Biometals (2015) 28:401–413
DOI 10.1007/s10534-015-9844-x

Role of monovalent and divalent metal cations in human ribokinase catalysis and regulation

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Received: 9 September 2014 / Accepted: 3 March 2015 / Published online: 8 March 2015

Abstract Human ribokinase (RK) is a member of the ribokinase family, and is the first enzyme responsible for D-ribose metabolism, since D-ribose must first be converted into D-ribose-5-phosphate to be further metabolized and incorporated into ATP or other high energy phosphorylated compounds. Despite its biological importance, RK is poorly characterized in eukaryotes and especially in human. We have conducted a comprehensive study involving catalytic and regulatory features of the human enzyme, focusing on divalent and monovalent metal regulatory effects. Mg^{2+}, Mn^{2+}, and Co^{2+} support enzyme activity although at different rates, with Mn^{2+} being the most effective. Analysis of the divalent cation requirement in the wild type enzyme demonstrates that in addition to that chelated by the nucleotide substrate, an activating cation (either Mn^{2+} or Mg^{2+}) is required to obtain full activity of the enzyme, with the affinity for both divalent cations being almost the same (4 and 8 μM respectively). Besides metal cation activation, inhibition of the enzyme activity by increasing concentrations of Mn^{2+} but not Mg^{2+} is observed. Also the role of residues N199 and E202 of the highly conserved NXXE motif present at the active site has been evaluated regarding Mg^{2+} and phosphate binding. K^+ (but not Na^+) and PO_4^{3-} activate the wild type enzyme, whereas the N199L and E202L mutants display a dramatic decrease in k_{cat} and require higher free Mg^{2+} concentrations than the wild type enzyme to reach maximal activity, and the activating effect of PO_4^{3-} is lost. The results demonstrated a complex regulation of the human ribokinase activity where residues Asn199 and Glu202 play an important role.

Keywords Human ribokinase · Enzyme regulation · Divalent metal cation · Manganese · NXXE motif

Introduction

D-ribose is one of the most abundant sugars used in metabolic pathways, being a basic component of important macromolecules such as DNA, RNA and ATP (Tozzi et al. 2006). Ribokinase (RK) plays a key role in metabolism phosphorylating D-ribose into D-ribose-5-phosphate (D-ribose-5-P), since D-ribose-5-P is then converted into phosphoribosyl-5-pyrophosphate (PRPP), used for synthesis of cofactors and in particular in the purine and pyrimidine synthesis. It has been reported that in pathological heart conditions, like ischemia, the availability of PRPP is limited, hindering nucleotide synthesis, and incrementing

Electronic supplementary material The online version of this article (doi:10.1007/s10534-015-9844-x) contains supplementary material, which is available to authorized users.

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cellular damage. By D-ribose supplementation, the regeneration of ATP can be accelerated and cardiac functions are restored in animals (Zimmer 1998; Balestri et al. 2007; MacCarter et al. 2009). Moreover, in humans, the supplementation of D-ribose enhanced diastolic function in congestive heart failure patients (Omran et al. 2004), and increased ischemic threshold in patients with stable severe coronary artery disease (Pliml et al. 1992). In spite of the crucial role of D-ribose in metabolism very little is known regarding human RK regulation. Kinetic studies of human RK are scarce and preliminary and although Park et al. (2007) reported the identification and preliminary characterization of the enzyme, a comprehensive study of the elements involved in its catalysis in and regulation is not addressed.

Structurally, RK present a dimeric structure (Sigrell et al. 1997, 1998) and belongs to the ribokinase superfamily (Merino and Guixé 2011), which can be subdivided into three major groups: the ATP-dependent sugar kinases, the ATP-dependent vitamin kinases, and the ADP-dependent sugar kinases. The ribokinase enzyme belongs to the ATP-dependent sugar kinases group which comprises enzymes that 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 ribose (Wu et al 1991; Bork et al. 1993).

For most kinases studied the true substrate is an ATP-divalent metal cation complex and for this reason, many phosphotransferases require divalent metal cations for their activity. Nonetheless, in some cases a divalent cation concentration that exceeds the one needed to complex ATP is required to obtain full activity, supporting the idea of an additional role for the metal cation (Knowles 1980; Yang 2008).

Ribokinase superfamily members show a strictly requirement of a divalent metal, in most cases Mg^{2+}, for their activity (Chuvikovsky et al. 2006; Maj et al. 2002; Parducci et al. 2006). Also, the requirement of a Mg^{2+} ion besides the one present in the metal–nucleotide complex has been reported for full activity of some members, such as *E. coli* Pfk-2 (Rivas-Pardo et al. 2011) and adenosine kinase (Maj et al. 2002). Also, the activity of ribokinase family members is regulated by monovalent cations and phosphate. For example, *E. coli* RK can be activated by K^+ and Cs^+, whilst Li^+ and Na^+ have no effect in RK activity (Andersson and Mowbray, 2002). It has been proposed that this activation corresponds to an allosteric mechanism, where binding of monovalent ions results in a conformational change and enzyme activation. More recently Li et al. (2012) proposed that monovalent ions shape the nucleotide binding pocket thereby enhancing substrate binding affinity as well as the catalytic activity. Nevertheless, the regulatory role of monovalent cations in ribokinase family members is not restricted to activation, since K^+ binding in Pfk-2 enhances the allosteric inhibition induced by MgATP (Baez et al. 2013). On the other hand, pentavalent ions such as phosphate, arsenate and vanadate also regulate enzyme activity. Addition of PO_4^{3-} to bacterial RK and AK from several sources correlates with an increase in the V_{max} values and a diminution in the K_m values for the ribose and adenosine substrates (Maj and Gupta 2001; Maj et al. 2002) whereas in *E. coli* Pfk-2 phosphate acts as an inhibitor (Parducci et al. 2006). It has been reported that the conserved NXXE motif, located at the active site of all the known structures of members of this superfamily, is involved in Mg^{2+} and phosphate binding (Maj et al. 2002; Parducci et al. 2006). Mutation of the E190 residue of the NXXE motif in *E. coli* Pfk-2 produces an enzyme with a greatly modified behavior respect to free Mg^{2+} concentration, which suggests the involvement of this motif in the proper orientation of a free divalent cation, not the one present in the ATP-metal complex (Parducci et al. 2006; Rivas-Pardo et al. 2011).

