dependent kinases is an adaptation to high temperatures. Later, homologs of these enzymes were identified in the genomes of mesophilic and thermophilic methanogenic archaea and even in the genomes of higher eukaryotes, suggesting that the presence of these proteins is not related to the hyperthermophilic life. The ADP-dependent kinases are very restrictive to their ligands being unable to use triphosphorylated nucleotides such as ATP. However, it has been shown that they can bind ATP by competition kinetic experiments. The hyperthermophilic methanogenic archaeon *Methanocaldococcus jannaschii* has a homolog of these genes, which can phosphorylate glucose and fructose-6-phosphate. For this reason, it was proposed as an ancestral form for the family. However, recent studies have shown that the ancestral activity in the group is glucokinase, and a combination of gene duplication and lateral gene transfer could have originated the two paralogs in this member of the *Euryarchaeota*. Interestingly, based on structural comparisons made within the superfamily it has been suggested that the ADP-dependent kinases are the newest in the group. In several members of the superfamily, the presence of divalent metal cations has been shown to be crucial for catalysis, so its role in the ADP-dependent family was investigated through molecular dynamics. The simulation shows that, in fact, the metal coordinates the catalytic ensemble and interacts with crucial residues for catalysis. © 2009 IUBMB

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Keywords ADP-dependent kinase family; ribokinase superfamily; modified Embden–Meyerhof pathway; glucokinase; phosphofructokinase.

Abbreviations ADP-GK, ADP-dependent glucokinase; ADP-PFK, ADP-dependent phosphofructokinase; ThzK, 4-Methyl-5- β -hydroxyethylthiazole kinase; HmppK, 4-amino-5-hydroxymethyl-2-methylpyrimidine kinase.

INTRODUCTION

The Embden–Meyerhof pathway is the most common route for the degradation of glucose. Although, in general, only small variations for this route have been identified, several archaea of the *Euryarchaeota* present major modifications in this pathway, because they have only four of the classical enzymes present in the canonical pathway (1). Amongst these differences, one of the most striking is the presence of ADP-dependent kinases (glucokinase and phosphofructokinase) instead of the classical ATP-dependent kinases. These ADP-dependent kinases are homologous to each other and they show no sequence similarity to any of the hitherto known ATP-dependent enzymes. However, the three-dimensional structure determination of several ADP-dependent kinases allowed classifying them as members of the ribokinase superfamily (2). The ribokinase-like fold is basically composed of an eight-stranded β -sheet surrounded by eight α -helices, three on one side and five on the other which constitutes a single domain (the large domain). This superfamily was first proposed based only on sequence data including ATP-dependent kinases of adenosine, fructose, tagatose-6-P, fructose-6-P, and fructose-1-P among others, besides ribokinase, the canonical enzyme (3). Later, with more structural information available, it has been possible to recognize that the ribokinase superfamily also contains enzymes that can transfer the γ -phosphate of ATP to some vitamins involved in B6 synthesis, such as pyridoxal kinase (4). Thus, this superfamily can be subdivided into three major groups: the ATP-dependent sugar kinases, the ATP-dependent vitamin kinases, and the ADP-dependent sugar kinases. The main structural difference between these groups is related to the presence of a small domain. The

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vitamin kinase enzymes present only the $\alpha\beta\alpha$ ribokinase-like fold (the large domain), whereas the enzymes of the other two groups also present a small domain composed by a β -sheet, in the case of the ATP-dependent kinases, and a β -sheet with some α -helical insertions in the case of the ADP-dependent kinases and ATP-dependent adenosine kinases. This small domain acts as a lid for the active site and has been proposed to be a good phylogenetic marker for the evolution of this superfamily (5).

Although the presence of ADP-dependent homologs has been proposed in several archaea of the *Euryarcheota*, the operation of the modified Embden–Meyerhof pathway has only been demonstrated in some hyperthermophilic microorganisms that belong to the *thermococcales*. However, based on genomic data, it has been proposed that this modified glycolysis could be operative also in some methanogenic archaea (6). In particular, these microorganisms present either two copies of ADP-dependent kinases in their genomes or just one, but with dual glucokinase/phosphofructokinase activity (7). The only two exceptions are *Methanotherix thermohila* that presents only one divergent copy of these enzymes and *Methanococcoides burtonii* whose genome presents two ADP-dependent homologs, but the glucokinase-like gene has a big C-terminal deletion that should make the product of this gene nonfunctional. This suggests that the modified Embden–Meyerhof pathway could be irrelevant for these methanogenic archaea.

