Archives of Biochemistry and Biophysics 505 (2011) 60–66

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Divalent metal cation requirements of phosphofructokinase-2 from *E. coli*. Evidence for a high affinity binding site for Mn^{2+}

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ARTICLE INFO

Article history:
Available online 29 September 2010

Keywords:
Phosphofructokinase
Electron paramagnetic resonance
Ribokinase family
FRET
Divalent cations

ABSTRACT

The reaction catalyzed by *E. coli* Pfk-2 presents a dual-cation requirement. In addition to that chelated by the nucleotide substrate, an activating cation is required to obtain full activity of the enzyme. Only Mn^{2+} and Mg^{2+} can fulfill this role binding to the same activating site but the affinity for Mn^{2+} is 13-fold higher compared to that of Mg^{2+}. The role of the E190 residue, present in the highly conserved motif NXXE involved in Mg^{2+} binding, is also evaluated in this behavior. The E190Q mutation drastically diminishes the kinetic affinity of this site for both cations. However, binding studies of free Mn^{2+} and metal–Mant-ATP complex through EPR and FRET experiments between the ATP analog and Trp88, demonstrated that Mn^{2+} as well as the metal–nucleotide complex bind with the same affinity to the wild type and E190Q mutant Pfk-2. These results suggest that this residue exert its role mainly kinetically, probably stabilizing the transition state and that the geometry of metal binding to E190 residue may be crucial to determine the catalytic competence.

Introduction

The transfer of the γ-phosphate of ATP to fructose-6-P in *E. coli* is achieved by two isoenzymes, phosphofructokinase 1 and 2, which belong to non related phylogenetic families. Pfk-1[1] belongs to the PfkA family while Pfk-2 is a member of the ribokinase superfamily of sugar kinases. The members of this superfamily catalyze the phosphorylation of substrates containing a secondary hydroxyl group and include kinases of adenosine, fructose, tagatose-6-P, fructose-6-P and fructose-1-P, in addition to ribokinase the canonical enzyme. Folding conservation revealed by structure determination allowed the recognition of distant homologs, including kinases involved in coenzyme metabolism, ADP-dependent glucokinases and ADP-dependent phosphofructokinases [1]. A divalent cation metal requirement has been established for all of these enzymes.

There are two general mechanisms of activation in the case of enzymes that require magnesium for their activity [2]. One is through ligand binding, thereby making it a suitable substrate (e.g., ATP–Mg). The other mechanism is through activation by binding to a different site from the one where the metal–substrate binds. Free ATP is not a substrate for phosphotransferases, and the true substrate is an ATP-divalent metal cation complex. For this reason, phosphotransferases require divalent metal cations for their activity [3]. However, in many cases a magnesium concentration much higher than the one needed to ATP complexation is required for full activity, which suggests an additional role for the cation. For example, most enzymes that catalyze displacement at phosphoric esters (kinases and phosphatases) along with enzymes that carry out the synthesis and degradation of phosphodiester bonds (RNA and DNA polymerases and restriction endonucleases) have a requirement for two-metal ions [4]. Also, a role for divalent metal cation other than ATP binding has been established for the cAMP-dependent protein kinase [5], pyruvate kinase, phosphoenolpyruvate kinase [6,7] and extracellular regulated protein kinase [8].

Pfk-2 from *E. coli* catalyzes a bisubstrate phosphoryl-transfer reaction, with MgATP complex as the phosphate-donating substrate and the 1-hydroxyl group present in fructose-6-P as the phosphate acceptor. It has been proposed that the conserved NXXE motif, whose residues are located at the active site in all of the known structures of members of this superfamily, plays a role in the mechanism by which Mg^{2+} and phosphate affect the activity of adenosine kinase [9]. In the case of Pfk-2, we proposed the requirement of a Mg^{2+} ion besides the one present in the metal–nucleotide complex for catalysis. Also, mutation of the E190 residue (E190Q mutant) of the NXXE motif of Pfk-2 greatly affects the behavior of the enzyme respect to free Mg^{2+} concentration, suggesting that this residue is required for the proper binding of the catalytic ion, that is the metal not bound to the nucleotide [10]. Since the second Mg^{2+} appears

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[1] *Abbreviations used:* Pfk, phosphofructokinase; EPR, electron paramagnetic resonance; FRET, Förster resonance energy transfer; Me^{2+}, divalent metal; Mant-ATP, (*N*-methylantranoyl) adenosine 5′-triphosphate.

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doi:10.1016/j.abb.2010.09.020

to be a key component of the catalytic machinery, understanding its structural environment and how it activates Pfk-2 will provide an important probe to the catalysis of the kinases of this family.

In this work we demonstrate the existence of a second binding site for divalent metal cations in Pfk-2 and by means of substitution studies establish that, besides Mg^{2+}, Mn^{2+} can interact at this site and with a higher affinity than Mg^{2+}. In addition, kinetic, fluorescence and binding studies, allow us to establish the involvement of the E190 residue of the NXXE motif in the interaction with these cations.

