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ORIGINAL PAPER

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Molecular and biochemical characterization of a fructose-6-phosphate-forming and ATP-dependent fructokinase of the hyperthermophilic archaeon *Thermococcus litoralis*

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Abstract Close to an operon encoding an ABC transporter for maltose and trehalose, *Thermococcus litoralis* contains a gene whose encoded sequence showed similarity to sugar kinases. We cloned this gene, now called *frk*, and expressed it as a C-terminal His-tag version in *Escherichia coli*. We purified the recombinant protein, identified it as an ATP-dependent and fructose-6-phosphate-forming fructokinase (Frk) and determined its biochemical properties. At its optimal temperature of 80°C, the apparent K_m and V_{max} values of Frk were 2.3 mM and 730 U/mg protein for fructose at saturating ATP concentration, and 0.81 mM and 920 U/mg protein for ATP at saturating fructose concentration. The enzyme did not lose activity at 80°C for 4 h. Under denaturating conditions in SDS-PAGE, it exhibited a molecular mass of 35 kDa. Gel-filtration chromatography revealed a molecular mass of 58 kDa, indicating a dimer under nondenaturating, in vitro conditions.

Keywords Ribokinase family · Sucrose utilization · Trehalose/maltose ABC transporter · TrmB-dependent regulation

Introduction

Thermococcus litoralis is a hyperthermophilic marine archaeon that grows optimally at 85°C on peptone under anaerobic conditions (Neuner et al. 1990). The organism can utilize carbohydrates such as maltose, trehalose, and sucrose as well as starch, but no monosaccharides except mannose (Rinker and Kelly 1996; Xavier et al. 1996). A binding protein-dependent maltose/trehalose ABC transporter in *T. litoralis* has been described (Diederichs et al. 2000; Diez et al. 2001; Greller et al. 2001; Horlacher et al. 1998; Xavier et al. 1996), and the enzymology for maltose metabolism has been studied (Xavier et al. 1999). Next to the operon harboring the ABC transporter genes, a divergently oriented single gene encodes a putative sugar kinase. This gene as well as the operon is contained in a 16-kb DNA fragment that shows the typical features of a composite transposon. This whole cluster of genes was found in nearly identical sequence in *Pyrococcus furiosus*, and the functional form indicated a recent lateral gene transfer between the two organisms (DiRuggiero et al. 2000).

The deduced amino acid sequence encoded by the putative sugar kinase gene, now called *frk*, revealed similarity to ATP-fructokinases that are part of the ribokinase family of sugar kinases (Bork et al. 1993) such as the *Escherichia coli* ScrK protein (Schmid et al. 1988). The latter is involved in the metabolism of fructose, which is internally released from sucrose phosphate taken up by a specific phosphotransferase system (PTS). Sucrose has been reported to be also taken up by *T. litoralis* (Rinker and Kelly 1996). In archaea, specifically in the hyperthermophilic *Pyrococcus* species that are related to *T. litoralis*, no PTS transporter has been recognized so far, indicating that sucrose transport in *T. litoralis* would be performed by an active transport system. *frk* and the trehalose/maltose transport genes (*malE* operon) share a common divergent promoter, but no obvious promoter elements can be recognized in front of *frk*. A transcriptional regulator (TrmB) for the *malE* operon has been identified (Lee et al. 2003). Footprint analysis revealed that TrmB binds to two sites in this promoter region; binding to one of them results in transcriptional repression of the *malE* operon. The other site consists of an inverted repeat sequence positioned at 73 to 40 bp in front of *frk* and has not been analyzed so far. Binding of TrmB to these two sites is prevented by maltose, which supposedly acts as inducer, qualifying TrmB as a transcriptional repressor for the *malE* operon.

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However, TrmB also binds sucrose competitively with maltose (S.-J. Lee, unpublished results), indicating the involvement of TrmB in the regulation of sucrose (and therefore fructose) metabolism. Thus, it became relevant to determine the enzymatic activity of the *frk* gene product and its possible regulation by TrmB.