Surprisingly, it is possible to identify some homologs for these enzymes in the genomes of higher eukaryotes. The gene present in the genome of *Mus musculus* was cloned and heterologously expressed in *Escherichia coli*. The characterization of this enzyme showed that the enzyme is a monomer, quite specific for glucose, classifying it as an ADP-dependent glucokinase (8). Although the physiological relevance of this ADP-dependent kinase for the higher eukaryotes is still unknown, it has been suggested that would be an advantage during ischemia/hypoxia conditions by decreasing the priming cost for the phosphorylation of glucose, saving ATP (8). These genes are expressed in many mammalian tissues which have been suggested before as a sign for a housekeeping role. Interestingly, the ADP-dependent gene has been identified as highly represented in prostate cancer cells (8). However, to date, there are no studies addressing directly the ADP-dependent activity *in vivo*. It is also very interesting to note that it is very uncommon to find a true glucokinase in the genome of an eukaryote since they were believed to be restricted to prokaryotes.

EVOLUTIONARY CONTEXT

As mentioned earlier, among the ADP-dependent family members, there are enzymes with glucokinase and phosphofructokinase activities. Interestingly, the hyperthermophilic methanogenic archaeon *Methanocaldococcus jannaschii* presents an enzyme that shows both ADP-dependent glucokinase and phosphofructokinase activities. On the basis of this dual specificity, this enzyme was proposed as an ancestral form in the family

that later gave rise to the separated activities through a gene duplication event (7). Although it is a very suggestive hypothesis, it was never demonstrated. Recent studies about the evolution of this protein family using the Bayesian method of phylogenetic inference and real value evolutionary trace showed that the evolutionary story of this family is much more complex than it was proposed earlier (9). The root of the family is located inside the glucokinase group, showing that the bifunctional enzyme is not an ancestral form, but could be a transitional form from glucokinase to phosphofructokinase, due to its basal location within the phosphofructokinase group. This implies that besides the gene duplication event, a horizontal gene transfer had to occur to explain the presence of both activities in *thermococcales* and *methanosarcinales* (9). However, it was not possible to demonstrate this hypothesis through the codon usage bias probably because this event is too ancient for this methodology.

Although the structure of around 40 members of the ribokinase superfamily has been deposited in the PDB database (and many of them in the presence of their substrates or analogs) little has been published about the issue of the evolution of this protein superfamily from a structural perspective. The main efforts in this respect have been focused in the relationships within the ATP-dependent sugar kinases such as ribokinase, adenosine kinase, and 6-phosphofructokinases. Zhang et al. (5) had proposed an evolutionary hypothesis for the superfamily. On the basis of the structural complexity of the monomers (mainly the presence/absence of the small domain) and the quaternary structure of the enzymes of the superfamily with known structure, they proposed that the more ancient activities would be those related with the phosphorylation of coenzymes (like pyridoxal kinase) which are the enzymes with the most simple monomer architecture. Then, the tendency in time was to increase the monomer complexity with a concomitant decrease in the aggregation state of the proteins. In this scenario, the ATP-dependent kinase family is the more ancient in the group. However, the issue of the aggregation state seems to be loosely related with the evolutionary hypothesis proposed. For example, the ADP-dependent kinase family presents enzymes with monomeric (7, 10, 11), dimeric (10, 12), and tetrameric aggregation state (13, 14) with no relationship between the number of subunits and the sugar specificity or the growth temperature of the organisms. Interestingly, it has been published that the phosphofructokinase from *Archeoglobus fulgidus* (15) elutes as dimers and tetramers from size exclusion experiments.

Among the structures available for superfamily members, the simplest fold that contains all the necessary elements for catalytic activity is the 4-methyl-5- β -hydroxyethylthiazole kinase (ThzK) monomer. However, as ThzK lacks a lid for shielding the active site from solvent it forms trimers in which each subunit contains one active site shielded by contacts with adjacent subunit. Comparison of the different folds in the superfamily led to Cheng et al. (16) to suggest, as Zhang's group did, that divergence of substrate specificity and quaternary structure may

Table 1
Nucleotide and metal specificities for all the ADP-dependent kinases characterized to date