Materials and methods

Enzyme expression and purification

Expression of the E190Q mutant was performed as described in Parducci et al. [10]. Wild type and E190Q mutant were purified essentially as described by Babul [11] replacing the AMP-agarose step with a second chromatography in Cibacron blue-Sepharose.

Reagent concentrations

Fructose-6-phospahte concentration was estimated spectrophotometrically using the aldolase coupling assay. The concentration of NADH was calculated using a molar extinction coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$. ATP concentrations were determined spectrophotometrically by the fructose-1,6-bisP formed in the presence of Pfk-2, fructose-6-P, aldolase, triose phosphate isomerase, α-glycerophosphate dehydrogenase and NADH. The $MgCl_2$ was atomic absorption grade. The protein concentration was determined using the Bradford assay.

Activity measurements and kinetics studies

The activity measurements were determined spectrophotometrically by coupling the fructose-1,6-bisphosphate production to the oxidation of NADH at pH 8.2 as described previously [11]. The standard assay reaction contained 0.05 M Tris-HC1, pH 8.2, 1 mM dithiothreitol, 0.2 mM NADH, and a mixture of 40 μg of aldolase, 3 μg of triose phosphate isomerase, and 30 μg of α-glycerophosphate dehydrogenase in a final volume of 0.7 ml. Fructose-6-P, ATP and $MgCl_2$ were varied as indicated.

The kinetic parameters were determined as a function of the concentration of $MgATP^{2-}$ or fructose-6-P while keeping the concentrations of the other substrates at fixed saturating levels. Free Mg^{2+} concentration was held constant at 5 mM unless otherwise was indicated. Determination of K_{act} for Mg^{2+} and Mn^{2+} was performed by measuring the activity of the enzyme as a function of the free divalent cation concentration at fixed saturating concentrations of $MgATP^{2-}$ and fructose-6-P. In each case, the concentration of Me^{2+}, ATP^{4-} and $MeATP^{2-}$ was calculated from the total concentration of the nucleotide (ATP_t) and divalent cation (Mg_t or Mn_t) used in the assay, assuming a dissociation constant of 14 μM [12,13] for the ionic equilibria $MgATP^{2-} \Longleftrightarrow ATP^{4-} + Mg^{2+}$ and $MnATP^{2-} \Longleftrightarrow ATP^{4-} + Mn^{2+}$. Protonated forms of the nucleotide, such as $HATP^{3-}$ or H_2ATP^{2-}, are poorly represented at pH 8.2, so their concentrations were not considered here. The experimental curves were fit to hyperbolic curve by using Sigma-plot software 10.0 (Systat Software, Inc.).

Prior to kinetic and EPR experiments, the enzyme was buffer-exchanged to 25 mM Hepes pH 8.2 and 1 mM DTT using two Hi-Trap desalting columns (Amersham Biosciences, Uppsala, Sweden) having a-4 cm layer of Chelex-100 in a Econo-column Chromatography 0.7 × 30 cm (Bio-Rad laboratories, CA, USA) on the top. The enzyme was concentrated to 60–70 mg/ml using an Amicon® Ultra-4 Ultracel-10k concentrator (Millipore Corporation, Billerica, USA).

Electron paramagnetic resonance spectroscopy measurements

Binding experiments of Mn^{2+} to the wild type and mutant Pfk-2 were measured by EPR spectroscopy on a Bruker Biospin GmbH EPR Spectrometer (model EMX-1572 X-band) at a frequency of 9.2–9.9 GHz with the cavity thermostatized at 25 °C (Rheinstetten, Germany). Stock of enzyme solutions (≈2 mM) were prepared in 25 mM Hepes and 1 mM DTT buffer (pH 8.2) previously treated with Chelex-100. The total concentration of protein was varied in each experiment over a range of 0–1000 μM at a fixed Mn^{2+} concentration of 100 μM. Each sample was prepared in a 70–80 μL volume, and the signal of free Mn^{2+} was measured. Data were obtained and processed with the software WinEPR and Bruker EMX. Calculation of the area for each measure was performed with GRAMS software. The data were fit to a hyperbola using Sigma-Plot software 10.0 (Systat Software, Inc.).

Fluorescence measurements

All fluorescent titrations were performed using a Shimadzu RF-5301 PC fluorometer. The titrations of Pfk-2 with Mant-ATP and the subsequent fluorescence measurements were performed as previously described by Guixé et al. [14]. Briefly, Pfk-2 samples (1.6–2 μM) were incubated in 20 mM Hepes, pH 8.0, 1 mM DTT and 5 mM $MgCl_2$ and subsequent aliquots of Mant-ATP added from stock solutions. For the recording of emission spectra of tryptophan fluorescence (300–550 nm) the excitation wavelength was set to 295 nm to minimize both the inner-filter effect of nucleotides and the contribution of tyrosine to the emission spectra. Binding of Mant-ATP to Pfk-2 was monitored by measuring the quenching of the intrinsic fluorescence emission of Pfk-2 tryptophan. All measurements were corrected for the dilution brought about by the addition of ligands (never exceeding 5% of total volume).