In this paper, we report that the gene product of *frk* in *T. litoralis* is a fructokinase (Frk)-forming fructose-6-phosphate (P) in an ATP-dependent manner. We purified the enzyme and determined its kinetic parameters. We found that the enzyme exhibited a high specific activity only for fructose.

Materials and methods

Cloning, overexpression, purification and molecular mass determination

Chromosomal DNA of *Thermococcus litoralis* was prepared as previously described (Lee et al. 2003). PCR was performed with chromosomal DNA as template and primers encompassing *frk*. Based on the annotated gene sequence (sugar kinase, AF307053), the two primers were 5′-CG**GAATTC**CATGCTATTTATTTACTTTAA-3′ (forward primer with *Eco*RI restriction site in bold) and 5′-CG**GGATCC**CGGAAGAAAGCTGAATTTCCT-3′, (reverse primer with *Bam*HI recognition site in bold). The PCR product was digested with *Eco*RI and *Bam*HI and cloned into plasmid pCS19 (Spiess et al. 1999) encoding a C-terminal 6×His tag. The resulting plasmid was named pSL149 and transformed into *Escherichia coli* strain DHB4 (Boyd et al. 1987). The transformant was cultivated at 30°C in 2 l NZA medium [10 g NZ-amine A (Sheffield Products), 5 g yeast extract, and 7.5 g NaCl in 1 l distilled water] containing 200 μg/ml ampicillin. When the OD_{600} reached 0.5–0.7, the cells were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside, followed by growth for another 5 h.

Cells were harvested by centrifugation and resuspended in 20 ml 50 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$ and 500 mM NaCl. The suspension was passed three times through a French pressure cell at 16,000 psi and centrifuged at 19,000 *g* for 10 min. The supernatant was heated at 80°C for 10 min, and the precipitant removed by centrifugation (19,000 *g*, 20 min).

The supernatant was loaded onto an Ni-NTA affinity column equilibrated with 50 mM Tris-HCl, pH 7.5, and washed with the same buffer containing 20 mM imidazole. Bound protein was eluted with 200 mM imidazole in this buffer. For final purification, 250 μl samples were applied to a Superdex 200 HR 10/30 column (24-ml bed volume, Amersham Biosciences) equilibrated with phosphate buffered saline (PBS), pH 7.4, and 5 mM $MgCl_2$. The protein was eluted with the same buffer. This preparation showed a single band on 12% SDS-PAGE and was stored at −80°C in PBS without loss of activity.

Molecular mass determination of Frk was done by gel-filtration chromatography at 4°C on a Superdex 200 column (Amersham Biosciences) equilibrated with PBS containing 1 mM β-mercaptoethanol and 5 mM $MgCl_2$. The column was calibrated with the low molecular mass calibration kit (Amersham Biosciences) containing bovine pancreas chymotrypsinogen A (25 kDa), hen egg albumin (43 kDa), bovine pancreas serum albumin (67 kDa), and rabbit muscle aldolase (158 kDa). The dimeric nature of Frk was corroborated by chemical crosslinking: 2.4 μg purified Frk was incubated with 10 mM dimethyl suberimidate for 1 h in 50 mM HEPES buffer (pH 7.5) in a total volume of 18 μl. Two microliters of 1 M Tris-HCl buffer, pH 8.0, was added to stop the reaction. The preparation was analyzed by SDS-PAGE for dimer formation.

HPLC analysis

Sugars, sugar phosphates, and sugar bisphosphates were analyzed by high pH–anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD). The HPAEC/PAD was performed with a Dionex BioLC-system (Dionex Corporation, Mississauga, Ontario, Canada) equipped with a quaternary gradient pump (GS50), a pulsed electrochemical detector (PED) with gold electrode (ED-50), and an autosampler (AS50). Samples were separated on a Dionex CarboPac PA-1 analytical column (2×250 mm) equipped with an amino trap precolumn previously equilibrated in 100 mM NaOH. The response factors of the PAD for the sugars and sugar phosphates were determined by repeated injections (20 μl) of standard mixtures (10–200 nmol). The enzyme reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 10 mM fructose, 5 mM ATP, and 20 mM $MgCl_2$ were incubated for 2–60 min at 60°C. The reaction was stopped by cooling to 4°C and removing the enzyme by ultrafiltration through a microcon YM-10 (Millipore).