Enzyme	Phosphoryl group donor							Divalent metal cation							Reference
	GDP	CDP	UDP	IDP	ATP	GTP	Ace-P	Mn ²⁺	Ca ²⁺	Co ²⁺	Ni ²⁺	Zn ²⁺	Pb ²⁺	Cu ²⁺	
<i>P. furiosus</i> ADP-GK	3	111	2	2	ND ^a	ND	NT ^b	47	ND	93	11	4	ND	ND	(10)
<i>T. litoralis</i> ADP-GK	2	122	3	2	ND	ND	NT	6	ND	22	7	ND	ND	ND	(10)
<i>A. fulgidus</i> ADP-GK	ND	ND	ND	NT	ND	NT	ND	167	92	42	50	25	NT	25	(11)
<i>P. furiosus</i> ADP-GK	ND	NT	NT	NT	ND	NT	NT	77	17	1	NT	5	NT	NT	(18)
<i>P. furiosus</i> ADP-GK	<0.3	66	NT	NT	<0.3	NT	NT	114	17	67	68	60	NT	NT	(19)
<i>M. musculus</i> ADP-GK	55	12	ND	NT	ND	ND	ND	NT	NT	NT	NT	NT	NT	NT	(8)
<i>M. jannaschii</i> ADP-GK/PFK	43	14	NT	NT	ND	ND	NT	77	117	77	38	ND	67	NT	(7)
<i>M. jannaschii</i> ADP-GK/PFK	33	ND	NT	NT	ND	ND	NT	40	120	80	40	ND	42	NT	(7)
<i>M. jannaschii</i> ADP-GK/PFK	1,4	NT	NT	NT	0,3	8,1	83	54	120	78	NT	ND	NT	NT	(6)
<i>T. zilligii</i> ADP-PFK	6	ND	ND	NT	5	ND	ND	65.6	20.1	NT	NT	NT	NT	NT	(20)
<i>P. furiosus</i> ADP-PFK	28	NT	NT	NT	<10	<6	ND	43	8	81	NT	NT	NT	NT	(13)
<i>A. fulgidus</i> ADP-PFK	8	3	3	NT	ND	NT	ND	62	2	30	33	6	NT	4	(15)

The table includes all the substrates where at least in one enzyme it was detected significant activity. Values are expressed as percent of the activity measured with Mg²⁺ and ADP. The upper half of the table contains information about enzymes with glucokinase activity, while the lower half contains information about enzymes with phosphofructokinase activity.

^aND, not detected.

^bNT, not tested.

be correlated with the evolution of an active site lid that shields the substrate from the solvent. Then evolution progressed from Thz kinase (no lid) to ribokinase (lid formed by four β -strands from one subunit and one β -strand from the two-fold-related subunit) to adenosine kinase (lid formed by five β -strands and two α -helices) and finally ADP-glucokinase (lid formed by five β -strands and four α -helices).

The question of whether proteins tend to increase or decrease its complexity during evolution has been treated in a much more general fashion by using several protein families as models (17). The results of this study showed that protein families tend to increase structural complexity over time showing that the evolutionary proposal of Zhang's group and Cheng's group is in good agreement with the general trend of proteins.

Interestingly, the ATP-dependent sugar kinases of the superfamily and those related with vitamins ATP-dependent phosphorylation are present in the three cellular domains whereas the ADP-dependent kinases are only present in Eukarya and Archaea, and the ADP-dependent phosphofructokinase activity is only present in some archaea of *Euryarchaeota*, giving support to the evolutionary proposal shown earlier for the superfamily. This also suggests that the modified Embden–Meyerhof pathway and, in particular, its “ADP-dependence” is an evolutionary novelty rather than an ancestral character. However, the reason for this is still not clear (see later).

SUBSTRATE SPECIFICITY

To date, ADP-dependent glucokinases and phosphofructokinases have been characterized kinetically only in a preliminary form. Unfortunately, while many substrates have been tested with these enzymes just a few of them have been fully characterized. Most of the information available is presented as percent of activity compared with the most probable physiological substrates making impossible the comparison using a true specificity constant (k_{cat}/K_M). Nevertheless, the k_{cat} comparison seems to be a good starting point for a specificity searching with these proteins.

With respect to phosphoryl donor specificity, the ADP-dependent glucokinases and phosphofructokinases from several sources (Table 1) are not able to use triphosphorylated nucleotides as substrates. The low relative activity detected with ATP, reported in some articles, is most probably due to ADP contamination present in the ATP solution, because in all those cases the detection was performed using the sugar coupled assay (coupling the production of the phosphorylated sugar to a redox reaction) which cannot discriminate the identity of the phosphoryl donor. In our group, detection of enzyme activity using the Pyruvate Kinase/Lactate Dehydrogenase method (which consumes specifically the ADP produced) for the glucokinases from *Pyrococcus furiosus* and *Thermococcus litoralis* and the phosphofructokinase from *Pyrococcus horikoshii* was unsuccessful (Guixé and coworkers, unpublished results). However,

Table 2
Kinetic parameters for ADP and CDP for two ADP-dependent glucokinases from different sources