Results

Effects of divalent cations on the activity of Pfk-2 and determination of kinetic parameters

Fig. 1 shows the effect of several divalent cations tested to support Pfk-2 activity. The data indicated that while Mg^{2+} and Mn^{2+} are able to support the enzymatic activity, Cu^{2+}, Zn^{2+} and Cd^{2+} could not significantly do so. Co^{2+} and Ca^{2+} exhibit about 17% and 5% of the activity compared to Mg^{2+}. Also, we tested $MgSO_4$ as a control for the counterion and found that sulphate has no effect. Since Mg^{2+} plays two roles that are essential for the catalytic activity of Pfk-2, as demonstrated previously, the ability of Mn^{2+} to support Pfk-2 activity in the absence of Mg^{2+} indicates that it can substitute for Mg^{2+} at the activating and metal–nucleotide sites. Then, this activity is the composite measure of its ability to serve as metal–nucleotide complex and activating ion. Considering that the experiment was performed at a single and fixed concentration of the cation (1 mM free metal), it was not possible to distinguish if there is any affinity difference between both cations at both sites. In order to address this question, the kinetic parameters of wild type and E190Q mutant Pfk-2 were determined for both divalent metal cations. Table 1 shows that for wild type Pfk-2 there is almost no difference in the kinetic parameters obtained for both divalent metal cations, except for a modest diminution in the K_m value for the Mn^{2+}–nucleotide complex. Instead, the E190Q mutant presents K_m values for fructose-6-P around 4-fold lower for either Mg^{2+} or Mn^{2+} compared to the wild type enzyme, and K_m values for

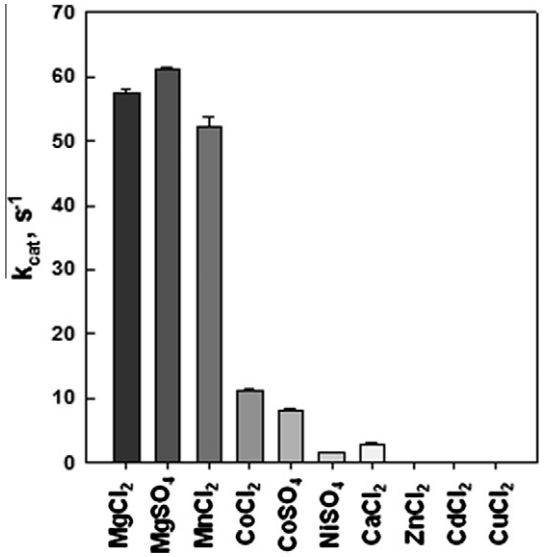

Fig. 1. Divalent metal cation dependence of wild type Pfk-2. Enzyme assays were performed at 25 °C in the presence of 1.8 mM of total divalent metal cation, 0.8 mM of total ATP and 0.8 mM fructose-6-P. The assay mixture contained 25 mM Tris/HCl pH 8.2, except for $ZnCl_2$, $CdCl_2$, $CuCl_2$ where 25 mM Tris/HCl pH 7.0 was used.

Table 1
Kinetic parameters for wild type and E190Q mutant Pfk-2 in the presence of Mg^{2+} or Mn^{2+}.

Kinetic parameter	Wild type		E190Q	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
K_m fructose-6-P (μM)	18 ± 2	37 ± 3	7 ± 4	7 ± 1
K_m metal ATP (μM)	12 ± 2	8 ± 4	117 ± 15	49 ± 10
k_{cat} (s^{-1})	62 ± 2	56 ± 3	0.24 ± 0.02	9 ± 1
k_{cat}/K_m metal–ATP(s^{-1} μM^{-1})	5.2	7.0	0.002	0.18

When assaying enzymatic activity versus fructose-6-P, MgATP2- was held constant at 0.2 and 1.5 mM for wild type and the E190Q mutant, respectively; when assaying versus fructose-6-P, $MnATP^{2-}$ was held constant at 0.2 and 0.8 mM for wild type and the E190Q mutant, respectively; when assaying versus $MgATP^{2-}$ or $MnATP^{2-}$, fructose-6-P was held constant at 0.8 mM. In all cases, the concentration of free divalent cation was 5 mM.

the metal–ATP complex around 10- and 6-fold higher than those obtained for the wild type enzyme for Mg^{2+} or Mn^{2+}, respectively. Also, a difference of about 260-fold in the k_{cat} value between the wild type and mutant enzymes was observed in the presence of Mg^{2+}. However, this value depends strictly on the concentration of free cation used in the kinetic experiments [10]. On the other hand, it seems that Mn^{2+} is much more effective for catalysis than Mg^{2+} in the mutant enzyme, since the catalytic efficiency (k_{cat}/K_m metal–ATP) is 90-fold higher for this cation as compared to Mg^{2+}.