Thin-layer chromatography

Samples (10 μl) from the reaction mixture (30 μg Frk, 20 mM fructose, 25 mM ATP, 100 mM $MgCl_2$ in 50 mM Tris-HCl, pH 7.5, in a total volume of 200 μl) were applied after different time intervals onto silica gel plates (type 60, Merck) and developed with butanol:ethanol:water (5:3:2) as solvent. To visualize the spots, the plate was dipped into methanol containing 5% H_2SO_4. The carbohydrate spots were visible after drying and charring at 120°C for 5 min.

Enzymatic assays

ATP–fructokinase activity was determined by measuring the formation of fructose-6-P in a two-step assay (assay 1). We used 100-μl samples of 50 mM Tris-HCl, pH 7.5, 2.5 mM ATP, 12.5 mM $MgCl_2$, and varying fructose concentrations. After preincubation at 80°C or at the

different temperatures for 5 min, the reaction was started by adding purified enzyme and stopped with 50 mM (final concentration) cold EDTA. Fructose-6-P was then quantified at 25°C by adding 0.4 mM $NADP^+$, 0.5 U glucose-6-P dehydrogenase (EC 1.1.1.49), and 0.5 U glucose-6-P isomerase (EC 5.3.1.9) to a final volume of 1 ml. The NADPH produced was determined at 340 nm. Using this method, the K_m and V_{max} values at 80°C for the formation of fructose-6-P and the temperature dependence of the enzymatic activity were determined. All assays were performed in duplicate.

The K_m and V_{max} values for ATP hydrolysis were determined by measuring ADP formation. In a two-step assay (assay 2) the reformation of ATP from ADP by pyruvate kinase was followed spectrophotometrically via the oxidation of NADH by lactate dehydrogenase. The assay mixture contained 50 mM Tris-HCl, pH 7.5, 10 mM fructose, and varying amounts of ATP. The assay was started by the addition of Frk and stopped by chilling. Then, 5 mM phosphoenolpyruvate, 0.15 mM NADH, 5.0 U lactate dehydrogenase, and 3.5 U pyruvate kinase were added, and the reaction was allowed to proceed at 25°C. The decrease in the absorption at 340 nm was measured. It was ensured that all auxiliary enzymes were not rate limiting. One unit of enzyme activity is defined as 1 μmol of fructose-6-P formed per minute. Specific activity is referred to as units per milligram of protein. Protein determination was done with the BCA (*b*i *c*inchoninic *a*cid) protein assay kit from Sigma.

Temperature dependence and thermal stability

The temperature dependence of Frk activity was measured from 30 to 100°C in the standard reaction system (assay 1). To test thermostability, the enzyme was incubated in sealed vials at 80°C for 10, 20, 30, 40, 50, 60, 120, 180, and 240 min. The vials were then cooled on ice for 1 h. The remaining enzyme activity was tested at 80°C. Untreated enzyme was used as control.

pH dependence and specificity of substrate, cation, and phosphoryl group donor

Possible substrates were tested by measuring ADP formation as described above (assay 2). The following sugars were used: D-fructose, D-glucose, D-glucosamine, D-mannose, D-galactose, 2-deoxy-D-glucose, D-fructose-6-P, D-fructose-1-P, and D-glucose-6-P.

To test divalent cation specificities, $CoCl_2$, $ZnCl_2$, $MnCl_2$, $NiCl_2$, $SrCl_2$, and $CaCl_2$ were used instead of $MgCl_2$ at equal concentration at 80°C (12.5 mM, assay 1). To analyze the specificity of phosphoryl group donors, GDP, CTP, ADP, GTP, pyrophosphate, and polyphosphate were used instead of ATP at equal concentration at 80°C (2.5 mM, assay 1).

For pH dependence, the enzyme was assayed in two buffer systems. From pH 4–8, 100 mM Tris base was adjusted to the desired pH with phosphoric acid. For the pH range of 9–10 a 1:1 mixture of 50 mM Tris base and 50 mM CAPS buffer (cyclohexylamino-1-propanesulfonic acid) was used and adjusted with NaOH to the desired pH. Both buffers contained 0.1 mM $CaCl_2$. The pH of these buffers was measured at room temperature and at 80°C; the difference was at most 0.5 pH units.