Source	k_{cat} ADP (s^{-1})	K_{M} ADP (mM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1} \text{M}^{-1}$)	k_{cat} CDP (s^{-1})	K_{M} CDP (mM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1} \text{M}^{-1}$)
<i>P. furiosus</i>	211 ± 15	0.055 ± 0.004	$3.8 \times 10^6 \pm 3.9 \times 10^5$	252 ± 15	2.21 ± 0.21	$1.1 \times 10^5 \pm 1.2 \times 10^4$
<i>T. litoralis</i>	71.1 ± 3.5	0.043 ± 0.002	$1.6 \times 10^6 \pm 1.1 \times 10^5$	65.7 ± 8.7	1.11 ± 0.04	$5.9 \times 10^4 \pm 8.1 \times 10^3$

The enzymatic activity was measured in all cases at 40°C coupling the production of glucose-6-P to the reduction of NAD^+ by the glucose-6-P dehydrogenase from *Leuconostoc mesenteroides* and using a constant excess of 1 mM of MgCl_2 over the nucleotide concentration. The enzymes were cloned in the pET17b expression vector and expressed in the *E. coli* BL21(DE3)pLysS strain. Proteins were purified with a protocol similar to that published in ref. (23), but without the final size exclusion step.

although ATP cannot be used as phosphoryl donor in these enzymes it has been demonstrated, using kinetic competition experiments, that they can bind this nucleotide (13); Guixé and coworkers, unpublished results).

In the past, it has been argued that ADP can replace ATP in the phosphoryl transfer reaction since they have a similar ΔG^0 of hydrolysis and then they are "energetically equivalent" (21). Considering that all cells are open systems in stationary state far from equilibrium, the application of this analysis, based on the thermodynamics of closed systems in equilibrium conditions, is highly misleading. In fact, to ensure the metabolic coupling it is extremely important to avoid the nucleotide phosphate hydrolysis, going again against the sentence cited earlier. Also, some authors have discussed the presence of these unusual ADP-dependent enzymes in terms of metabolic adaptation to high temperatures and to conditions of starvation (21), but several facts indicate that extreme growth temperature is not the selection factor for the occurrence of ADP-dependent sugar kinases in the central metabolism of Archaea. Among others are: (i) presence of ATP-dependent sugar kinases in several hyperthermophiles and ADP sugar kinases in mesophiles, (ii) presence of ATP- and ADP-dependent kinases in the same organism, and (iii) although ATP is less thermostable than ADP, the half life for ATP is much higher than that for several intermediates of the Embden–Meyerhof pathway (22). Moreover, if ADP were indeed the least thermostable intermediate in the pathway the assumption falls in the same mistake that the free energy statement shown above. As mentioned earlier, cells are not deposits of intermediates, they are systems in stationary state far from equilibrium and thus the molecules of a particular intermediate are changed in time although its concentration could be always the same. In this fashion, if the exchange rate for ADP and ATP are much faster than its hydrolysis rate, the kinetic stability hypothesis is irrelevant. However, to the best of our knowledge, there are not available studies of this kind for members of the *thermococcales*.

Phosphofructokinases seem to be more restrictive than glucokinases with respect to its ability to use diphosphorylated nucleotides (Table 1). Besides ADP, only the enzymes from *P. furiosus*, the bifunctional enzyme, and the glucokinase from *Mus musculus* are able to use GDP as substrate, although noticeably, the bifunctional enzyme is also able to use acetyl-P (6). In con-

trast, many of the ADP-dependent glucokinases studied presents relative activity with CDP similar to that obtained with ADP (Table 1). Analysis of the kinetic parameters of the glucokinases from *P. furiosus* and *T. litoralis* from our group, indicate that in the presence of CDP the same maximum velocity is obtained for both enzymes, while the K_{M} for the nucleotide increase 40- and 25-fold for *P. furiosus* and *T. litoralis*, respectively (Table 2). These values give a decrease in the specificity constant ($k_{\text{cat}}/K_{\text{M}}$) of approximately 30-fold in both the cases. This means that for two competing substrates such as these, in an equimolar mixture of both nucleotides, the rate at which glucose is phosphorylated with ADP is approximately 30 times higher than the rate at which glucose is phosphorylated with CDP, at any substrate concentration. Although there is no information about the intracellular concentrations of these nucleotides in archaea, one can speculate that ADP is most probably the physiological substrate. On the other hand, comparison of the catalytic efficiencies of *pyrococcal* sugar kinases for galactose (ATP-dependent kinase) and glucose (ADP-dependent kinase) resemble those of the respective sugar kinases from mesophilic bacteria and eukaryotes. However, the catalytic efficiencies of these kinases for the phosphoryl donor are 10–150 times higher, when compared to their mesophilic counterparts. Verhees et al. (19) suggest that a high catalytic efficiency for adenine nucleotides and a normal catalytic efficiency for carbohydrates might suggest that the availability of certain nucleotides in the hyperthermophilic *P. furiosus* cells is lower than that present in the cells of mesophilic organisms.

