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# Ribokinase from *E. coli*: Expression, purification, and substrate specificity

Dmitry V. Chuvikovsky,[a] Roman S. Esipov,[a,*] Yuri S. Skoblov,[a] Larisa A. Chupova,[a] Tatyana I. Muravyova,[a] Anatoly I. Miroshnikov,[a] Seppo Lapinjoki[b] and Igor A. Mikhailopulo[b,*]

[a]*Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya 16/10, Moscow 117997, Russia*
[b]*Department of Pharmaceutical Chemistry, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland*

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**Abstract**—Ribokinase (RK) was expressed in the *Escherichia coli* ER2566 cells harboring the constructed expression plasmid encompassing the *rbsK* gene, encoding ribokinase. The recombinant enzyme was purified from sonicated cells by double chromatography to afford a preparation that was ca. 90% pure and had specific activity of 75 μmol/min mg protein. Catalytic activity of RK: (i) is strongly dependent on the presence of monovalent cations (potassium ⋙ ammonium > cesium), and (ii) is cooperatively enhanced by divalent magnesium and manganese ions. Besides D-ribose and 2-deoxy-D-ribose, RK was found to catalyze the 5-*O*-phosphorylation of D-arabinose, D-xylose, and D-fructose in the presence of ATP, and potassium and magnesium ions; L-ribose and L-arabinose are not substrates for the recombinant enzyme. A new radiochemical method for monitoring the formation of D-pentofuranose-5-[$^{32}$P]phosphates in the presence of [γ-$^{32}$P]ATP and RK is reported.


## 1. Introduction

Transfer of a pentofuranosyl moiety of pyrimidine nucleosides to purine bases catalyzed by nucleoside phosphorylases ('transglycosylation reaction') is a very efficient methodology for the synthesis of a number of analogues of natural purine nucleosides (Scheme 1).[1] Chemical prerequisite for this transformation consists in that diverse sugar-modified pyrimidine nucleosides can be easily prepared from readily available natural nucleosides through intermediate formation of $O^2$,2′(3′,5′)-anhydro derivatives followed by an anhydro ring opening on treatment with nucleophilic agents.[2–6] However, similar approach to the synthesis of 2′- or 3′-modified purine nucleosides has very limited application from the structural viewpoint and the transglycosylation reaction is a reasonable alternative.

It is obvious that the use of these tandem enzymatic reactions for the synthesis of purine nucleosides is limited by the substrate specificity of the participating enzymes. Unfortunately, a number of sugar-modified uracil and thymine nucleosides display low or no substrate activity for uridine (UP) and thymidine (TP) phosphorylases precluding the preparation of the corresponding purine nucleosides of biological importance catalyzed by purine nucleoside phosphorylase (PNP). The following question has arisen: if a donor of the pentofuranose residue is not substrate for UP or TP, will the corresponding α-D-pentofuranose-1-phosphates (α-PFP-1) be recognized by and reacted with the same enzymes or PNP? The most impressive positive answer for this question was recently disclosed by Komatsu and Araki,[7] who showed that chemically synthesized 2,3-dideoxy-3-fluoro-α-D-ribofuranose-1-phosphate is a good substrate for PNP and was successfully used in an enzymatic synthesis of 9-(2,3-dideoxy-3-fluoro-β-D-ribofuranosyl)guanine (FLG) in ca. 63% yield, whereas 3′-fluoro-3′-deoxythymidine (FLT) is not substrate for TP and consequently cannot be employed as a donor in an enzymatic transglycosylation.

An enzymatic glycosylation of heterocyclic bases employing α-PFP-1 as pentofuranose donors has attracted attention during recent years (see, e.g., Refs.

