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Identification and characterization of human ribokinase and comparison of its properties with *E. coli* ribokinase and human adenosine kinase

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Abstract **The gene responsible for ribokinase (RK) in human/eukaryotic cells has not yet been identified/characterized. Blast searches with *E. coli* RK have identified a human protein showing significant similarity to the bacterial RK. The cDNA for this protein was expressed in *E. coli* and the recombinant protein efficiently phosphorylated ribose to ribose-5-phosphate using ATP, confirming its identity as RK. In contrast to ribose, the enzyme exhibited very little to no phosphorylation of D-arabinose, D-xylose, D-fructose and D-galactose. The catalytic activity of human RK was dependent upon the presence of inorganic phosphate, as observed previously for *E. coli* RK and mammalian adenosine kinases (AK). A number of activators and inhibitors of human AK, produced very similar effects on the human and *E. coli* RKs, indicating that the catalytic mechanism of RK is very similar to that of the AKs.**

Keywords: Ribokinase; Adenosine kinase; PfkB family of proteins; Phosphate dependency; Inhibitors and activators of adenosine kinase and ribokinase; Ribose metabolism

1. Introduction

Ribokinase is a carbohydrate kinase, which catalyzes the phosphorylation of ribose to ribose-5-phosphate (R-5-P). Exogenous ribose has shown beneficial effects in a number of studies. It has been reported to enhance cardiac and muscular performance and to accelerate the repletion of ATP in rat and canine myocardium as well as in isolated cardiomyocytes and endothelial cells [1–4]. Ribose addition also helps maintain higher levels of ATP in rat heart and dog kidney during transplantation experiments [5], and it led to improvement of neurological symptoms in a patient with adenylosuccinase deficiency [6]. In order for ribose to be incorporated into ATP or other high energy phosphorylated derivatives, ribose must first be converted into ribose-5-phosphate [1]. Hence, it is of much interest to identify and characterize the enzyme responsible for this critical first step.

The enzyme RK belongs to the PfkB family of carbohydrate kinases [7], which includes enzymes such as adenosine kinase (AK), inosine-guanosine kinase, fructokinase, 1-phosphofructokinase, and 6-phosphofructokinase minor. The overall sequence identity between PfkB family members is less than 30%, but remarkably high structural similarity is seen between RK [8] and AK [9]. Most of the biochemical and structural studies on RK have been carried out using enzyme from *E. coli* cells [10–12]. Although RK activity was first described in calf liver in 1956 [13], very little work on characterizing this activity in mammalian systems has been carried out. The identification of the gene encoding for human/eukaryotic RK, or detailed characterization of this activity in mammalian systems has not yet been reported. This communication describes, for the first time, definitive identification of the human RK gene and biochemical characterization of its encoded protein product. The substrate specificity of the enzyme as well as the effects of a number of activators and inhibitors of human AK on both human and *E. coli* RK were studied. Our results indicate that the catalytic mechanism of both human and *E. coli* RK is very similar to that of AK.

2. Methods and materials

2.1. Materials

D-[1-^{3}H] ribose (20 Ci/mmol), D-[1-^{3}H] arabinose (20 Ci/mmol), D-[^{14}C] fructose (300 mCi/mmol), D-[6-^{3}H] galactose (40 Ci/mmol), and D-[1-^{3}H] xylose (15 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). ATP was purchased from Pharmacia Biotech (Canada). All other chemicals were of analytical reagent grade.

2.2. Cloning, expression, and purification of E. coli and human RK

The *E. coli* RK gene (accession no. NC_000913) was amplified by PCR from the *E. coli* genomic DNA using specific oligonucleotide primers and cloned into pET22b expression vector. Human RK was identified by Blastp searches and its sequence was aligned with that of *E. coli* and other ribokinases. For expression of human RK cDNA, total mRNA from human HT1080 cells was prepared and reverse transcribed using reverse transcriptase. Using this cDNA, the cDNA for human RK was PCR amplified using specific primers based on the predicted start and end positions of the RK protein. After checking the sequence for its accuracy, it was cloned into the pET15b expression vector. For expression studies, the vector constructs were transformed into *E. coli* BL21 cells, which were induced with 0.1 mM IPTG for 6 h at room temperature. The $(His)_6$-tagged protein was purified to >90% purity by means of nickel affinity chromatography. Further purification was carried out by means of gel-filtration chromatography and the purified enzyme was stored at −20 °C in 10% glycerol.