Comparison of the three-dimensional structures of members of the superfamily reveals several motifs important in both structure and function are conserved between the ADP- and ATP-dependent kinases. The recognition of the α - and β -phosphates in glucokinase from *T. litoralis* is almost identical with the recognition of the β - and γ -phosphates of ATP in the ATP-dependent kinases of the superfamily. The adenosine and ribose moieties of ATP/ADP and the α -phosphate of ATP are mainly recognized by side chains, which are not conserved between the ATP/ADP-dependent kinase families. In contrast, the two terminal phosphate moieties are mainly recognized by main chain atoms, which are invariable. Among the residues involved in recognition of the adenine moiety, the presence of Y357 in glucokinase from *T. litoralis* seems to be prominent. This residue

Table 3
Sugar specificity for the ADP-dependent kinases characterized to date

Enzyme	Phosphoryl group acceptor								Reference
	Glucose	Fructose-6-P	Galactose	Fructose	Mannose	2-Deoxy-D-glucose	Glucosamine	1,5-Anhydroglucitol	
<i>P. furiosus</i> ADP-GK	100	NT ^a	<0.3	<0.3	2	8	<0.3	NT	(19)
<i>P. furiosus</i> ADP-GK	100	NT	ND ^b	ND	ND	9.2	NT	NT	(18)
<i>P. furiosus</i> ADP-GK ^c	100	NT	NT	ND	ND (13)	3 (9.2)	NT	NT	(22)
<i>P. furiosus</i> ADP-GK	100	NT	7	ND	13	3	72	68	(10)
<i>A. fulgidus</i> ADP-GK	100	ND	ND	ND	ND	ND ^d	ND ^d	NT	(11)
<i>T. litoralis</i> ADP-GK	100	NT	9	2	13	4	67	166	(10)
<i>M. musculus</i> ADP-GK	100	NT	ND	10	20	ND	NT	NT	(8)
<i>M. jannaschii</i> ADP-GK/PFK	100	32	ND	14	ND	19	ND	NT	(7)
<i>A. fulgidus</i> ADP-PFK	ND	100	NT	NT	NT	NT	NT	NT	(15)

The table includes all the sugars where at least significant activity was measured in one enzyme. Values are expressed as a percent of the maximal activity.

^aNT, not tested.

^bND, not detected.

^cValues outside parenthesis for this enzyme were measured at 50°C while those inside parenthesis were measured at 37°C.

^dActivity here was informed as very low.

is replaced by residues with a smaller side chain in the ATP-dependent ribokinase family, making room for the ribose moiety to enter. In particular, the side chain of this residue has been pointed out as the key residue for the ADP-dependent kinases to lose their ATP activity, since impair the proper position of the nucleotide to donate the γ -phosphate (2).

In opposition to nucleotide specificity, the sugar specificity problem has received much less experimental attention being almost related with the specificity of ADP-dependent glucokinases. Table 3 contains the information about phosphoryl acceptor specificity available for the enzymes of the ADP-dependent family. With respect to this, glucokinases from *P. furiosus*, *M. musculus*, and *T. litoralis* only phosphorylate mannose besides glucose, while the former enzyme can also phosphorylate 2-deoxy-glucose to a little extent, and the one from mouse can also poorly use fructose between several sugars tested (8, 18, 19, 22, Guixé and coworkers, unpublished results). Also, it has been published that the glucokinases from *P. furiosus* and *T. litoralis* can phosphorylate glucosamine and 1,5-anhydroglucitol at a high rate compared with glucose (10), but other publications show that the enzyme from *P. furiosus* cannot use glucosamine (19) suggesting a revision of this point.

Interestingly, although most of the residues involved in sugar binding are highly conserved between the ADP-dependent phosphofructokinases and glucokinases, the enzymes are highly specific for one of the two sugars suggesting that the sugar specificity should be related to just a few residues. Recently, it has been demonstrated that the capability to bind fructose-6-phosphate is mainly related to the presence of two positive charged residues in the bottom of the binding pocket that help to stabilize the negative charge of the phosphate group in the ligand.

On the other hand, glucose specificity is related to the presence of a highly conserved glutamic acid residue, which makes a hydrogen bond with the C2 hydroxyl group in this sugar (9).

To perform catalysis, all the ADP-dependent kinases require the presence of a divalent metal cation. The metal specificity for several members of the family has been assayed, as it is shown in Table 1. Although, in general, the most effective ion is magnesium, several different uncommon metals such as nickel, cobalt, and manganese can replace it to a significant extent, presenting in some cases activities higher than the one obtained with magnesium. In addition, the bifunctional enzyme from *M. jannaschii* can also use calcium and lead. This suggests that the main role of the ions is not related to the specific electronic properties of a particular metal, but to its capability to give a special geometry to the phosphates in the ADP substrate (see later).