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* Corresponding authors. Tel.: +358 17 163 128; fax: +358 17 162 456 (I.A.M); e-mail: Igor.Mikhailopulo@uku.fi

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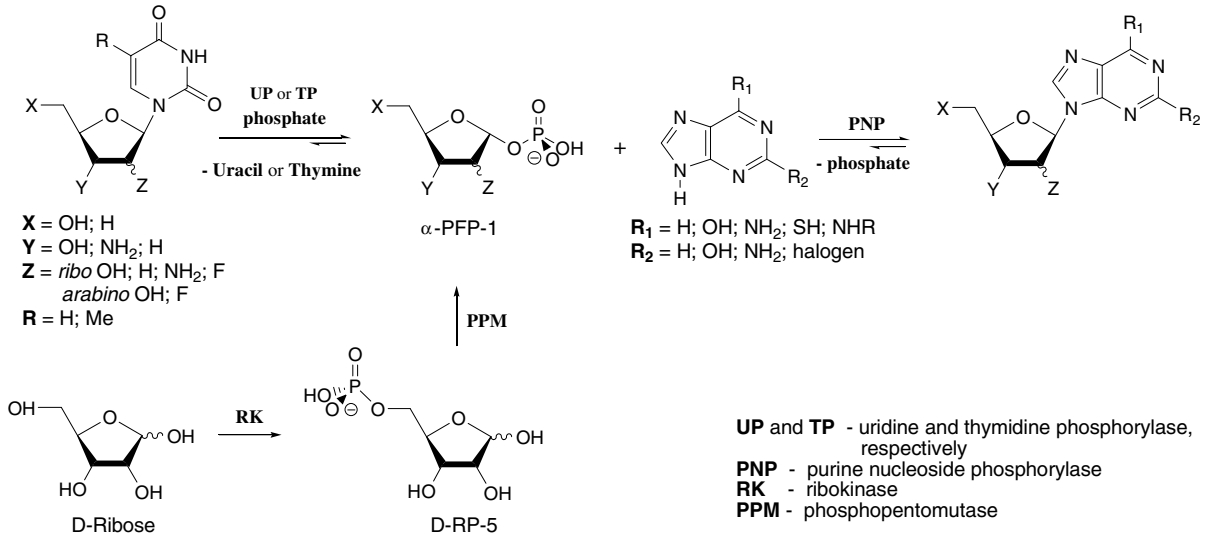


**Scheme 1.** Chemo-enzymatic syntheses of nucleosides using nucleoside phosphorylases and enzymatic syntheses from pentoses.

7–9). Presently, chemical syntheses of α-PFP-1 are rather complicated in order to find a broad application for the nucleoside preparation.[10–16] For the synthesis of 2-deoxy-α-D-ribofuranose-1-phosphate (α-dRFP-1) was, therefore, employed triosephosphate isomerase (TRI)/2-deoxy-D-ribose-5-phosphate (dRP-5) aldolase (DERA) system followed by the phosphopentomutase (PPM) catalyzing the transformation of dRP-5 into the desired α-dRFP-1.[8,9] The first step of this route appears to be suitable only for the synthesis of α-dRFP-1. Aiming at the search for more universal and efficient approach to diverse α-PFP-1, we have prepared the recombinant ribokinase (RK; EC 2.7.1.15) and phosphopentomutase (PPM; EC 2.7.5.6). The former enzyme catalyzes the 5-*O*-phosphorylation of D-ribose using ATP as a donor of the phosphate group.[17,18] In the present communication, we describe the preparation of RK, its properties and substrate specificity.

## 2. Results and discussion

Our interest to the synthesis of pentofuranose-5-phosphates (PFP-5) emanates from their possible use as precursors in an enzymatic transformation into α-D-pentofuranose-1-phosphate (α-PFP-1) under the action of phosphopentomutase (PPM; EC 2.7.5.6). The literature contains a number of reports on chemical synthesis of PFP-5, which are characterized by a diversity of approaches and most of them are rather laborious.[19,20] In a comprehensive study by Whitesides and co-workers three chemical and one enzymatic methods for the preparation of D-ribofuranose-5-phosphate (RFP-5) on a multigram scale have been investigated.[19] The most efficient chemical method was found to be an acid-catalyzed hydrolysis of adenosine-5′-phosphate (AMP) affording the desired RFP-5 of 85% purity in 94% yield. The use of a crude enzymatic (nuclease $P_1$) RNA hydrolysate for an acid-catalyzed hydrolysis of AMP and GMP, on the one hand, and of methyl 2,3-*O*-isopropylidene-β-D-ribofuranose in $POCl_3$ phosphorylation, on the other, was less efficient and more laborious. Application of partially purified RK from *Lactobacillus plantarum* (D-ribose was in growth medium; specific activity 0.3 U/mg protein; immobilized in PAN) for an enzymatic synthesis of RFP-5 afforded a mixture of 5-phosphates of D-ribose (36%), D-ribulose (15%), and D-xylulose (38%). Two undesired 5-phosphates resulted from RFP-5 under the consecutive action of phosphoriboisomerase and ribulose-5-phosphate epimerase that are present in the RK preparation.[19]