In this work we address a comprehensive study involving catalytic and regulatory features of the human enzyme, making emphasis in divalent and monovalent cation regulation. We demonstrated, for the first time, the presence of an activating divalent cation site where Mg^{2+} and Mn^{2+} can interact with equal affinity, besides the site where the nucleotide-complex bind and also demonstrated the presence of a regulatory inhibitory site where Mn^{2+} but not Mg^{2+} can bind. Finally, we establish that the N199 and E202 residues from the conserved NXXE motif play a key role in RK catalysis and regulation.

Materials and methods

Enzyme expression and purification

Human RK gene with a N-terminal $(His)_6$ tag was overexpressed in the *E. coli* BL21(DE3) strain

transformed with the EX-W1395-B01 expression vector (Genecopeia). Cells were cultured at 37°C in LB broth containing 100 μg/mL ampicilin until the OD at 600 nm reached 0.5. Protein expression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside, incubating at 37 °C for 4 h. Thereafter, cells were collected by centrifugation, resuspended in buffer 25 mM Tris-HCl, pH 7.8, and disrupted by sonication. The crude extract was centrifuged at 8230 g for 15 min and the supernatant was loaded in a DEAE-cellulose column, equilibrated with 4 mM K_2HPO_4 buffer, 6 mM KH_2PO_4, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 %, glycerol pH 7.4 (buffer A). After washing with 150 mL of buffer A, the protein was eluted with a linear gradient of KCl (0–0.5 M) in buffer A. The active fractions were pooled and loaded in a nickel affinity column (His Trap HP 5 mL, General Electric), equilibrated with 20 mM K_2HPO_4, 0.5 M NaCl and 20 mM imidazole, pH 7.4 (buffer B). The column was washed with 200 mL of buffer B, and the protein was eluted with 50 mL of a linear gradient of imidazole (20–500 mM) in buffer B. The active fractions were pooled, concentrated and dialyzed into 25 mM Tris buffer, pH 7.2, 2 mM $MgCl_2$. The purified enzyme was stored at −20 °C in 20 % glycerol.

Reagent concentrations

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

Activity measurements and kinetic studies

All kinetic experiments were performed on a Hewlett Packard 8453 spectrophotometer. RK activity was determined spectrophotometrically by coupling the ADP production to the oxidation of NADH at pH 7.2, using piruvate kinase (PK) and lactate dehydrogenase (LDH) as auxiliary enzymes, as described previously (Ogbunude et al. 2007), with some modifications. The standard assay reaction contained 25 mM Tris-HCl buffer, pH 7.2, 0.5 mM D-ribose, 1 mM ATP, 3 mM $MgCl_2$, 125 mM KCl, 0.3 mM phosphoenolpyruvate (PEP), 0.2 mM NADH, 0.4 U of PK, and 1.2 U of LDH, in a final volume of 0.7 mL. The reaction was initiated by adding ATP and the activity was measured at 37 °C.

The effect of divalent and monovalent cations was determined using a discontinuous assay. The ribokinase catalysis was carried out in an eppendorf test tube (reaction mixture detailed below), and the reaction was stopped with $HClO_4$ reaching a final concentration of 3 %. After 5 min in ice, the medium was neutralized with $KHCO_3$ and the test tube centrifugated at 10,000 g during 20 min at 4 °C. Then, a supernatant aliquot was taken and poured into a reaction mixture containing the reactants and enzymes of the coupled assay: 125 mM KCl, 0.3 mM PEP, 0.2 mM NADH, 2 mM $MgCl_2$, 0.4 U of PK, and 1.2 U of LDH, in a final volume of 0.7 mL. The ADP formed in the RK reaction was tittered, following the reaction by the oxidation of NADH at 340 nm.

For the divalent cation effect assays, the reaction mixture contained, in a final volume of 0.5 mL, 25 mM Tris-HCl buffer, pH 7.2, 0.5 mM D-ribose, ATP and $MeCl_2$ at variable concentrations depending on the final free metal concentration desired. The reaction was measured in the presence of Mn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+} and Cu^{2+}, as the unique metal in the reaction mixture.

For the assay of the monovalent cation effect, the reaction mixture contained 25 mM Tris-HCl buffer, pH 7.2, 0.5 mM D-ribose, 1 mM ATP and 3 mM $MgCl_2$, in a final volume of 0.5 mL. The reaction velocity was measured in the presence of KCl, NaCl, K_2HPO_4, and Na_2HPO_4, separately.

The kinetic parameters, Michaelis constant (K_m), catalytic constant (k_{cat}), and inhibition constant (K_i) were determined adjusting the initial velocity data, independent for each substrate, varying one substrate concentration keeping fixed the co-substrate concentration. The concentrations of D-ribose, MgATP and free Mg^{2+} were varied as indicated in each assay. The kinetic data were fitted to a hyperbolic model (Segel 1975):

$$v = \frac{V_{max} \times S}{K_m + S}$$

Otherwise, when substrate inhibition was observed the kinetic data were fitted to the following equation:

$$v = V_{max} \times S/\left(K_m + S + \left[S^2/K_i\right]\right)$$

The concentration of the inhibitory substrate S is varied holding constant the co-substrate concentration. Otherwise, kinetic data were fitted to hyperbolic curves using Sigma-plot software 10.0 (Systat Software, Inc.) and plots constructed using the same software.

Competitive, mixed and uncompetitive inhibition mechanism were tested for the Mn^{2+} inhibition by performing global fits using GraphPad Prism statistics software (GraphPad software, Inc.) version 5.0. Additionally, the data were fitted to linear regressions by Lineweaver–Burk double-reciprocal plots using Sigma-plot software 10.0 (Systat Software, Inc.).

Ionic equilibria

The concentration of Me^{2+} (divalent metal), ATP^{4-} and $MeATP^{2-}$, were calculated from the total concentration of the nucleotide (ATP_t) and divalent cation (Me_t) used in each assay, assuming a specific dissociation constant (K_d) value for the ionic equilibria: $MeATP^{2-} \leftrightarrow ATP^{4-} + Me^{2+}$. In the assays were Mg^{2+} was the metal used, a K_d of 45.7 μM was assumed, and when Mn^{2+} was the employed metal a K_d constant of 12.9 μM was employed. The K_d values for each $MeATP^{2-}$ ionic equilibria were taken from NIST Critically database. The ionic species present in solution were calculated by using the quadratic equation:

$$\left[MeATP^{2-}\right] = \left\{ \left([ATP]_t + [Me]_t + K_d\right) - \sqrt{\left([ATP]_t + [Me]_t + K_d\right)^2 - 4\,[ATP]_t\,[Me]_t} \right\} \Big/ 2$$

$$\left[Me^{2+}\right] = [Me]_t - \left[MeATP^{2-}\right]$$

$$\left[ATP^{4-}\right] = [ATP]_t - \left[MeATP^{2-}\right]$$

Protonated forms of the nucleotide, such as $HATP^{3-}$ or $H2ATP^{2-}$, are poorly represented at pH 7.2, so their concentration is not considered.