CATALYTIC MECHANISM

Among the different reactions catalyzed by the enzymes that belong to a particular superfamily, one would expect that a similar catalytic mechanism would be present in all these proteins.

Sequence alignment based on comparison of the three-dimensional structures revealed that structurally significant motifs are conserved in the ribokinase superfamily, especially some containing active site residues (Fig. 1). Among these, we can find a strictly conserved aspartic acid residue, inside a motif called GXGD, which is proposed to act as a catalytic base that activates the sugar substrate for the nucleophilic attack (Fig. 1B). Mutation of this residue in the ADP-dependent glucokinase from *T. litoralis* (D451) reduces the activity of the enzyme a

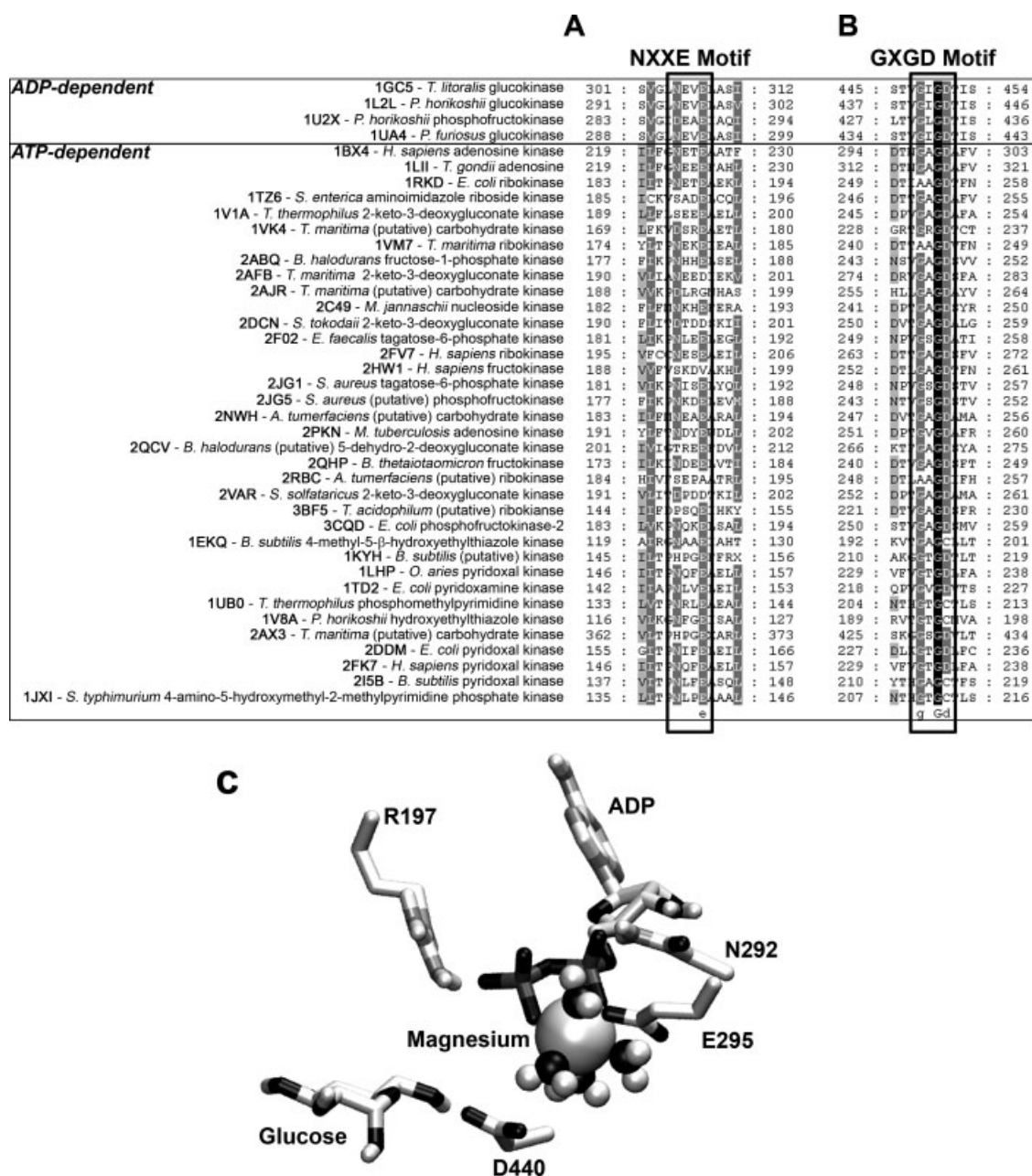


Figure 1. Conserved residues within the ribokinase superfamily involved directly in catalysis. (A) The NXXE motif, the asparagine residue is related with phosphate binding while the glutamic acid residue is related with the binding of the metal to the enzyme. (B) The GXGD motif, the aspartic acid here is proposed to act as the catalytic base activating the hydroxyl acceptor. In bold letters is shown the PDB code for these enzymes. (C) Average of frames in the simulation of the catalytic ensemble of the ADP-dependent glucokinase from *P. furiosus*. The simulation was performed with NAMD 2.6 (24) using the CHARMM force field (25). Briefly, the system was softly thermalized after minimization between 50 and 320 K increasing the temperature by 10 K every 2 picoseconds. After that, the system was equilibrating using Langevin dynamics to complete 1 nanosecond. Later, the system was simulated for another nanosecond. Temperature and pressure were targeted to a value of 320 K and 1 bar, respectively. A time step of 2 femtoseconds was used. The protein was putted in a box of water were with an extension of 1.2 nm far from the last protein atom in each direction and periodic boundary condition was used. The system was neutralized with NaCl to a final concentration of 0.1 M.

thousand times when mutated to alanine or asparagine and a hundred times when mutated to serine (26). In addition, mutation of this aspartic residue (D316) in mammalian adenosine kinase into either asparagine or glutamic acid, leads to a complete loss of activity (27). Similar results were obtained by mutation of the corresponding residue in the adenosine kinase from *Leishmania donovani* (D299) (28) and *Homo sapiens* (D300) (23). Also, the crystal structure of *Toxoplasma gondii* adenosine kinase shows that the side chain of the conserved D318 is well-positioned for proton subtraction of the adenosine 5'-hydroxyl group (29). On the basis of the crystallographic data and by comparison with the catalytic mechanism of *E. coli* ribokinase, D251 was also postulated as the catalytic base in the 2-keto-3-deoxygluconate kinase from *Thermus thermophilus* (28). As a conserved feature, a contact has always been seen by X-ray crystallography between this conserved aspartic residue and the hydroxyl group that will receive the phosphate group in the phosphoryl acceptor molecule. (26, 29–32).