A common drawback of chemical methods consists in that they are not universal and strongly depend on the availability of the starting selectively protected pentofuranoses with free 5-hydroxyl group. On the other hand, the main limitation of the application of bacterial RK for the syntheses of PFP-5 is connected with its substrate specificity. It has been shown that the use of 2-deoxy-D-ribose as the major carbon source in fermentation of *L. plantarum* leads to the production of RK with higher specificity for 5-*O*-phosphorylation of 2-deoxy-D-ribose ($K_m$ of 1.8 mM) versus D-ribose.[21] This preparation was, however, unable to phosphorylate D-xylose, D- and L-arabinose, D- and L-fructose, D- and L-ribulose, and L-xylulose; D-xylulose displayed moderate substrate activity. Later, Schimmel et al. have purified similar RK from *Salmonella typhimurium* with high affinity for 2-deoxy-D-ribose ($K_m$ of 0.1 mM), which was also able to accept D-ribose and 2-deoxy-D-ribitol as substrates.[22] It is noteworthy that several ribo- and deoxyribonucleotides have been shown to be good phosphoryl donors.[22] To the best of our knowledge, more detailed investigation of the substrate specificity of RK has not been undertaken.

Aiming at the use of RK for the synthesis of PFP-5, we focused on the elaboration of optimal conditions of RK functioning and on assessment of the scope and limitations of this enzymatic reaction.

### 2.1. Activation of ribokinase by mono- and divalent cations

The dependence of the catalytic activity of the RK obtained on different factors was studied under standard conditions. Effect of temperature on activity of RK was studied in a range of 25–75 °C and the enzyme manifested the highest activity within 30–50 °C. By going from 50 to 60 °C, a fast drop of activity was observed, which is likely due to denaturation of the enzyme. Within pH 8.0–9.0 values, the highest activity (95–100%) of the enzyme was observed, at pH 7.8 it declined by 30%, and the further pH lowering to 6.0 resulted in a gradual dropping to 50% of maximal value. It is noteworthy that the presence of Tris–HCl in concentrations 0–100 mM did not influence the RK activity, but further increase of its concentration to 0.3 M is accompanied by a 70% drop of activity. The effect of temperature on the RK stability was determined by incubating RK for 20 min at temperatures ranging from 25 to 70 °C in Tris–HCl buffer (70 mM; pH 7.8). Residual activity was afterwards determined using the method 2 at 37 °C. RK was found to be stable within this temperature interval.

RK catalyzes the 5-*O*-phosphorylation of D-ribose in the presence of ATP and magnesium that is the first step in a D-ribose metabolism. It is assumed that magnesium binds to the triphosphate group of ATP giving rise to the chelate, which, in turn, facilitates the phosphoryl group transfer. Nonetheless, magnesium was not observed in the crystal structure of RK from *Escherichia coli* and ADP, although magnesium was included in the crystallization mixture.[23] It has also been shown that monovalent cations[24] and inorganic phosphate[25] activate RK. In a comprehensive study on the RK activation by monovalent cations it was concluded that the cesium ion binds between two loops adjacent to the anion hole of the active site.[24] From a viewpoint of practical application of RK for the synthesis of D-pentofuranose-5-phosphates (PFP-5), an activation of RK is of importance. Therefore, we have studied the effects of monovalent and divalent cations on activity of RK. Influence of monovalent and divalent cations was studied in the presence of 5 mM $MgCl_2$ and 100 mM KCl, respectively; activity of RK in the absence of both potassium and magnesium ions was ca. 1% of the maximal value.