Site-directed mutagenesis of RK

Site-directed mutagenesis of residues Asn199 and Glu202 was carried out using the GeneTailor™ site-directed mutagenesis system (Invitrogen) using as a template the EX-W1395-B01 plasmid carrying the human RK gene as template. Three pairs of oligonucleotides were used to construct the mutants, each pair complementary. to opposite strands of the template. The underlined bases indicate the codon for the new amino acid. Only forward oligonucleotides are shown:

N199L: 5′-CAGATGTGTTCTGCTGCCTTGAAAGTGAGGCTG-3′.

E202L: 5′-GTTCTGCTGCAATGAAAGTCTTGCTGAGATTTTAAC-3′.

Electron paramagnetic resonance spectroscopy measurements

Binding affinity of human RK by Mn^{2+} was determined by electron paramagnetic resonance spectroscopy (EPR) measurements on a Bruker Biospin GmbH (model EMX 1572) at a frequency of 9.2–9.9 GHz, with the cavity thermostatized at 310 K (37 °C). Data were obtained and processed with the software WinEPR and Bruker EMX. An enzyme solution (12.4 mg/mL) of human RK was prepared in 25 mM HEPES buffer, pH 7.8, previously treated with Chelex-100, to eliminate the interfering metals. The total enzyme concentration was varied (for over a range) between 0–350 μM holding constant Mn^{2+} concentration at 50 μM. Each sample was prepared in a 60 μL volume and was incubated in the thermostatized cavity for three min, before the signal of free Mn^{2+} was recorded. Determination of the area for each measure was performed with GRAMS software. The data were fitted to a hyperbola using Sigma-Plot software 10.0 (Systat Software, Inc.).

Results

Effect of divalent cations

Figure 1a shows the effect of several divalent cations tested to support RK activity. The reaction velocity was measured in the presence of Mn^{2+}, Co^{2+}, Cd^{2+},

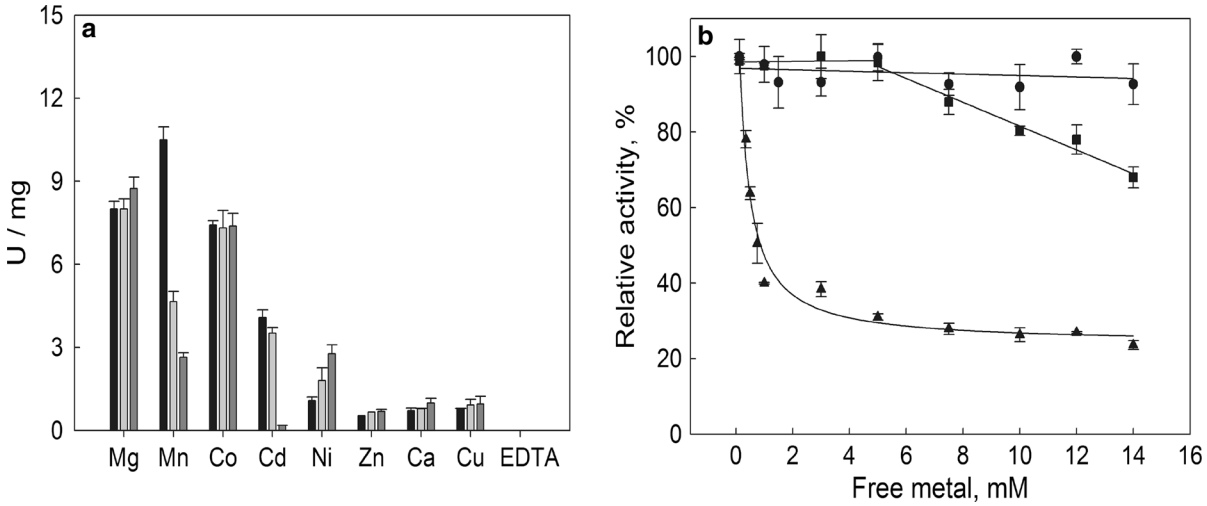

Fig. 1 Effect of divalent metal cation on the human RK activity. **a** Divalent metal cation dependence of the human RK. *Black bars* represent 0.1 mM free metal, *light grey bars* represent 1 mM of free metal, and *dark grey bars* represent 5 mM of free metal (all concentrations have been calculated). **b** Effect of free metal concentration on human RK activity. The metals assayed were: Mg^{2+} (*filled circles*), Co^{2+} (*filled squares*) and Mn^{2+} (*upright filled triangles*). The points in the graphs represent the average of three determinations ± SE. The concentration of D-ribose and Me-ATP were held constant at 0.5 mM in all the assays

Ni^{2+}, Zn^{2+}, Ca^{2+} and Cu^{2+}, as the unique metal and at different concentrations of free metal (0.1 mM, 1 and 5 mM calculated). The results show that among the cations tested only Mg^{2+}, Mn^{2+}, Co^{2+} and in a less extent Cd^{2+} and Ni^{2+} are able to support RK activity although at different rates. In presence of Mn^{2+} human RK has the highest activity, approximately 1.4 fold higher than in the presence of Mg^{2+}. Also, no activity was detected when the assay was performed in the presence of EDTA, which strongly suggests that the true substrate is the nucleotide–metal complex. However, the magnitude of the effect observed is highly dependent on the relative concentrations of metal and nucleotide used. Mg^{2+} and Co^{2+} present only minor differences in the enzyme activity under the three conditions tested; however, in the presence of a free Mn^{2+} concentration of 1 or 5 mM, a strong inhibitory effect was observed. Inhibition was also observed in the presence of free Cd^{2+} although with this cation the activity is lower than the one measured with Mg^{2+} or Mn^{2+}. To further investigate the specificity of the divalent metal cations in the inhibition of RK activity, the effect of increasing concentrations of free Mg^{2+}, Mn^{2+}, and Co^{2+} was assayed (Fig. 1b). In the concentration range tested only Mn^{2+} significantly inhibits the enzyme with an IC_{50} near 250 μM. A slightly inhibitory effect is observed over 5 mM free Co^{2+}, while Mg^{2+} did not affect the enzyme activity in the entire concentration range studied.