Binding of Mg^{2+} and Mn^{2+} to the activating site of the wild type and E190Q mutant Pfk-2

Since the K_m values for both substrates in the wild type enzyme were not significantly different in the presence of Mg^{2+} or Mn^{2+}, the binding affinity of the activating site for both divalent cation metals was assessed. In the case of Mn^{2+} substitutions studies were performed in the wild type enzyme where a saturating concentration of MgATP and fructose-6-P and different and fixed concentrations of free Mg^{2+} were used. This condition ensures saturation of the catalytic site with MgATP and that the effect of other divalent cation metals would be through interaction with the activating site. Under these conditions, only Mn^{2+} is able to stimulated Pfk-2 activity (data not shown) and the stimulation effect depends on the concentration of the free Mg^{2+} (Fig. 2A). In all the conditions studied the same value of k_{cat} was reached, indicating that both divalent cations bind to the same site (Fig. 2B). The rate of a metal-activated enzyme catalyzed reaction at constant substrate concentration is typically a hyperbolic function of the free metal ion concentration [15]. Then, in order to compare the binding affinities of Mn^{2+} at the activating site in both enzymes (wild type and E190Q mutant), with the corresponding ones for Mg^{2+}, the reaction velocity of these enzymes as a function of free metal concentration, at saturating concentrations of metal–ATP complex and fructose-6-P (Fig. 3A and B) was measured. The data were adjusted to a single hyperbolic fit for both enzymes. The activating constant (K_{act}), was defined as fifty percent of change in enzymatic activity and reflects the kinetic affinity of the enzyme for the activator metal. For wild type Pfk-2 K_{act} values of 7 μM and 89 μM were obtained for Mn^{2+} and Mg^{2+}, respectively, whereas corresponding ones for E190Q were 6.5 mM and 33 mM. At a saturating concentration of free Mn^{2+} a k_{cat} value of 17 s^{-1} was obtained for the mutant enzyme, which represents a 3.6-fold decrease compared to the value obtained for wild type Pfk-2. These results demonstrate the requirement of a second divalent metal for Pfk-2, Mg^{2+} or Mn^{2+}, and that the E190 residue participates in the correct positioning of this activating cation.

Mn^{2+} binding affinities for the wild type and E190Q mutant Pfk-2

Because of its value as a paramagnetic probe, Mn^{2+} was used to determine the binding affinity and the number of divalent metal

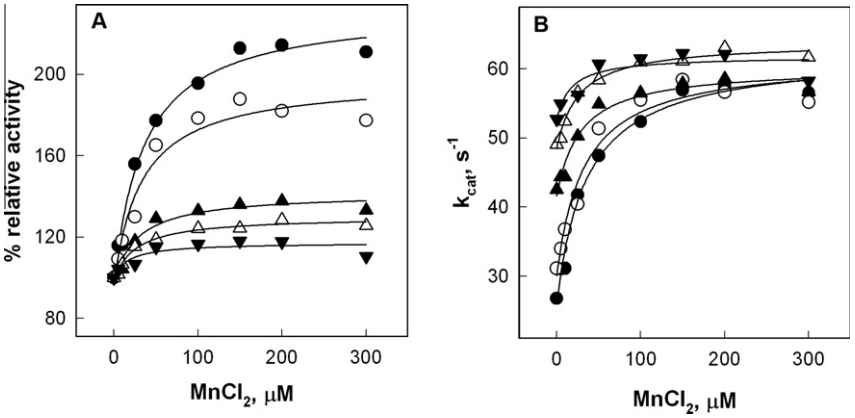

Fig. 2. Effect of Mn^{2+} concentration on the Mg^{2+}-supported wild type Pfk-2 activity. (A) Activities were measured as a function of the total Mn^{2+} concentration at 0.2 mM MgATP, 0.8 mM fructose-6-P and (●) 0.08 mM, (○) 0.12 mM, (▲) 0.26 mM, (Δ) 0.4 mM and (▼) 0.8 mM free Mg^{2+}. Results are expressed as percentage of the velocity obtained in the absence of added Mn^{2+}. (B) Results expressed as k_{cat} values (s^{-1}).