An enhancement of the KCl concentration from 0 to 30 mM resulted in a jump-like increasing of the 5-*O*-phosphorylation velocity up to 90% and reached maximum at 0.1 M concentration of KCl. Further increase of the KCl concentration to 0.5 M gave rise to decreasing of activity to ca. 60%. An enhancement of the RK activity in the presence of ammonium and cesium cations at 0.1 M concentration amounted to ca. 60% and 40%, respectively (Fig. 1). Sodium and lithium cations showed no influence on the RK activity that is in harmony with the data by Andersson and Mowbray[24] who showed that the *E. coli* RK favors larger ions and disfavors smaller ones.

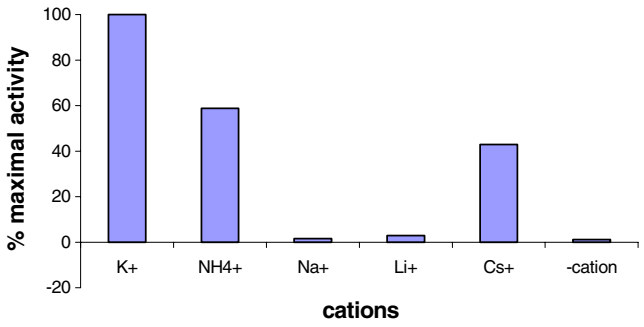


**Figure 1.** The effect of monovalent cations at 0.1 M concentration on activity of RK (−cation means that the reaction mixture did not contain a monovalent cation).

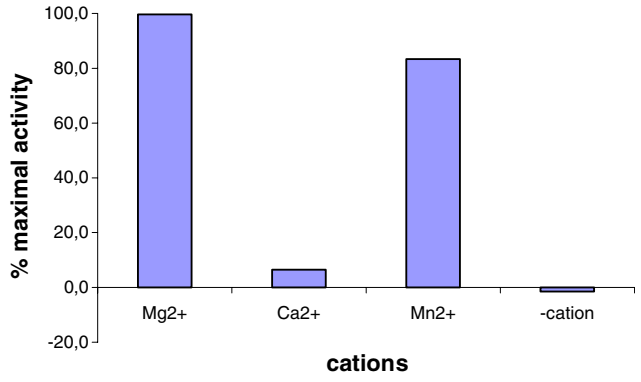


**Figure 2.** The effect of divalent cations at 5 mM concentration on activity of RK (−cation means that the reaction mixture did not contain a divalent cation).

More pronounced effect on the velocity of the 5-*O*-phosphorylation of D-ribose was observed in the case of magnesium ion. Indeed, a jump-like increase of the activity up to 85% was observed at 0.5 mM concentration of magnesium chloride and maximal activity was achieved at 5 mM. Similar effect on the RK activity was observed in the case of manganese ion that was about 80% of that of magnesium ion at 5 mM concentration; calcium showed very low influence on the RK activity (Fig. 2).

### 2.2. Substrate specificity of ribokinase

Substrate specificity of RK was investigated by means of kinetic studies and the results and procedures are presented in Table 1.

It is noteworthy that the $V_{max}$ values published by Maj and Gupta[25] are presented as pmol of D-ribofuranose-5-phosphate formed/min without indication of the quantity of the enzyme precluding the direct comparison with those obtained by us.

The X-ray crystallographic studies of the *E. coli* RK in a complex with D-ribose and ADP showed that oxygen atoms of the α-D-pentofuranose ring are involved in numerous hydrogen bonding interactions.[23,24,26] As might be expected on the basis of these results, replacement of one of C2 and C3 hydroxyls by proton or

**Table 1.** Kinetic data for the 5-*O*-phosphorylation of D-pentoses by RK[a]

| Substrate | $K_m$ (mM) | $V_{max}$ (μmol/min mg) | $k_{cat}$ ($s^{-1}$) [%] |
|---|---|---|---|
| D-Ribose | 0.279 (0.18)[b] | 81 (0.193)[b] | 86.4 [100] |
| 2-Deoxy-D-ribose | 1.41 | 25 | 26.7 [31] |
| D-Arabinose | 32.0 | 0.60 | 0.64 [0.74] |
| D-Xylose | 31.6 | 0.86 | 0.92 [1.06] |
| D-Fructose | 8.20 | 0.226 | 0.24 [0.28] |
| ATP[c] | 0.213 | 122 | 130 |