2.3. Activity assay

RK activity was measured by a radiochemical method using D-[1-^{3}H] ribose, as described previously [11]. Assays were performed at 37 °C in 20 mM Tris–maleate buffer (pH 7.0) containing 125 mM KCl. The kinetic data were fitted to the equation:

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$v_0 = V_{max}/(K_m + A + (A^2/K_i))$

which describes the rate of a bisubstrate reaction where the concentration of an inhibitory substrate A is varied at a fixed concentration of the other substrate [14,15]. For the K_m determination of ribose, the concentration of ATP was held fixed at 5 mM, whereas the K_m determination of ATP was carried out in the presence of 2 mM ribose. Saturating concentrations of ribose were avoided in these experiments due to significant enzyme inhibition by the substrate at high concentration. In all cases, the concentration of free magnesium was fixed at 2 mM, which produced maximal enzyme activity. Magnesium also showed inhibitory effects on the enzyme activity at higher concentrations (not shown).

3. Results

3.1. Identification of the human RK gene and expression of the corresponding cDNA

The gene responsible for RK activity and the corresponding protein has not yet been identified or biochemically characterized in human and other eukaryotic cells. Our Blastp searches of the NCBI nr database with bacterial (*E. coli*) RK sequence identified a protein in the human genome that has been annotated as ribokinase (protein length 322 aa, accession number NP_071411){Lander, 2001 276 /id}. Significant blast hits with low E values ($<1e^{-25}$) were also observed for a variety of other eukaryotic organisms including rat, mouse, dog, chicken, frog, *Drosophila*, nematode, *Leishmania*, *Trypanosoma*, *Entamoeba*, *Giardia*, various plants (e.g. *Arabidopsis thaliana*) and fungi (e.g. *Saccharomyces cerevisiae*), etc. Interestingly, when Blast searches were performed with either the human (or mouse) RK sequence (instead of the *E. coli* RK sequence), the blast hits with the lowest E values were for the animal homologs followed by those of the bacterial homologs. The hits from plants and fungi had much higher E values in comparison to the bacterial homologs indicating that they were more distantly related to the human or animal homologs in comparison to the bacterial proteins.

Table 1 presents a pair-wise sequence identity/similarity matrix of RK sequences from representative prokaryotic and eukaryotic species. As seen, the human RK shows a much higher degree of sequence identity to the RK sequences from protist species (columns F and G) and various bacteria (columns H and I) as compared to that observed for the homologs from plants and fungi (columns D and E). These results are unexpected as the fungi and plants are more closely related to the animals than the bacteria [16]. The human RK also shows significant sequence similarity to the human as well as other eukaryotic adenosine kinases. However, this sequence similarity is lower than that seen for any of the ribokinases. In Fig. 1A, a multiple sequence alignment of RK from some representative prokaryotic and eukaryotic species is presented. There are several short stretches that are conserved in all of the ribokinases, and overall about 12–15% of the residues in divergent species are completely conserved. The human RK gene, which is located on chromosome 2 (locus NM_022184), is approximately 110 kb in length and is comprised of 8 exons of varying lengths (Fig. 1B).

The PfkB family of proteins include many carbohydrate kinases including RK and AK [7]. Based upon the observed sequence similarity between *E. coli* RK and the human protein (Table 1), one cannot be certain that the human protein in question corresponds to ribokinase and not some other sugar kinase. The lower similarity of the human protein to fungi and plant homologs as compared to bacteria highlights this problem. To determine whether the protein product of this putative human gene indeed corresponds to RK, the cDNA for this gene was PCR amplified from human HT1080 cells and expressed in *E. coli* cells. In cells transformed with the recombinant plasmid, a protein of the expected molecular mass (~30 kDa) was expressed at high level upon induction with IPTG. This protein was purified to near homogeneity by nickel-affinity and gel-filtration chromatography.