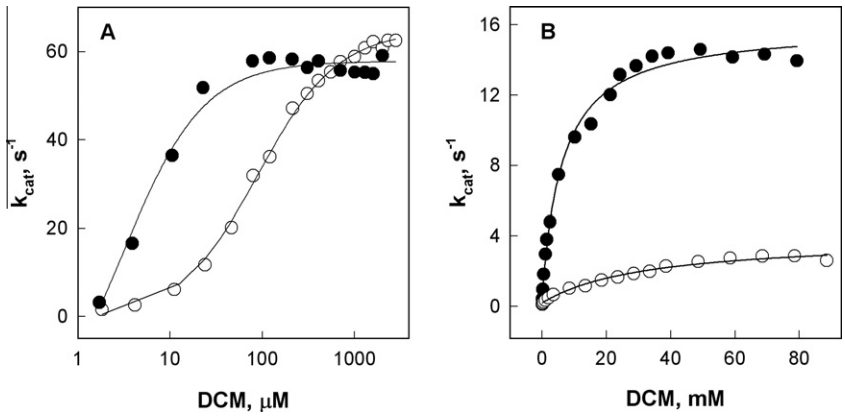

Fig. 3. Effect of free divalent metal cation (DMC) on the activity of wild type and E190Q mutant Pfk-2. (A) The initial velocity of wild type Pfk-2 was measured as a function of the free (○) Mg^{2+} and (●) Mn^{2+} concentration at 0.2 mM metal–ATP. For clarity, the free divalent cation concentration is shown in a logarithmic scale. (B) The initial velocity of the E190Q Pfk-2 was measured as a function of the free (○) Mg^{2+} and (●) Mn^{2+} concentration at 1.5 mM and 0.8 mM of metal–ATP, respectively. Results are expressed as k_{cat} values (s^{-1}).

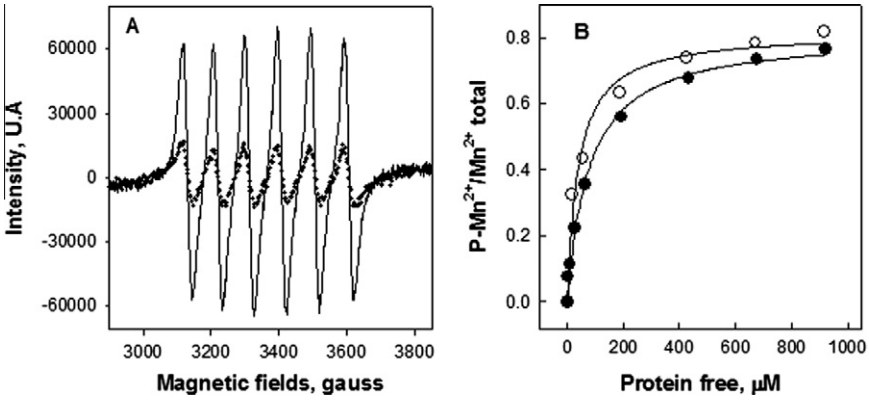

Fig. 4. Mn^{2+} binding to wild type and E190Q mutant Pfk-2. The binding of Mn^{2+} was measured at 25 °C in the presence of 25 mM HEPES pH 8.2, 1 mM DTT by EPR spectroscopy, as described under Materials and methods. (A) Absorption curve of free Mn^{2+} in absence (solid line) and presence (dotted line) of wild type Pfk-2 (1200 μM). (B) Binding curve of Mn^{2+} to (○) wild type and (●) E190Q mutant Pfk-2.

cations bound to wild type and E190Q mutant Pfk-2 in the absence of ligands. Addition of either wild type or E190Q mutant Pfk-2 to Mn^{2+} solutions causes a diminution of the Mn^{2+} EPR signal when compared to a standard in the absence of protein. This diminution is due to a change in the electronic environment of the Mn^{2+} ion that is caused by interaction of the Mn^{2+} with the protein and can be quantitated to obtain the number of Mn^{2+} associated with the added ligand. The Mn^{2+} EPR spectra in the absence and presence of the wild type Pfk-2 are shown in Fig. 4A as an example, and the binding isotherms of the wild type and E190Q mutant constructed from the EPR data are shown in Fig. 4B. The data were analyzed by nonlinear least squares fit to the binding isotherms as described under Experimental Procedures. The results show one Mn^{2+}-binding site in both enzymes (stoichiometry of 0.83 for Pfk-2 and 0.81 for E190Q) in the absence of other ligands, with a K_d value of 41 μM for wild type Pfk-2 and 78 μM for the E190Q mutant (Table 2).