Since Mn^{2+} is also able to support catalytic activity we determined if there is any preference in the use of Mn^{2+} or Mg^{2+} at the active site. To address this question the kinetic parameters for RK in the presence of MnATP were determined. Table 1 show that the K_m value for the MnATP complex is about 5.5 fold lower than the one obtained in the presence of MgATP, while the specificity constant (k_{cat}/K_m) is 4.2 fold higher for Mn^{2+} compared to Mg^{2+}. These results indicate that Mn^{2+} is slightly more effective than Mg^{2+} for catalysis.

Since the participation of two Mg^{2+} ions seems to be a general feature of the catalytic mechanism of ribokinase family members we decide to gain further insights regarding this feature in human RK. For example in *E. coli* Pfk-2 Mg^{2+} plays two roles that are essential for the catalytic activity; one forming part of the metal–nucleotide complex substrate and other as an activating ion (Parducci et al 2006; Rivas-Pardo et al. 2011). However, in order to elucidate the different roles that the metal can play in enzymes that use a metal–nucleotide as a substrate, the concentration of each species has to be calculated considering

Table 1 Kinetic parameters of human RK using MeATP substrate in complex with Mg^{2+} or Mn^{2+}

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_i (mM)
MgATP	160 ± 40	9.8 ± 0.8	0.06	16.9 ± 2.6
MnATP	29 ± 6	7.3 ± 1.6	0.25	10.3 ± 1.6

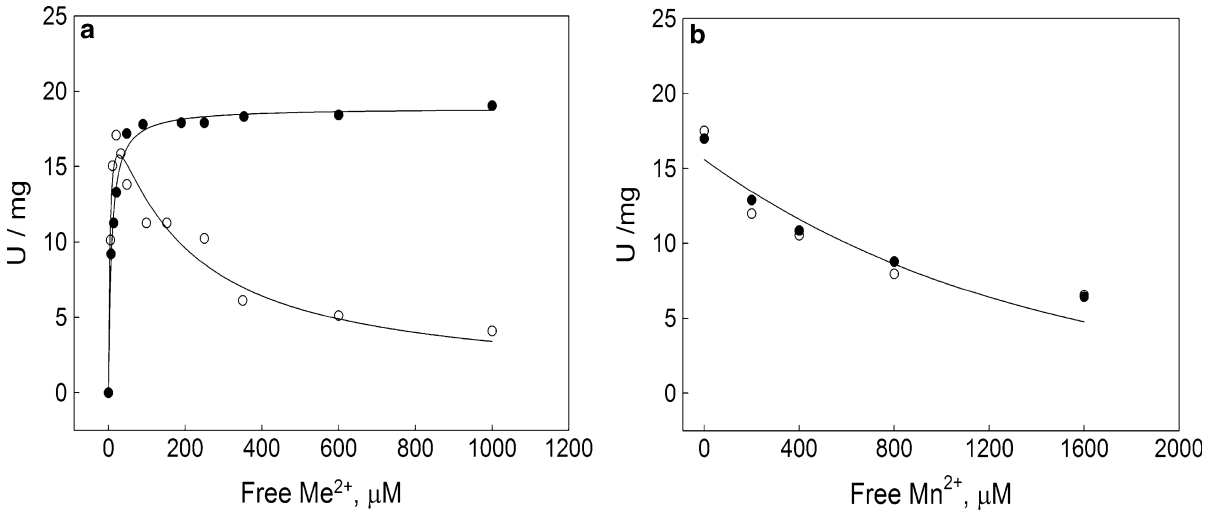

Fig. 2 Effect of free Mg^{2+} and free Mn^{2+} on the human RK activity. **a** The reaction velocity was measured as a function of free Mg^{2+} concentration (*filled circles*), at saturating concentrations of the MgATP complex (1280 μM) and as a function of free Mn^{2+} concentration (*empty circles*), at saturating concentrations of the MnATP complex (232 μM). **b** The reaction velocity was measured as a function of free Mn^{2+} concentration at saturating concentrations of the MgATP complex (1280 μM), and at free Mg^{2+} concentrations of 1.5 mM (*empty circles*) and 3.0 mM (*filled circles*). For all the assays, the concentration of D-ribose was held constant at 0.5 mM. Each point represents the mean of two determinations

the different equilibriums between the several possible ionic species present at the pH employed (Sigel 2004). For this reason we assay the activity of human RK as a function of the free metal concentration (Mg^{2+} or Mn^{2+}) at a saturating concentration of the metal nucleotide complex (~8 times the respective K_m). The results show that at the lowest free Mg^{2+} or Mn^{2+} concentration assayed (8–10 μM), RK activity increases reaching the same V_{max} (20 U/ mg with Mn^{2+} and 19 U/ mg with Mg^{2+}), being the K_{act} for Mn^{2+} and Mg^{2+} about the same (4 and 8 μM, respectively). Nonetheless, above 30 μM a significant inhibition was observed only in the presence of Mn^{2+} (Fig. 2a). It is important to stress out that for this kind of experiments it is impossible to perform experiments where the concentration of the free metal is zero due to the finite value of the dissociation constant for the formation of the metal–nucleotide complex and because a very low free Mg^{2+} concentration implies concomitantly an increase in the ATP^{4-} concentration. This is important, considering that ATP^{4-} inhibits human RK activity (Fig. S1). However, the ribokinase activity declines with the decrease of the free metal concentration (either Mg^{2+} or Mn^{2+}) and the trend points to zero activity when the concentration of free metal approaches zero, allowing us to infer that this divalent cation is essential for enzyme activity.