[a] Reaction conditions: reaction mixture (50 μL) containing Tris–HCl (70 mM; pH 7.8), KCl (0.1 M), 5 mM $MgCl_2$ (5 mM), ATP (0.1 mM), [γ-$^{32}$P]ATP (6 mCi), and RK ($1.1 \times 10^{-3}$ μg) was incubated at 37 °C. Substrate concentrations: D-ribose and 2-deoxy-D-ribose—0.01 to 10 mM; D-arabinose, D-xylose, and D-fructose—0.33 to 100 mM, ATP (3.3 mM), and 3.3 μg RK.

[b] Data in parentheses have been taken from the paper by Maj and Gupta[25] for the reaction at 37 °C [100 mM Tris–maleate buffer (pH 7.4), 125 mM KCl, 5 mM ATP, and 0.04 μg of the purified *E. coli* RK] and included in table for comparison purpose. Note that the $V_{max}$ value in the paper[25] is presented as pmol/min without indication of the enzyme quantity used in calculation.

[c] Kinetic parameters have been determined under the experimental conditions indicated in footnote (a) in the presence of D-ribose and RK in fixed concentrations of 3 mM and $1.1 \times 10^{-3}$ μg, respectively; ATP concentration varied within 0.01–5 mM.

changing its configuration from *ribo* to *arabino* or *xylo* can essentially influence the substrate properties of such pentoses. Indeed, as seen from Table 1, by going from D-ribose to 2-deoxy-D-ribose the affinity of the substrate to the enzyme, $K_m$, and the processing of the enzyme–substrate complex to products, $V_{max}$, decrease fivefold and ca. threefold, respectively. More remarkable decrease of the 5-*O*-phosphorylation efficiency was observed in the case of D-xylose, D-arabinose, and D-fructose. D-Lyxose, 2,3-dideoxy-D-ribose, L-ribose, L-arabinose, D-glucose, saccharose, and glycerin did not reveal the substrate properties under the experimental conditions employed for D-xylose, D-arabinose, and D-fructose.

It is noteworthy that GTP displayed activity as a donor of the phosphate group in the reaction catalyzed by RK, which was, however, ca. two orders of magnitude lower versus ATP. On the contrary, ribokinase from *S. typhimurium* was able to accept several ribo- and deoxyribonucleotides as good phosphoryl donors.[22] Polyphosphate showed no ability to serve as donors of the phosphate residue.

## 3. Conclusions

In conclusion, the *E. coli* RK was expressed in the *E. coli* ER2566 cells and purified by double chromatography of the cell lysate to afford a preparation of ca. 90% purity with specific activity of 75 μmol/min mg protein. The dependence of the enzyme activity on monovalent and divalent cations was studied and the conditions for the efficient 5-*O*-phosphorylation of D-ribose have been found. The substrate specificity of the RK preparation using new radiochemical method for monitoring the formation of D-ribofuranose-5-[$^{32}$P]phosphate in the presence of [γ-$^{32}$P]ATP and RK was investigated. Preliminary studies revealed that, besides D-ribose and 2-deoxy-D-ribose, RK in the presence of ATP, and potassium and magnesium ions catalyzes the 5-*O*-phosphorylation of D-arabinose, D-xylose, and D-fructose. More detailed studies are needed to determine the scope and limitations of this enzymatic 5-*O*-phosphorylation of pentofuranoses with diverse modifications at C2 and C3 carbons.

## 4. Materials and methods

### 4.1. Plasmid construction, expression and purification

The *rbsK* gene, encoding *E. coli* RK, was amplified by PCR employing synthetic oligonucleotides for the RK flanks: a 35-mer primer CTGCGTAAAAATCTGT CCGTCTCCACTTCGAACCC for the (−) strand contained the *Hin*dIII restriction site (underlined) at the 5′-end, and a 34-mer primer CATGCCATGGAAA ACGCAGGCAGCCTCGTTGTTC for the (+) strand contained the *Nco*I restriction site (underlined) for the 3′-end.