3.2. Biochemical characterization of human ribokinase

The protein purified as outlined above was shown to carry out efficient phosphorylation of ribose to ribose-5-phosphate in presence of ATP (under standard conditions containing 2 mM ribose, 5 mM ATP and 10 mM Pi) confirming its identity as RK. Although ribose metabolism and transport has

Table 1
Pair-wise identity/similarity between RK and AK sequences from different species

Source of sequence	% Identity or similarity in amino acid sequences[a]											
	A	B	C	D	E	F	G	H	I	J	K	L
RK												
(A) *H. sapiens*		69.6	42.8	26.1	29.1	41.9	37.1	39.1	35.5	21.0	23.5	24.1
(B) *X. tropicalis*	81.4		44.1	25.9	27.9	39.8	37.1	39.0	31.9	21.7	23.4	22.5
(C) *D. melano.*	60.9	60.3		26.9	30.6	36.2	31.5	39.4	36.0	24.1	26.1	22.9
(D) *A. thaliana*	41.6	39.3	37.7		28.3	24.0	26.4	27.4	29.7	21.0	23.3	22.4
(E) *S. cerevisiae*	43.4	42.6	44.6	41.5		28.0	27.4	31.9	30.7	26.9	25.3	22.1
(F) *T. cruzi*	56.9	54.5	52.3	39.2	45.8		38.7	39.4	36.1	23.5	26.9	23.7
(G) *En. histolytica*	55.3	54.7	48.3	40.7	43.5	55.0		30.3	31.3	20.7	19.3	21.8
(H) *B. cepacia*	50.9	52.7	51.4	41.4	46.4	51.5	49.5		42.5	22.0	21.7	27.5
(I) *E. coli*	53.2	48.0	48.8	45.7	48.9	51.8	49.5	55.3		23.2	22.6	20.3
(J) *H. sapiens*	37.8	40.0	40.1	37.4	39.5	35.9	36.6	34.6	40.1		57.2	39.9
AK												
(K) *A. thaliana*	41.1	40.0	41.6	38.1	40.8	40.2	35.9	37.4	41.2	74.6		39.4
(L) *S. cerevisiae*	41.0	38.7	37.1	40.8	38.0	39.4	38.7	40.9	36.9	59.8	55.7	

[a]Sequence alignment and identity/similarity between different pairs of sequences were carried out by using the BioEdit program. Upper and lower triangles indicate the percent identity and percent similarity, respectively, between pairs of sequences. Abbreviations in the sequence names are: A., *Arabidopsis*; B, *Burkholderia*; *D. melano.*, *Drosophila melangaster*; *En.*, *Entamoeba*; *E.*, *Escherichia*; *H.*, *Homo*; *S.*, *Saccharomyces*; *T.*, *Trypanosoma*; *X.*, *Xenopus*. RK, ribokinase; AK, adenosine kinase.

Fig. 1. (A) Sequence alignment of ribokinases from five species: *Homo sapiens*, *Drosophila melanogaster*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae* and *E. coli*. Symbols: semi-conserved (·); conserved (:) and absolutely conserved (∗). The asparagine and glutamic acid residues highlighted in red are part of the conserved NXXE motif (boxed) indicated to be involved in the binding of inorganic phosphate (14). (B) The gene structure of RK in human. The 8 exons are presented as boxes of corresponding lengths. The total length of the introns not shown here is approximately 108 kb.

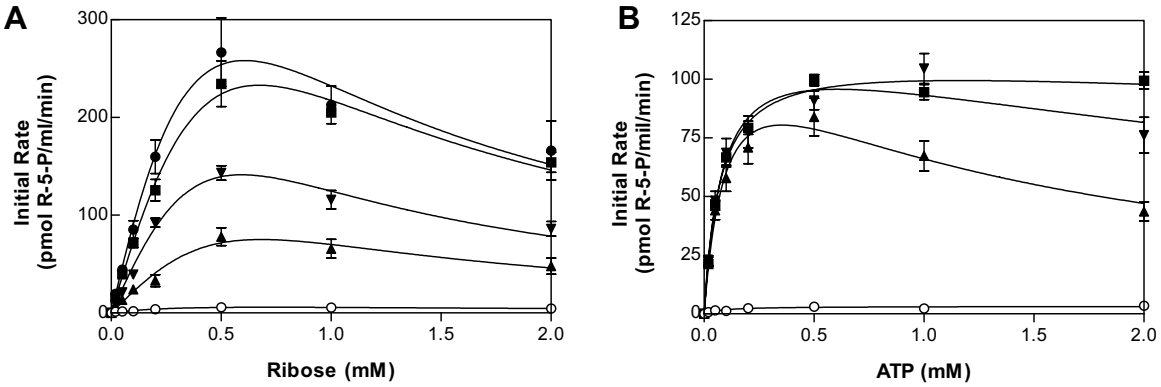
been extensively studied in various systems, the biochemical properties of eukaryotic RK have never been studied in detail in any of the earlier studies. The availability of purified recombinant protein has facilitated examination of its biochemical properties in detail. Earlier studies on *E. coli* RK have shown that its catalytic activity is dependent upon the presence of phosphate or other pentavalent ions [11]. In the absence of inorganic phosphate, the bacterial RK enzyme showed only a low, basal level of catalytic activity, which was greatly stimulated by added phosphate. In the present work, we have observed that the catalytic activity of human RK also showed marked dependency upon the presence of inorganic phosphate. Under standard conditions, if Pi was omitted from the reaction mix, the activity of RK was reduced to <2% of that seen in the presence of 10 mM Pi (data not shown; see below).