Interestingly, two members of the ATP-dependent vitamin kinases ThzK and 4-amino-5-hydroxymethyl-2-methylpyrimidine kinase (HmppK) have a conserved cysteine residue at the position corresponding to the aspartic acid residue of ribokinase and ADP-dependent kinases and no alternative catalytic base was found in its neighborhood. Mutation of C198 of ThzK from *Bacillus subtilis* into an aspartic acid gave a mutant enzyme with specific activity 9-fold higher than the native enzyme, while mutation of this cysteine by serine or alanine gave enzymes with specific activities of 20% and 40% that of the native enzyme. These results raise some doubts on the importance of the cysteine residue and leads to Campobasso et al. (33) to propose that one of the γ -phosphate oxygen atoms of ATP may function as the alcohol activating base. This may be also the case for HmppK from *Salmonella typhimurium*, since C213 is 4.5 Å from the hydroxyl group, too far away to serve as the general base that activates the hydroxyl group (16).

For several members of the ribokinase superfamily, it has been shown that it is not possible to transfer the phosphoryl group in the absence of a divalent metal cation. As mentioned earlier, members of the ADP-dependent family can use several uncommon metals such as nickel, cobalt, or calcium. Then, if the presence of a divalent cation is required for the reaction in all the members of the superfamily, there is probably a conserved motif related to metal binding.

Figure 1A shows the presence of a conserved motif called NXXE, which has been related with metal binding in some member of the superfamily. Mutation of the glutamic acid residue in this motif in the phosphofructokinase-2 from *Escherichia coli* leads to an enzyme that appears to bind magnesium with a very low affinity compared with the wild-type enzyme (34) and the same effect was seen when this residue was mutated into aspartic acid or leucine in adenosine kinase (35).

It has been not possible to identify a metal cation by crystallography in any of the structures of the ADP-dependent family. However, in the X-ray structure of the glucokinase from *T.*

litoralis it can be seen some electron density in the place where the magnesium ion has been seen in other members of the superfamily (2). Interestingly, in this structure the two phosphates in ADP are not in an alternated geometry. Instead, they are eclipsed in an energetically unfavorable fashion that could be related with the presence of a metal. As it was not possible to see clear electron density for an ion it is possible that the metal is loosely bound and that its role is just to maintain the geometry of the phosphates to favor catalysis explaining the promiscuity of this enzymes.

To explore the catalytic mechanism in more detail we decided to simulate the catalytic ensemble using molecular dynamics. Briefly, we used as starting point the ADP-dependent glucokinase from *P. furiosus* whose crystallographic structure presents glucose and AMP in its active site. The position of ADP was inferred by homology using the structure of the ADP-glucokinase from *T. litoralis* as a guide. All the crystallographic waters were kept except those who crashed with the extra phosphate included by ADP. Finally, one water molecule close to the expected site for the metal (based on the location of magnesium in other member of the superfamily) was transformed into a magnesium ion and the behavior of this system was studied for about 1 nanosecond.