A 944-base pair amplified fragment was cut off from agar gel, digested with *Nco*I and *Hin*dIII restriction endonucleases, and ligated with plasmid pET23d (contained T7 promoter; Novagen) treated with the same restrictases.[27] The plasmid resulted was transformed into the *E. coli* ER2566 cells (New England Biolabs). Nucleotide sequence of the cloned fragment according to the F. Sanger method revealed no mutations.

The *E. coli* ER2566 cells harboring the expression plasmid have been cultivated in yeast–tryptone broth in the presence of ampicillin (100 μg/mL) at 37 °C to $OD_{600} = 0.5$ (ca. 6 h). Then, *i*-propyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and cultivation was continued for an additional 6 h.[28] The cells were harvested by centrifugation at 5000 rpm for 15 min to yield 0.8 g of wet paste, which was re-suspended in 0.1 M Tris–HCl buffer (2 mL; pH 7.8) and disrupted by sonication at 4 °C for 5 min. Cell debris was pelleted by centrifugation (14,000 rpm, 15 min), the supernatant was diluted with three volumes of water and applied onto a Q Sepharose High Performance column [HiTrep 5 mL (Amersham Bioscience)] that was equilibrated with 25 mM Tris–HCl/5 mM EDTA buffer (pH 7.8; standard buffer), prior to chromatography. After washing with standard buffer, column was eluted with a linear 0–0.5 M gradient of NaCl solution (50 mL), the fractions containing the desired activity were pooled (4.6 mL) and applied onto a Superdex 200 column [16 × 70 mm (Amersham Bioscience)] that was equilibrated with standard buffer prior to chromatography. Elution with the same buffer gave the solution of RK (8.0 mL), which was used for characterization of the enzyme. The protein concentration was measured by the Lowry method,[29] employing BSA as a standard. RK migrated as ca. 32 kDa monomeric protein in the SDS–PAG electrophoresis (Fig. 3) that is in accord with previous studies.[18,23] Note that RK exists

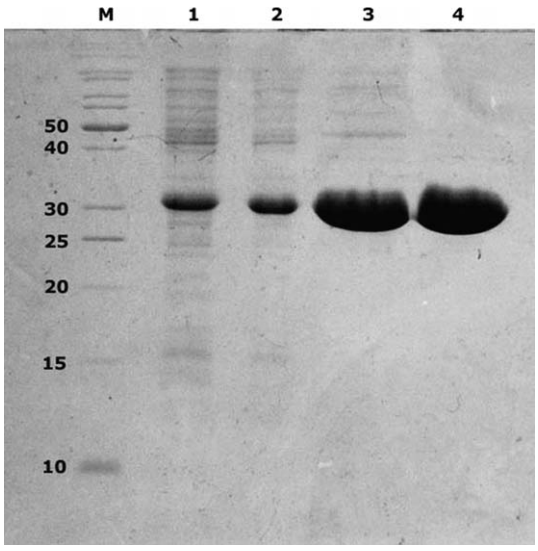


**Figure 3.** Progress of RK purification by the SDS–PAG electrophoresis. Lane M, molecular mass standards; lane 1, total cell lysate of the *E. coli* cells; lane 2, supernatant obtained after separation of cell debris; lane 3, combined fractions from a Q Sepharose column; lane 4, combined fractions from a Superdex 200 column.

in solution as a homodimer and is functioning in this form.[22,30] Progress of purification and molecular mass determination are illustrated by Figure 3 and summarized in Table 2. Gels were stained with Coomassie R-250 and scanned using Microtek scanner. Peak integration was performed using SigmaGel (Jandel—Scientific Software) program and purity of the enzyme was not less than 90% after Superdex 200 chromatography.