Binding of metal–Mant-ATP complex to the wild type and E190Q mutant Pfk-2

Since binding affinities of Mn^{2+} to wild type and E190Q mutant Pfk-2 show no significant differences we wanted to asses if there is any difference in the binding affinity of the metal–nucleotide complex. For this purpose we used the ribose-modified fluorescent analog (*N*-methylantranoyl) adenosine 5′-triphosphate (Mant-ATP), since binding of Mant- derivatives to proteins can be monitored easily by following either the increase of the Mant-group (extrinsic) fluorescence or the (intrinsic) fluorescence quenching of tryptophan residues which is strengthened by the large overlap between the emission spectrum of tryptophan residue and the absorption spectrum of the Mant-group. In Pfk-2 there is a single tryptophan residue located at position 88, sensitive to conformational changes induced by ligands [14]. This spectral overlap gives rise to a Förster resonance energy transfer (FRET), the degree of which depends on the location of the Mant-nucleotide binding site. Binding of Mant-ATP to Pfk-2 resulted in a significant quenching of the intrinsic tryptophan fluorescence of the protein and in an enhancement in Mant-ATP fluorescence consistent with the presence of FRET between the tryptophan residue and bound Mant-nucleotide [16] (inset Fig. 5). The large emission value of Mant-ATP prevented us from using the appropriate concentration range for K_d determination, so the dissociation constant for Mant-ATP was measured using the energy transfer signal resulting from enzyme association. In these experiments, the binding of Mant-ATP to wild type and E190Q mutant Pfk-2 in the presence of Mg^{2+} or Mn^{2+} was monitored by measuring the quenching of the intrinsic fluorescence emission of the tryptophan residue (Fig. 5). The data was fitted to a hyperbolic function and the corresponding K_d values are summarized in Table 2. These values are very similar for the wild type and mutant enzymes, either in the presence of Mg^{2+} or Mn^{2+}.

Table 2
Binding constants for wild type and E190Q mutant Pfk-2 determined by EPR (A) and FRET (B).

	[A] Mn^{+2} (μM)	[B] Mant-ATP–Mg^{+2} (μM)	[B] Mant-ATP–Mn^{+2} (μM)
WT	41	38	21
E190Q	78	28	18

Discussion

Metal ions can act as essential cofactors through an indirect structural mode in which they facilitate the formation of the transition state, release of products, or through a mechanistic mode in which they or a water molecule bound to the metal play a direct role in catalysis [17]. In a previous work we proposed that a catalytic Mg^{2+} ion, additional to that present in the MgATP complex, is important for Pfk-2 enzymatic activity and that the E190 residue participates in the interaction with this ion. We here report that besides Mg^{2+}, Mn^{2+} can support Pfk-2 activity at both sites, as forming part of the phosphate-donating substrate and as an activator, being much more effective than Mg^{2+} in this later role. A dual-cation requirement has been reported for several kinases. In such cases, metal ions may accelerate reactions by lowering the chemical transition state energy through polarization of the electrophile, charge reduction, and stabilization of the leaving group [18]. In addition, ions may confer tight binding or proper orientation of the substrate's functional groups. Polymerases and nucleases using two-metal ions for catalysis share a common active site structure. In the enzyme–substrate complexes, the two-metal ions are jointly coordinated by the scissile phosphate and an active site Asp [4,19].

Pfk-2 can use either Mg^{2+} or Mn^{2+} with the same catalytic efficiency; the kinetic parameters of Pfk-2 obtained either in the presence of Mg^{2+} or Mn^{2+} show no significant differences, and Mn^{2+} can efficiently replace Mg^{2+} as a metal–nucleotide complex substrate and as an activator metal. However, when comparing the activation constants, Mn^{2+} is more effective (K_{act} 89 versus 7 μM). Also, substitution experiments show that the enzyme can use both divalent metal cations simultaneously but in different sites; that is, Mg^{2+} forming part of the metal–nucleotide complex and Mn^{2+} bound at the activating metal binding site. Thus, Mg^{2+} and Mn^{2+} can bind to both sites either when they are mixed or alone. An example for this kind of differential metal binding is phosphoenolpyruvate carboxykinase from *E. coli*, where the enzyme can bind the MgATP complex and Mn^{2+} in different sites at the same time [7]. This is not unexpected considering that Mn^{2+} can replace Mg^{2+} in the majority of enzymes where MgATP is used as substrate, since both cations have similar ligand preferences, exhibit both inner and outer sphere complexes, and usually prefer hexacoordinate ligations. However, in a previous report regarding the kinetics of Pfk-2 with Mg^{2+} and Mn^{2+} the authors claimed that the results show conclusively that there is a definite concentration dependence for MnATP rather than a catalytic effect from free Mn^{2+}, although they emphasize that under its experimental conditions it is not possible to conclude binding of free Mn^{2+} to the protein [20]. By means of EPR experiments we definitively show that free Mn^{2+} can bind to Pfk-2 and also the kinetic data demonstrate the existence of a second binding site for divalent metal cations, where Mn^{2+} can bind with higher affinity than Mg^{2+} acting as an activator metal. Nevertheless, although the activation constant for Mn^{2+} in the wild type enzyme is approximately 10-fold smaller than the activation constant for Mg^{2+}, the intracellular concentrations of both metal are very different (~10 μM for Mn^{2+} and in the millimolar range for Mg^{2+}) which raises the question if under physiological conditions Mn^{2+} binding plays a role in the regulation of Pfk-2 activity [17,21].