We have subsequently examined the effect of increasing concentrations of inorganic phosphate on the steady-state kinetics of human RK in presence of different concentrations of the two substrates, ribose and ATP. Fig. 2 shows the summary of the results from this study. As seen, in the absence of Pi, very little activity is seen at different concentrations of the two substrates. The addition of inorganic phosphate serves to greatly increase the catalytic activity of the enzyme, as well as decrease the K_m for both substrates, ribose and ATP, in a concentration-dependent manner (Fig. 2). Increased phosphate concentration also correlates with increased K_i values for both ribose and ATP, suggesting that presence of phosphate somehow relieves substrate inhibition of the enzyme by both substrates (Fig. 2). Decreased K_m and increased K_i values associated with increasing phosphate concentration have been also seen in *E. coli* RK and mammalian AKs previously [11,17,18].

We have also examined whether the human RK can phosphorylate other sugar substrates. Kinetic studies were carried out and k_{cat}/K_m values were calculated to determine the substrate specificity of the enzyme for other simple sugars including D-arabinose, D-fructose, D-galactose and D-xylose. The results of these studies showed that human RK was highly specific for ribose phosphorylation. Besides ribose, very weak activity was also observed for D-arabinose (k_{cat}/K_m, 40.58 and 1.77 s^{-1} mM^{-1}, D-ribose and D-arabinose, respectively, in presence of 10 mM Pi), but no significant phosphorylation was observed of other sugars such as D-fructose, D-galactose and D-xylose, (k_{cat}/K_m; 0.059, 0.08 and 0.15 s^{-1} mM^{-1}, respectively). These results are very similar to those reported recently for *E. coli* RK where the phosphorylation efficiency of the enzyme for D-arabinose, D-fructose and D-xylose was found to be 0.74%, 1.06% and 0.28%, respectively of that observed for D-ribose [10].

3.3. Effects of AK activators and inhibitors on human and E. coli RK

Adenosine kinase and RK are among the few enzymes known to show phosphate dependency [11]. Earlier studies on AK have identified several phosphorylated metabolites and related compounds, which can either activate AK in place of inorganic phosphate or inhibit the enzyme in the presence of phosphate [17,18]. Kinetic and structural studies indicate that these compounds and inorganic phosphate exert their effects by acting on the same regulatory site on the enzyme



Pi (mM)	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_i (mM)
1 (▲)	30.43	9.32	0.02
2 (▼)	6.62	20.85	0.05
5 (■)	3.39	31.84	0.13
10 (●)	2.17	40.58	0.17

Pi (mM)	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_i (mM)
1 (▲)	0.10	53.59	1.20
2 (▼)	0.08	63.91	4.40
5 (■)	0.07	66.83	18.91

Fig. 2. Effect of inorganic phosphate on the initial velocity of human RK at varying (A) ribose concentrations and (B) ATP concentrations. The concentrations of phosphate used were: 0 mM (○); 1 mM (▲); 2 mM (▼); 5 mM (■) and 10 mM (●). Kinetic parameters in the table were determined from the plot using the same software. The kinetic parameters at 0 mM phosphate could not be determined reliably due to low enzyme activity.

[14,17,18]. Therefore, it was of great interest to determine whether AK activators and inhibitors had similar effects on RK, which presumably shares a similar catalytic mechanism with AK.

The AK activators whose effect on RK activation was tested included acetyl phosphate, creatine phosphate, phosphoribosyl pyrophosphate, dihydroxyacetone phosphate, phosphoenol pyruvate, and carbamoyl phosphate (refer to [17] for their chemical structures). All of these compounds caused a dose-dependent stimulation of the activities of both human and *E. coli* RK, although the level of activation was lower then that seen with inorganic phosphate. The effects of some of these compounds on activity of the *E.coli* and human enzymes are shown in Fig. 3. The degree of stimulation or activation elicited by each compound differed from each other, and also for the human and *E. coli* enzymes. As seen in Fig. 3, phosphate and AK activators generally produced maximal enzyme activity in the concentration range of 5–10 mM, and lead to a decrease in activity at higher concentrations.