The results can be interpreted as the presence of three metal cation binding sites in human RK, one activating where Mg^{2+} and Mn^{2+} can bind with almost the same affinity (K_{act} 8 and 4 μM, respectively), one for the metal–nucleotide complex and other inhibitory where only Mn^{2+} can bind. To further explore the specificity of the inhibitory site we assay the RK activity in the presence of a saturating concentration of MgATP (8 times K_m) at two free Mg^{2+} concentration (1.5 and 3.0 mM). Figure 2b shows that irrespective of the high free Mg^{2+} concentration employed, Mn^{2+} is able to inhibit RK activity with the same affinity, which supports the presence of an inhibitory site for Mn^{2+} and the fact that both metal cations do not compete

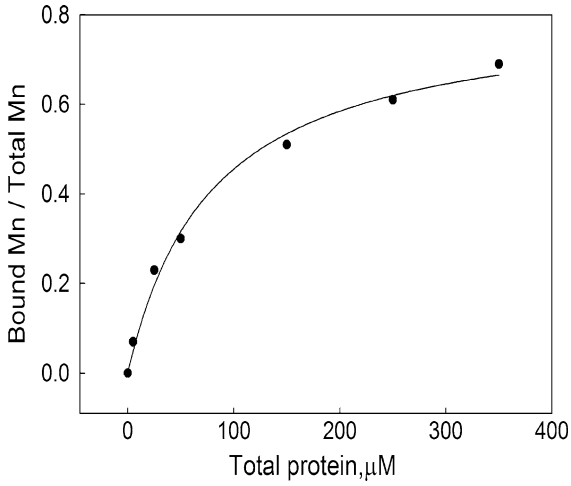

Fig. 3 Binding of Mn^{2+} to human RK. The binding of Mn^{2+} was measured at 37 °C in the presence of 25 mM HEPES, pH 7.8, by EPR spectroscopy, as described in materials and methods. The Mn^{2+} concentration was held constant at 50 μM and the concentration of protein was varied between 0 and 350 μM

for it. The regulatory nature of the site for Mn^{2+} was also reinforced by the fact that free Mn^{2+} behaves as an uncompetitive inhibitor respect to the Mn-ATP concentration, which implies that the inhibitor bind to the enzyme only after the substrate binds (Fig. S2).

To determine the binding affinity and the number of divalent metal cations bound to human RK we took advantage of Mn^{2+} as a paramagnetic probe. Addition of RK to Mn^{2+} solutions causes a diminution of the Mn^{2+} EPR signal compared to a standard solution in the absence of protein (not shown). This diminution is consequence of a change in the electronic environment of the Mn^{2+} ion due to the interaction between the Mn^{2+} and the protein. The binding isotherm for RK constructed from the EPR data is shown in Fig. 3 from which one Mn^{2+} binding site (stoichiometry of 0.8) with a K_d of 50 μM in the absence of other ligands is obtained.

Effect of monovalent cations and phosphate

Monovalent cations and phosphate have also been described as modulators of the activity of ribokinase family members, being either activators, as in the case of ribokinases from *E. coli* (Andersson and Mowbray 2002) and *S. aureus* (Li et al. 2012) and in AK from different sources (Maj et al. 2002), or acting like inhibitors as in *E. coli* Pfk-2 (Parducci et al. 2006; Baez et al. 2013). Since the crystal structure of human RK is available we evaluated if human RK shares a monovalent binding site with other ribokinase members. The superimposition between the *H. sapiens* RK structure (PDB: 2FV7), the *E. coli* RK structure (PDB: 1GQT), and the *E. coli* Pfk-2 (PDB: 3UMO), shows that Na^+, Cs^+ and K^+ ions bind to the same site, suggesting that a monovalent cation binding site is a conserved property in several ribokinase family members (Fig. S3). In order to determine the effect of monovalent cations on human RK and their interplay with phosphate we assayed the activity in the presence of only K^+ or Na^+ or in a combination with phosphate as K_2HPO_4 or Na_2HPO_4 (Fig. 4). In all these cases a discontinuous assay was employed in order to avoid the interference of any compound present in the coupled assay. When the activity was assayed in the presence of K^+ or Na^+ as the unique cation present, activation was observed only with K^+ (twofold at 100 mM KCl). Although both ions are members of alkali metals and have similar ionic radii, Na^+ has no influence in RK activity. When activity is measured in the presence of K_2HPO_4 an additional activating effect was observed compared to the activity measured in the presence of KCl. In the

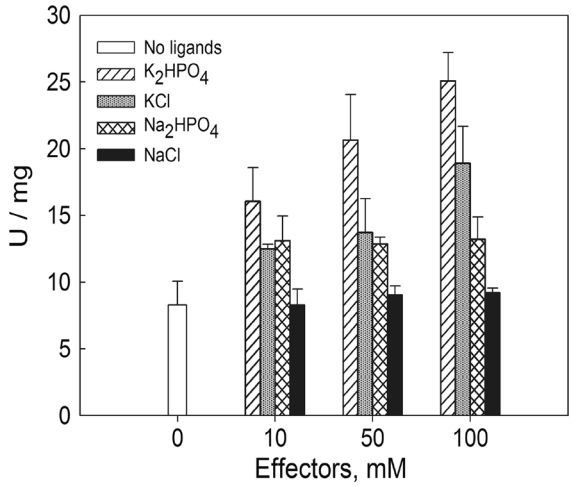

Fig. 4 Effect of monovalent cations on the human RK activity. The RK activity was determined in presence of increasing concentrations of four effectors: K_2HPO_4, KCl, Na_2HPO_4 and NaCl. For all the assays, the concentration of D-ribose was held constant at 0.5 mM and for MgATP at 1 mM. Free Mg^{2+} concentration was held constant at 2 mM. Each *bar* in the graph represents the mean of three determinations ± SE