Although the k_{cat} values for the wild type enzyme are about the same either in the presence of Mg^{2+} or Mn^{2+}, the effects (k_{cat} or k_{cat}/K_m) of the E190Q mutation were much more severe when Mg^{2+} was the metal analyzed. Mg^{2+} binds predominantly to anionic moieties with high density of negative charge, such as carboxyl group of Asp or Glu, or O atoms negatively charged like oxygen of phosphorus group of ATP whereas Mn^{2+}, due to its borderline hard–soft character, prefers ligands such as ε-amine of Lys, NH of His or O of

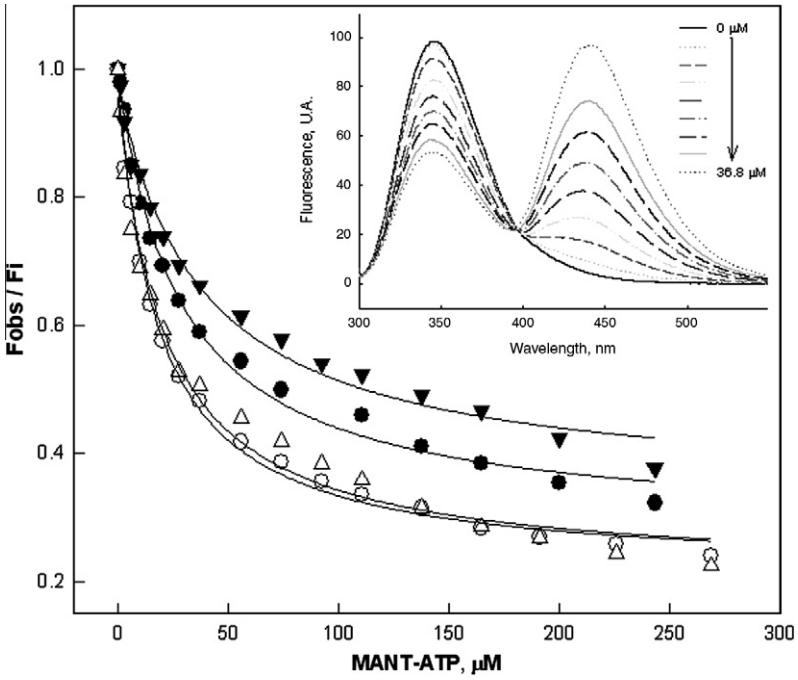

Fig. 5. Förster energy transfer between wild type and E190Q Pfk-2 Trp 88 and Mant-ATP. Quenching of intrinsic fluorescence of wild type Mant-ATP–$MgCl_2$ (●), wild type Mant-ATP–$MnCl_2$ (○), E190Q Mant-ATP–$MgCl_2$ (▼) and E190Q Mant-ATP–$MnCl_2$ (△) as a function of total Mant-ATP concentration. Inset: the emission spectrum of Pfk-2 Trp 88 was recorded upon excitation at 295 nm at different Mant-ATP concentrations (from 0 to 36.8 μM, as indicated by the arrow).

water. Although the side chain of Glu190 makes hydrogen bonds with water in the coordination sphere of one Mg^{2+}, maybe considering the borderline hard–soft character of Mn^{2+}, the presence of Gln in the mutant allows a more proper orientation of the catalytic metal when this cation is Mn^{2+} instead of Mg^{2+}.

On the other hand, the K_d value for Mn^{2+} binding to E190Q mutant obtained by EPR experiments is at most twice the K_d value for the wild type enzyme, whereas this mutant presents a severe diminution of its k_{cat} value. How can these two effects be reconciled? One possibility is conformational differences in the active form of the enzyme, since metal affinity of the mutant enzyme was performed in the absence of substrates. Alternatively, since geometric requirements for stabilization of the transition state are in general different and more stringent than those for ground states, small differences in the positioning of metal ions, water molecules, other ligands of the metal ions and other side chains, could be induced by the mutation. These perturbations might have only small destabilizing effects on the metal ion–protein interactions, but could have profound effects on the stabilization of the transition state. A similar situation was observed for bacteriophage T7 RNA polymerase where mutation of two aspartic residues provokes only a 2–5-fold increase in the K_d values for metal binding, while these mutants show no perceptible enzymatic activity [22]. Also, a similar scenario could apply to the effect of the mutation over the kinetic affinity of Mg^{2+} and Mn^{2+} in the metal-binding site of the enzyme, since the E190Q mutation increases the activation constant (K_{act}) by 1000-fold while having almost no effect on the equilibrium binding constant of Mn (approximately 2-fold change). This supports the idea that the role of the second metal is in transition state stabilization and that residue correctly positions the metal ion during catalysis.