We have also examined the effect of several AK inhibitors on the activity of *E. coli* and human RK. The effects of two of these compounds, phosphonoacetic acid and etidronate, on RK activity in the presence of 10 mM inorganic phosphate are shown in Fig. 4. As seen, both these compounds inhibited both of the enzymes in a similar fashion, with IC_{50} values in a low millimolar (1–8 mM) range. The other inhibitors tested (viz. 2-carboxyethylphosphonic acid, *N*-(phosphonomethyl)-glycine, *N*-(phosphonomethyl)iminodiacetic acid and clodronate) also inhibited both *E. coli* and human RK in a similar manner as shown previously for the human AK [17]. A subsequent kinetic study indicates that these compounds inhibit the RK in a competitive manner with respect to the activating phosphate (results not shown), as was found to be the case for human AK.

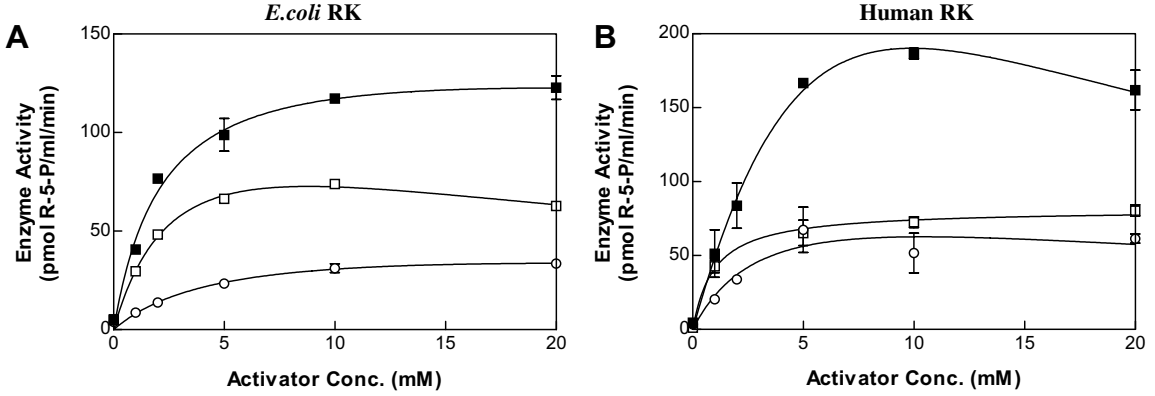


Fig. 3. Effects of various AK activators on the activity of (A) *E. coli* and (B) human ribokinase. Assays were carried out under standard conditions in the absence of any added phosphate, using either (A) *E. coli* or (B) human RK. The AK activators tested include: inorganic phosphate (■); acetyl phosphate (□) and dihydroxyacetone phosphate (○).

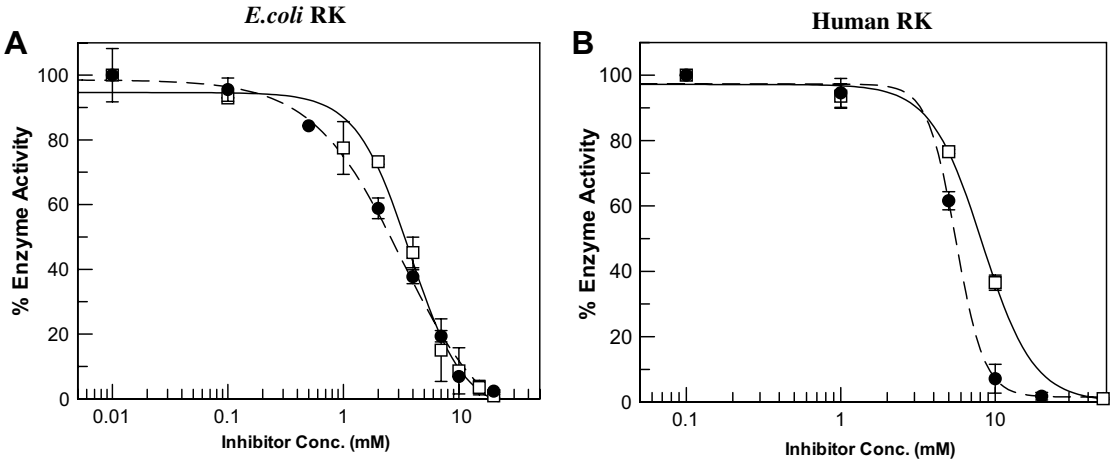


Fig. 4. Effect of AK inhibitors on (A) *E. coli* and (B) human RK. Assays were carried out under standard condition in the presence of 10 mM inorganic phosphate. Symbols: phosphonoacetic acid (–□–) and etidronate (--●--).