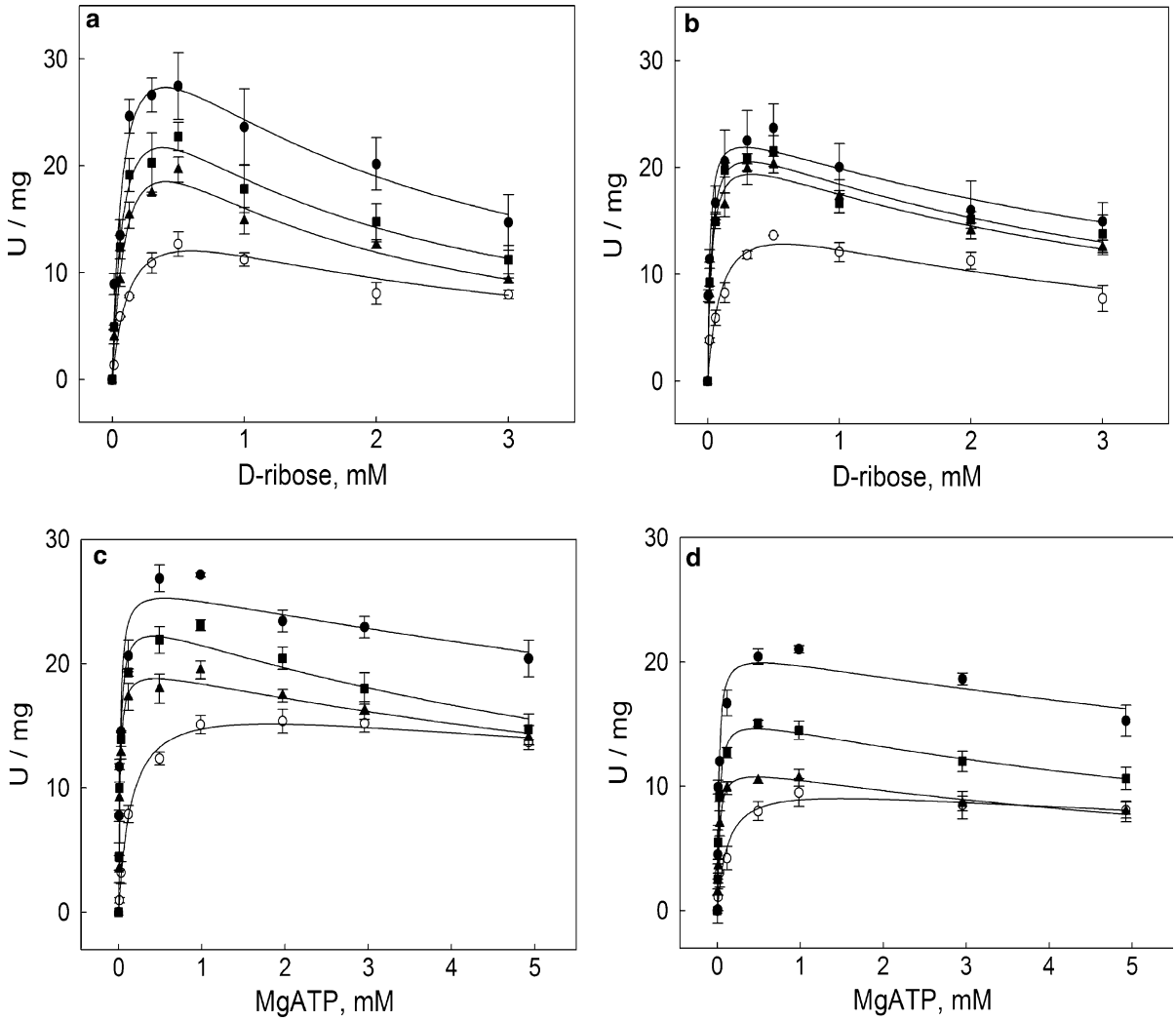

Fig. 5 Effect of K_2HPO_4 on the activity of human RK with varying D-ribose (**a** and **b**) and MgATP(**c** and **d**) concentrations. In **a** MgATP concentration was held constant at 0.5 mM and in **b** at 5 mM. In **c** the D-ribose concentration was held constant at 0.5 mM and in **d** at 2 mM. For all the assays, free Mg^{2+} concentration was held constant at 2 mM. The K_2HPO_4 concentrations used were: 0 mM (*unfilled circles*), 25 mM (*upright filled triangles*) 50 mM (*filled squares*) and 100 mM (*filled circles*). Each point in the graphs represents the mean of three determinations ± SE

presence of Na_2HPO_4, only a slight activation was seen, attributable to PO_4^{3-} activation, since 100 mM Na_2HPO_4 produces an activation of 1.6 fold. The activating effect observed in the presence of K_2HPO_4 is a consequence of the additive effect of phosphate and K^+ separately, since no evidence of synergisms between them is observed. Since the enzyme is still active in the absence of K^+ this situation would correspond to nonessential activation. The results demonstrate that activation of human RK by K_2HPO_4 is a consequence of a dual effect, since both the PO_4^{-3} and K^+ ions are able to activate the enzyme separately.

Role of the NXXE conserved motif in metal and phosphate regulation

The NXXE motif is considered a signature among the ribokinase family, and it has been involved in the mechanism by which metal cations and phosphate affect enzyme activity (Maj et al. 2002; Parducci et al. 2006). Since human RK activity is regulated by metals and phosphate we addressed the involvement of residues of this motif in this behavior. For this purpose we performed site directed mutagenesis of the N199 and E202 residues

of the NXXE motif by leucine. First we evaluated the effect of K_2HPO_4 in the kinetic parameters of wild type RK. Figure 5 shows that the enzyme is activated by K_2HPO_4. Although enzyme activity is observed in the absence of the effector, in disagreement with that reported before, this could be attributable to the presence of PEP in the assay mixture, considering that this compound can activate ribokinase (Park et al. 2007). Since human RK is inhibited by D-ribose and by MgATP kinetic parameters were determined at two concentrations of the co-substrate. In the absence of K_2HPO_4 the kinetic parameters for D-ribose are essentially the same at the two MgATP concentrations employed (0.5 and 5 mM), although at 0.5 mM MgATP the activating effect of K_2HPO_4 is more pronounced with a twofold increment in k_{cat} and a twofold diminution in the K_m values for D-ribose (Table 2). Additionally, kinetic parameters for MgATP were determined at two fixed D-ribose concentrations (0.5 and 2 mM) with K_2HPO_4 activation being similar at both co-substrate concentrations (Table 3). Nevertheless, the ability of K_2HPO_4 to relieve substrate inhibition of the enzyme was only detectable for D-ribose inhibition at both co-substrate concentrations with an increment in the K_i values near 1.7 fold in the presence of 100 mM of K_2HPO_4, compared to the value obtained in the absence of the effector. The opposite effect was observed for MgATP inhibition where addition of K_2HPO_4 provokes a decrease in the K_i values for the metal–nucleotide complex. Kinetic parameters of the NXXE mutants show that the replacement of the N199 residue by leucine provokes an increase in the K_m for MgATP by about 68-fold, a decrease in the k_{cat} between 573 and 1036 fold and almost no change in the K_m for D-ribose. The E202L mutant shows an increase in the K_m for MgATP of 52 fold, and a decrease in the k_{cat} value of around 200 fold (Table 4). Unlike the N199L mutant, substrate inhibition by D-ribose is observed in the E202L mutant, with a K_i value very similar to the one observed for the wild type enzyme (not shown). In both mutants the activation effect elicited by phosphate is absent and also the behavior of the enzyme respect to free Mg^{2+} concentration is greatly affected, since enzyme activity was only observable at a free Mg^{2+} concentration of 30 mM. For both mutants the CD spectra is superimposable with the one recorded for the wild type enzyme indicating absence of