The engagement of two Mg^{2+} ions at the active site may be a general feature of the catalytic mechanism of all the ribokinase family members. *In silico* calculations of the phosphoryl-transfer reaction of Thz kinase, a distant relative that belongs to the superfamily, lead to the conclusion that two magnesium ions seem to be important for preferential transition state stabilization and lowering the activation barrier [23]. Also, Maj et al. [9] based on the crystallographic data for adenosine kinase from *T. gondii* and *H. sapiens*, and ribokinase from *E. coli* made a composite model of the active site where two magnesium ions are present, one interacting with N and E side chains of the NXXE motif, and a second one assumed to bind to the α- and β-phosphates from ATP. Recently, determination of the high resolution structures of *S. aureus* D-tagatose-6-phosphate kinase in several ligation states shows that in the ternary complex, two Mg^{2+} ions are observed at the active site [24].

Also, the crystallographic structure of Pfk-2 in its inhibited tetrameric form (in the presence of the allosteric inhibitor MgATP and $MgCl_2$ in the crystallization set up), shows two ATP molecules and two Mg^{2+} ions [25]. Oxygens of the β- and γ-phosphates of the substrate ATP are engaged in bidentate coordination with both Mg^{2+} ions. The side chains of Glu190 and Asp166 make hydrogen bonds with water in the coordination sphere of one of these, which we call the "first Mg^{2+}" (M1). The coordination sphere of the second Mg^{2+} (M2) involves a further bidentate coordination, coming from the β- and γ-phosphates of a second ATP molecule, the allosteric ATP. The remaining coordination positions are occupied by waters, one of them interacts with the side chain of the conserved residue Asn187. Since the binding constants for Mant-ATP are essentially the same either in the wild type and the mutant enzyme, one can assume that this binding is through the metal present in M1, since free ATP is unable to bind to Pfk-2 [14]. The E190Q mutation does not affect binding of either the metal–ATP complex or the activating metal and then the role of this residue is more probably linked to the stabilization of the transition state and phosphoryl transfer.

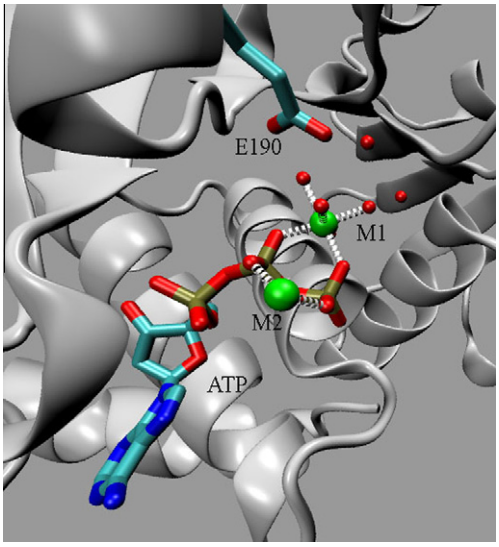

Fig. 6. Mg^{2+} coordination at the active site of *E. coli* Pfk-2 (PDB3CQD). The mutated residue E190 is labeled. Hydrogen bonding interactions are observed between M1, water molecules and phosphates groups of ATP. The side chain of E190 makes hydrogen bond with water in the coordination sphere of M1, while M2 presents coordination with the γ and β phosphates of ATP. Magnesium ions are shown in green. Water molecules are shown as red spheres. The side chain E190 and ATP are represented as sticks and colored by CPK. The figure was made using VMD [26].

From the crystallographic data of Pfk-2, it is not clear which Mg^{2+} corresponds to the catalytic one and which is complexed with the nucleotide (Fig. 6). Considering that one Mn^{2+} can bind to the enzyme in the absence of ATP (EPR studies), we propose that the catalytic metal is M1 since it coordinates with four water molecules and has two bonds with the phosphate groups of ATP, which means that this metal is able to bind to the protein and to the nucleotide. Instead, metal M2 presents a bidentate coordination with the two ATP molecules present in the structure, which makes it difficult to assume that this metal can bind to the protein in the absence of other ligands (Fig. 6).

Taking into account the crystallographic, kinetic and fluorescence data, we proposed that M2 is the metal present in the MgATP complex, that represents the true substrate, and that serves to facilitate nucleophilic attack at the γ-phosphate of the highly negative ATP substrate, while occupation of the secondary metal binding site (M1) causes an activation in k_{cat}. The E190 residue would exert its role mainly kinetically, probably stabilizing the transition state, and the geometry of metal binding to Asp190 residue may be crucial to determining the catalytic competence.

Conclusions

We here demonstrate that *E. coli* Pfk-2, member of the ribokinase family, presents a dual-cation requirement for full activity: as a metal–nucleotide complex and as activating cation. Mn^{2+} can bind to the activating site with higher affinity than Mg^{2+} and the E190 residue of the conserved NXXE motif is critical for catalytic competence. The presence of two metals at the active site may be a general feature of the catalytic mechanism of all the ribokinase family members.

Acknowledgments

This work was supported by a grant from Fondo Nacional de Desarrollo Científico y Tecnológico (Fondecyt 1070111). We are grateful to Dr. C. Aliaga from the Universidad de Santiago for the use of the EPR equipment and her support with these experiments.

JARP and CAMW are recipients of a Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) graduate fellowship.

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