4. Discussion

Currently, there is a great interest in the transport and metabolism of ribose for its potential as a metabolic supplement. For instance, administration of exogenous ribose accelerates the repletion of ATP and helps maintain its level high in various tissue types [2–4,19]. With these tissue-protective effects, ribose has potential therapeutic applications involving the cardiovascular as well as the central and peripheral nervous systems [5,6]. In spite of the importance of ribose metabolism, very few studies on ribose metabolism have been carried out in mammalian cells. Although RK activity was first reported in calf liver in 1956 [13], little further work has been done on characterizing this protein or its various biochemical properties. The RK gene from any eukaryotic organism has yet to be definitively identified. In this context, it is important to recognize that because RK belongs to the PfkB family of carbohydrate kinases [7], mere observance of significant similarity between a known bacterial RK and a eukaryotic (viz. human) gene/protein is not sufficient to infer that the latter gene/protein is RK. The higher degree of similarity of human (or other animals) RK to the protein from bacteria as compared to the putative ribokinases from either yeast or plants as seen in the present work is quite surprising. This result was unexpected as plants and fungi are more closely related to the animals than bacteria [16]. Since the annotation of proteins from plants and fungi as RK is presently based solely on the observed sequence similarity, it is possible that proteins from these species may represent some other kinds of sugar kinases rather than ribokinase. Thus, it is of interest to characterize the proteins from these species with regard to their substrate specificities.

In this work, we report for the first time definitive identification of the gene for human RK and biochemical characterization of its protein product. The human RK protein shows between 35% and 40% sequence identity to the known bacterial RKs and it efficiently catalyzes the conversion of ribose into ribose-5-phosphate in presence of ATP. Other pentoses and simple sugars were either not or very poorly phosphorylated by the enzyme. The K_m of the human RK for ribose was ~10-fold higher than that of the *E. coli* enzyme, but their k_{cat} values were comparable [10,11].

Human RK, similar to the *E. coli* enzyme, was also found to exhibit phosphate dependency, a property that these enzymes share with the AK, which is also a member of the PfkB family of proteins [7]. Several phosphorylated compounds, which have been previously identified as activators or inhibitors of AK [11,17,18], also produced similar effects on both the human, as well as *E. coli* RK, without a single exception. These activator and inhibitor compounds have been shown to regulate the function of AK by mimicking inorganic phosphate [11,17,18], and thus are useful tools to identify other phosphate-dependent enzymes. These results, along with the similar effects of the phosphate on the catalytic activity and K_m for the sugar-derived substrates for AK and RK, provide evidence that these two enzymes share a similar catalytic mechanism, in common with some other phosphate-dependent enzymes [11,14,17,20,21].

The structure of *E. coli* RK has been solved with a bound phosphate, as well as ribose and ADP [8]. This phosphate ion makes close contacts with asparagine and glutamic acid residues at sequence positions 187 and 190, whose corresponding residues are also conserved in the human form (see boxed in Fig. 1A). In earlier studies, we have shown that these amino acid residues, forming an NXXE sequence motif, are also completely conserved in other proteins belonging to the PfkB family of carbohydrate kinases [14]. Site-directed mutagenesis of these conserved residues in Chinese hamster AK has led to proteins with greatly altered phosphate stimulation and substrate inhibition characteristics [14]. Recently, site-directed mutation of the NXXE motif in *E. coli* phosphofructokinase-2 (also a member of the PfkB family) led to creation of mutant enzymes with altered $MgATP^{2+}$ and phosphate requirements [22]. Therefore, the sequence motif NXXE may represent the site of phosphate binding and activation for AK, RK, Pfk-2, as well as other enzymes belonging to the PfkB family of carbohydrate kinases. The identification and characterization of human RK as described in this work sets the stage for understanding the function of this enzyme in ribose metabolism and cell structure and function.

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