Table 2 Kinetic parameters of wild type RK, varying D-ribose concentration

K_2HPO_4 (mM)	K_m D-ribose (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_i (mM)	K_m D-ribose (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	K_i (mM)
MgATP 0.5 mM					MgATP 5 mM			
0	174 ± 12	11.04 ± 0.66	0.06	1.7 ± 0.07	120 ± 10	10.36 ± 0.38	0.09	3.3 ± 0.5
25	99 ± 8.7	14.89 ± 0.68	0.15	1.8 ± 0.0 2	31 ± 5	12.68 ± 0.56	0.41	3.5 ± 0.2
50	80 ± 5	18.81 ± 2.16	0.24	1.8 ± 0.04	27 ± 6	13.29 ± 0.36	0.49	3.6 ± 0.4
100	80 ± 6	21.37 ± 1.11	0.27	1.9 ± 0.10	17 ± 4	13.51 ± 1.74	0.79	5.6 ± 0.9

For all the assays, free Mg^{2+} concentration was held constant at 2 mM

Table 3 Kinetic parameters of wild type RK, varying MgATP concentration

K_2HPO_4 (mM)	K_m MgATP (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_i (mM)	K_m MgATP (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_i (mM)
D-ribose 0.5 mM					D-ribose 2 mM			
0	162 ± 40	9.81 ± 0.80	0.06	16.0 ± 2.6	109.3 ± 322	5.73 ± 0.71	0.05	15.7 ± 0.9
25	20 ± 6	11.07 ± 0.35	0.55	12.5 ± 0.6	23.1 ± 3	6.51 ± 0.25	0.28	9.5 ± 1.1
50	18 ± 3.7	13.03 ± 0.31	0.72	11.9 ± 0.7	22.3 ± 6	8.83 ± 0.25	0.40	8.5 ± 0.4
100	18 ± 6	15.33 ± 0.15	0.85	10.5 ± 0.8	19.7 ± 5	12.5 ± 30.30	0.64	7.1 ± 0.2

For all the assays, free Mg^{2+} concentration was held constant at 2 mM

Table 4 Kinetic parameters for wild type RK, N199L and E202L mutants

Enzyme	K_m D-ribose (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	K_m MgATP (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)
RK wild type	120 ± 10	10.36 ± 0.38	0.09	109.3 ± 22	5.73 ± 0.71	0.05
RK N199L	118 ± 30	$0.01 \pm 1 \times 10^{-3}$	0.08×10^{-3}	7480 ± 1490	$0.01 \pm 2 \times 10^{-3}$	1.34×10^{-6}
RK E202L	80 ± 20	$0.04 \pm 6 \times 10^{-3}$	0.5×10^{-3}	5730 ± 1120	$0.04 \pm 2 \times 10^{-3}$	6.98×10^{-6}

significant alterations in secondary structure (data not shown).

These results establish for the first time that human ribokinase requires a dual-cation requirement for full activity; as a metal–nucleotide complex and as an activating cation and support the role of the NXXE motif residues in phosphate regulation and in binding of the activating Mg^{2+}.

Discussion

Metal ions can play different roles in enzyme catalyzed reactions; 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. Recently, it has been reported that catalytically essential divalent cation binding residues may be targetable using drug-like small molecules in enzymes associated with cancer risk, representing a potential cancer therapeutic strategy (Deng et al. 2015).

Ribokinase superfamily members have a strict requirement of a divalent metal cation for catalysis. Moreover, the regulation of the activity of some members is strongly dependent on the amount of free divalent metal cation present which can act as inhibitor or activator (Maj et al. 2002; Parducci et al. 2006; Merino et al. 2012). Besides Mg^{2+}, human ribokinase can employ Mn^{2+} and Co^{2+} and in a less extent Cd^{2+} and Ni^{2+} for catalysis. The interchangeability between Mg^{2+} and Mn^{2+} is not unexpected considering that Mn^{2+} can replace Mg^{2+} in most of enzymes that employ MgATP as substrate (Maguire and Cowan 2002) and considering that both cations have similar ligand preferences and usually prefer hexacoordinate ligations. The use of cobalt in phosphotransferase reactions is quite uncommon, although the ADP-dependent sugar kinases from archaea are able to use this ion even better than Mg^{2+} or Mn^{2+} (Merino et al. 2012). In these archaeal enzymes it seems that the only considerations for metal assisted catalysis are related to the ionic radii and coordination geometry of the cations. On the other hand, we demonstrated that two metal cations are required for full ribokinase activity: one forming the metal nucleotide complex and one activating. In the activating site Mg^{2+} and Mn^{2+} can bind with a similar affinity being the K_{act} equal to 8 and 4 μM respectively, while at the active site binding of MnATP is more effective since the k_{cat}/K_m value is four times higher than the one obtained for the MgATP complex. Although the activation constants for Mg^{2+} and Mn^{2+} are rather the same, the intracellular concentrations of both metals are very different. It has been reported that the intracellular Mn^{2+} concentration is around 10 μM (Oulhote et al. 2014) while the intracellular total magnesium concentration in a typical cell is estimated to be 30 mM, with the concentration of free magnesium being 0.3–0.5 mM (Maguire and Cowan 2002). This means that under physiological conditions Mg^{2+} would be the main regulator at the activating site. Other scenario has to be considered at the inhibitory site, where only Mn^{2+} can exert an effect. The K_i for this site is around 200 μM which raises the question if physiologically this inhibition plays a role; however one has to consider that intracellular concentrations determinations represent a rough average and not excludes higher local concentrations of this metal.