### 4.2. Activity assays

The activity of RK was measured (1) by a coupled assay monitoring finally the oxidation of NADH into $NAD^{\oplus}$ at $\lambda = 340$ nm,[18] and (2) by a radiochemical method for monitoring the formation of D-ribofuranose-5-[$^{32}$P]phosphate in the presence of [$\gamma$-$^{32}$P]ATP and RK. The latter method was developed to measure directly the transformation of D-ribose into D-ribofuranose-5-[$^{32}$P]phosphate. Reaction mixture (50 μL; pH 7.8) containing 70 mM Tris–HCl, 0.1 M KCl, 5 mM $MgCl_2$, 1.0 mM D-ribose, 0.1 mM ATP, 6 μCi [$\gamma$-$^{32}$P]ATP, and ribokinase ($1.1 \times 10^{-3}$ μg) was incubated at 37 °C. Aliquots (1.0 μL) were withdrawn after 5, 10, and 20 min, and placed onto PEI-cellulose plates that have been developed in aq 0.25 M $KH_2PO_4$. The formation of D-ribofuranose-5-[$^{32}$P]phosphate was quantified on an InstantImager Electronic Autoradiography (Packard BioScience Co.). The values represent means of three separate experiments and error rates are within 5%. One unit of enzymatic activity is defined as the amount of RK that transformed 1 μmol D-ribose into 5-monophosphate per minute at 37 °C in the reaction mixture containing 70 mM Tris–HCl, 0.1 M KCl, 5 mM $MgCl_2$, and ATP (0.1 mM) (pH 7.8). Method 1 was used only for determination of activity during isolation of RK, whereas in all other experiments method 2 was employed. The latter is universal because it allows to test any sugars and, moreover, appears to be much simpler versus the one suggested by Maj and Gupta.[25]

### 4.3. Reaction conditions for experiments on activation of ribokinase

Standard conditions: reaction mixture (50 μL) contained RK ($1.1 \times 10^{-3}$ μg), Tris–HCl (70 mM), [Hepes (70 mM) in experiments with pH changing], $MgCl_2$ (7.5 mM), KCl (0.1 M), ATP (0.1 mM), and D-ribose (1.0 mM) at pH 7.8. Reaction mixture was incubated for 10 min at temperatures 25–70 °C.

### 4.4. Kinetic measurements

Reaction conditions: reaction mixture (50 μL) containing Tris–HCl (70 mM; pH 7.8), KCl (0.1 M), $MgCl_2$ (5 mM), ATP (0.1 mM), [$\gamma$-$^{32}$P]ATP (6 μCi), and RK ($1.1 \times 10^{-3}$ μg) was incubated at 37 °C. Substrate concentrations: D-ribose and 2-deoxy-D-ribose—0.01 to 10 mM; D-arabinose, D-xylose, and D-fructose—0.33 to 100 mM, ATP (3.3 mM), and 3.3 μg RK. The $K_m$ and $V_{max}$ values have been obtained according to Lineweaver and Burk[31] on the basis of three experiments for each concentration of substrate (the values are within ± 20%).

Kinetic parameters for ATP have been determined under conditions indicated above in the presence of D-ribose and RK in fixed concentrations of 3 mM and $1.1 \times 10^{-3}$ μg, respectively; ATP concentration was varied within 0.01–5 mM. In experiments with [$\gamma$-$^{32}$P]GTP and [$^{32}$P]polyphosphate as donors of a phosphate residue the following conditions were employed: Tris–HCl (70 mM; pH 7.8), KCl (0.1 M), $MgCl_2$ (7.5 mM), and D-ribose (1.0 mM). To this reaction mixture, RK (3.3 μg) and [$\gamma$-$^{32}$P]GTP (specific activity 1000 Ci/mmol; 1.0 μL) or [$^{32}$P]polyphosphoric acid (specific activity 1000 Ci/mmol of phosphate group; 1.0 μL) (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia) were added and allowed to react at 37 °C for 10 min. Control probes did not contain D-ribose.

**Table 2.** Summary of the purification of RK from the *E. coli* ER2566 cells

| Fraction | Volume (mL) | Concn of protein (mg/mL) | Total protein (mg) | Total activity (U μmol/min) | Specific activity (U/mg protein) |
|---|---|---|---|---|---|
| Supernatant of lysate | 1.5 | 26.70 | 40.1 | 2246 | 56 |
| Q Sepharose pool | 4.6 | 2.3 | 10.6 | 752 | 71 |
| Superdex 200 pool | 8.0 | 1.10 | 8.8 | 660 | 75 |

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.05.057.

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