Figure 1C shows the averaging of several frames in the simulation. It is possible to see the magnesium ion hexa-coordinated by two oxygens of ADP (one from each phosphate) and four water molecules. As it was expected, the two phosphate groups conserve their eclipsed conformation during the molecular dynamics simulation. In the second solvation sphere, the four water molecules make some hydrogen bonds with E295 (from the NXXE motif) and with D440 (from the GXGD motif). Also, N292 makes a hydrogen bond with the α -phosphate of ADP. The hydroxyl acceptor in glucose also makes a hydrogen bond with D440, in good agreement with the mechanism predicted. Together, these facts suggest that the metal cation is indeed needed for catalysis since it coordinates the entire catalytic ensemble in the enzyme and also positioned the phosphates groups in the nucleotide in a favorable conformation for the transfer reaction.

Also, a highly conserved arginine residue in the ADP-dependent family (R197 in *P. furiosus* glucokinase) appears to make a strong contact with the β -phosphate of ADP in the simulation (Fig. 1C). It has been suggested before that this residue interacts with the phosphoryl group to stimulate the transfer reaction, and it has been shown that mutation of the corresponding residue (R205) in the glucokinase from *T. litoralis* into lysine or alanine leads to an enzyme with no catalytic activity (26), in good agreement with the results of the simulation.

Taking the fact that these conserved residues within the superfamily play a vital role in function as it has been demonstrated, it is possible to conclude that the members of the group have acquired a great variety of substrate specificity (phosphoryl group donor and acceptor) without changing their reaction mechanism during evolution.

In some of the ATP-dependent enzymes of the superfamily that have the small domain, there is a large conformational change on phosphoryl acceptor binding (29, 31). Here, the two domains close the cleft between them, trapping the substrates and bringing the binding and catalytic residues to a correct conformation for catalysis. On the basis of indirect crystallographic data it has been suggested that the same conformational change occur in the ADP-dependent enzymes (36). However, in this case, the conformational change seems to be related with binding of both ligands. The glucokinase from *P. horikoshii*, crystallized in its apo form, presents an extended conformation (36). However, when the crystallization was performed in the presence of glucose and AMP the glucokinase from *P. furiosus* presents a closed conformation, as it can be expected (26). Interestingly, when crystals of the glucokinase from *T. litoralis* were soaked with ADP, the enzyme presents an intermediate conformation neither fully extended nor fully closed (2). However, the conformational change of the enzymes of the ADP-dependent family waits for a direct experimental demonstration.

A highly conserved di-glycine motif seems to be related to domain motion and is present in all the enzymes of the superfamily that have the small domain (ATP or ADP dependent kinases). The only exception is the 2-keto-3-deoxygluconate kinases that do not present the motif and do not show the conformational change as has been shown by determination of the crystallographic structures (30).

One of the most interesting features of the ADP-dependent phosphofructokinases is that, in opposition to the canonical ATP-dependent phosphofructokinases, they seem to be nonregulated. However, it has been shown that some divalent metal cations can produce inhibition in enzymes of the ADP-dependent family (8, 20; Guixé and coworkers, unpublished results) and also, as it was mentioned before, ATP produces competitive inhibition. Yet, the physiological relevance of these inhibitors is not clear because their *in vivo* concentration is unknown. Interestingly, it has been demonstrated by microarrays experiments that the expression of either ADP-dependent phosphofructokinase or fructose-1,6-bisphosphatase from *P. furiosus* is dependent on the carbon source given to the microorganism (glycolytic or gluconeogenic conditions, respectively) (37).

CONCLUDING REMARKS

The discovery of the ADP-dependent kinases in hyperthermophilic archaea led to some authors to suggest that this feature is an adaptation to high temperatures. However, several facts demonstrated that the presence of these proteins is not related to the hyperthermophilic life. In this way, part of the future work with this protein family should be aimed to reveal the real reason for the use of ADP instead of ATP by these kinases.

Studies about the evolution of this protein 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. Structural determination of ADP-dependent glucokinases and phosphofructokinases allowed classifying them as members of the ribokinase superfamily. Sequence alignments and structural superposition of members of this superfamily identifies several conserved motifs related to catalytic mechanism and substrate specificity. Specifically, the GXGD and NXXE motifs are conserved within the group suggesting a common catalytic mechanism for all the enzymes.

More studies about structure–activity relationship with different members of the superfamily can shed light about the evolution of the group and the structural determinants involved in nucleotide specificity.

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