A dual cation requirement has been reported for several kinases and also for ribokinase family members. In Thz kinase, a member of the ribokinase superfamily, *in silico* studies lead to the conclusion that two magnesium ions seem to be important for preferential transition state stabilization and lowering the activation barrier (Dyguda et al. 2004). Also, determination of the crystal structure of *S. aureus* D-tagatose-6-phosphate kinase shows the presence of two Mg^{2+} ions at the active site in the ternary complex

(Miallau et al. 2007). Moreover, a composite model of the active site based on the crystallographic data for adenosine kinase from *T. gondii* and *H. sapiens*, and ribokinase from *E. coli* suggest the presence of two magnesium ions; 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 (Maj et al. 2002). In the crystallographic structure of Pfk-2 in its inhibited tetrameric form, two ATP molecules and two Mg^{2+} ions are observed. Site directed mutagenesis of the NXXE motif of this enzyme led to the authors to propose that the Mg^{2+} ion (called M1) whose water coordination sphere make hydrogen bonds with the side chains of Glu190 and Asp166 of the NXXE motif would corresponds to the catalytic metal (Rivas-Pardo et al. 2011). In the ribokinase structure only one Mg^{2+} ion is observed, however, a structural superimposition of these two highly homologs enzymes, *H. sapiens* RK structure (PDB: 2FV7) and *E. coli* Pfk-2 structure (PDB: 3UMO), indicate that the Mg^{2+} ion observed in the ribokinase structure would correspond to the catalytic one and not the one forming the metal–nucleotide complex (Fig. S4). This idea is supported by the fact that mutants of the NXXE motif require a very high free Mg^{2+} concentration (30 mM) to obtain activity, which in turn can be interpreted as a dramatic loss of affinity of the activating site for Mg^{2+} in these mutants. This situation would be rather different than the one reported for *E. coli* Pfk-2 where the E190Q mutation does not affect binding of either the metal-ATP complex or the activating metal and then in this case the role of this residue can be more probably linked to the stabilization of the transition state and phosphoryl transfer (Rivas-Pardo et al. 2011). On the other hand, EPR studies show that only one Mn^{2+} ion binds to ribokinase in the absence of other ligands, with a K_d of 50 μM. Since the K_m for the Mn-ATP complex is 29 μM while the K_{act} for this ion is only 4 μM it would be possible that the metal detected in the EPR studies corresponds to the one present in the complex although the nucleotide is absent. The ribokinase activity is also inhibited by high concentrations of free Mn^{2+} as was the case for the ADP-dependent kinases of this superfamily, probably due to binding of this metal to a regulatory inhibitory site (Merino et al. 2012). In all the conditions assayed the K_i for the free Mn^{2+} is about 200 μM, much higher than the one detected in the EPR experiments. In addition the mechanism of this inhibition corresponds to an uncompetitve inhibition with respect to the metal–nucleotide complex, which implies that the inhibitory free Mn^{2+} binds to the enzyme only after substrate binding, which rules out that the binding site determined by EPR corresponds to the inhibitory site. Mutations of residues of the NXXE motif produced enzymes with dramatic alteration in its kinetic parameters, compared to the wild type RK. The most profound effect on the catalytic constant was seen in the N199L mutant (1036 fold diminution). The crystal structure of human RK in the presence of Mg^{2+} and ADP inside the active site shows both ligands in a position close to the NXXE motif which make feasible the interaction between them and the Asn199 and Glu202 residues. The uncharged polar side chain of residue Asn199 interacts with ADP through a hydrogen bond with the O1 atom of the β phosphate and also can interact with the Mg^{2+} ion through two indirect hydrogen bonds mediated by water molecules. When N199 was replaced by a nonpolar and similar size residue such as Leu, which is unable to form this contact, the k_{cat} diminishes by 1036 fold and the Km for MgATP increases near 70 fold. The carboxyl group of the side chain of E202 interacts with ADP through two indirect hydrogen bonds; O1 of the COO- group interact with the bridging oxygen between α and β phosphates, mediated by a water molecule, and O2 of COO- form a hydrogen bond with the O2 atom of the β phosphate of ADP, also mediated by a water molecule. This residue can interact with Mg^{2+} through two indirect hydrogen bonds, mediated by two water molecules, since the distance between this residue and the water molecules is ∼3Å, and the distance between the water molecules and the Mg^{2+} ion is ∼2.6 Å. Replacement of this residue by Leu precludes these interactions. This mutant also presents a severe diminution in k_{cat} (259 fold) and an increase in the K_m for MgATP (52 fold). The lack of phosphate activation in these mutants demonstrated that residues of the NXXE motif are also implicated in phosphate binding. A similar situation has been described in mammalian AK where mutants of the NXXE motif require much higher concentrations of Mg^{2+} for optimal activity and lack phosphate activation (Maj et al. 2002).

Other regulators described for members of the ribokinase family are monovalent cations such as Na^+, Cs^+ and K^+ (Andersson and Mowbray 2002; Baez et al. 2013) and phosphate and other pentavalent ions

(Maj et al. 2002; Parducci et al. 2006). The structural superimposition of the *H. sapiens* RK (PDB: 2FV7), the *E. coli* RK (PDB: 1GQT), and the *E. coli* Pfk-2 (PDB: 3UMO) structures, demonstrated that Na^+, Cs^+ and K^+ ions bind to the same site, suggesting that a monovalent cation binding site is a conserved property in several ribokinase family members. Our results indicate that PO_4^{3-} and K^+ are able to activate the ribokinase enzyme, alone or in combination, with no synergism between them, while Na^+ has no effect on enzyme activity. The intracellular concentration of K^+ fluctuates between 100 and 150 mM (Andersson and Mowbray 2002; Maguire and Cowan 2002), which is as 10 times higher than Na^+ concentration (between 5 and 15 mM) (Andersson and Mowbray 2002; Maguire and Cowan 2002). This indicates that K^+ is most probably the physiologically relevant regulator of RK activity. Since these experiments were done at substrates concentrations that give 80–85% of the maximal velocity, K^+ binding should exert its effect mainly by affecting the catalytic constant.

It has been proposed that in ribokinase from *E. coli* and *S. aureus* activation by monovalent cations proceeds by conformational changes that organize the nucleotide binding pocket which results in anion hole formation (Andersson and Mowbray 2002; Li et al. 2012). Considering the highly structural identity between the *E. coli*, *S. aureus*, and *H. sapiens* ribokinases, it is likely that a similar conformational change be responsible for the activation effect provoked by K^+ in human RK.

Acknowledgments The authors would like to thank to Dr. C. Aliaga (Universidad de Santiago) for using the EPR equipment and her support with our experiments. This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1110137, Chile) Grant.

Conflict of interest The authors declare that they have no conflicts of interest in the research.

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