Fructose binding

Purified enzyme (10 μM in 100 μl) was mixed with 0.14 μM^{14} C-fructose and unlabeled fructose (the final concentration ranging from 10 μM to 1 mM) in Tris-phosphate buffer, pH 7.5. Reaction mixtures were incubated at 80°C for 10 min, stopped by the addition of 2 ml ice-cold saturated ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.5, and kept on ice for 10 min (Richarme and Kepes 1983). They were then filtered through cellulose nitrate membrane filters (Schleicher and Schüll, pore size 0.45 μm) and washed with 2 ml 95% saturated ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.5. Bound radioactivity was measured in a scintillation counter. The K_d value was defined as the fructose concentration at half-maximal binding of Frk.

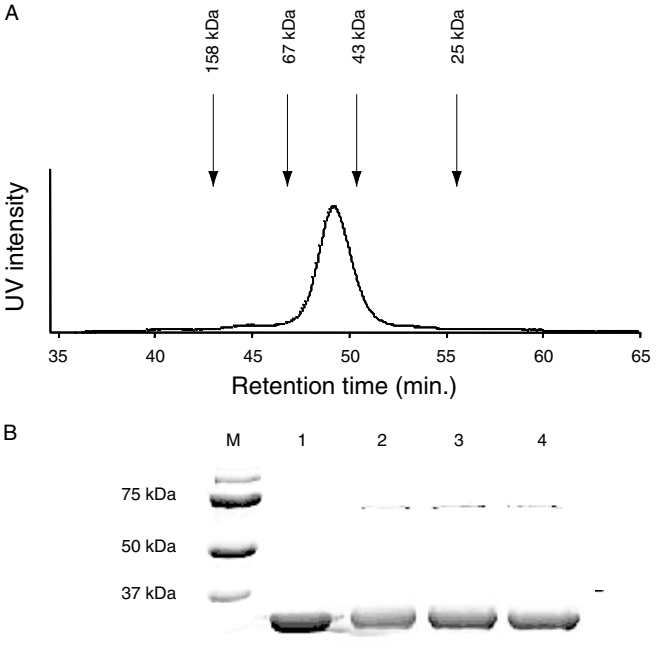

Fig. 1a, b Molecular mass determination of fructokinase (Frk). **a** Molecular-sieve chromatography on Superdex 200 at 4°C. The apparent molecular mass of Frk is 58 kDa. *Arrows* indicate molecular mass standards. **b** SDS-PAGE of purified Frk crosslinked with suberimidate. Crosslinked Frk has a molecular mass of about 70 kDa. *Lane M* Molecular mass standards, *lane 1* purified His-tag Frk without crosslinking, *lane 2* crosslinked protein without additions, *lane 3* Frk incubated with ATP/Mg^{2+} prior to crosslinking, *lane 4* Frk incubated with ATP/Mg^{2+} and fructose (5 mM) prior to crosslinking

Results

Purification of fructokinase

A 936-bp DNA fragment containing *frk* was amplified by PCR and cloned into plasmid pCS19, yielding pSL149. The plasmid-encoded protein was expressed in *Escherichia coli* DHB4 and purified from cellular extracts by heating to 80°C, followed by an Ni-NTA affinity chromatography and gel filtration. About 5 mg purified enzyme was obtained from 1 l bacterial culture. It was stored in PBS at −80°C and did not lose its activity for 3 months. The purified protein showed a single band on SDS-PAGE with an apparent molecular weight of 35,000, very close to the calculated molecular mass of 36,221 (His-tag version; see below, Fig. 1b).

Molecular mass

When gel-filtration chromatography was used to estimate the molecular mass, the protein appeared as a symmetrical peak calibrated at about 58 kDa, irrespective of the initial concentration (ranging from 0.05 to 1.0 mg/ml) and regardless of the presence of ATP (2.5 mM) and Mg^{2+} (10 mM) in the running buffer or of heating the protein prior to gel filtration (Fig. 1a). Crosslinking with suberimidate followed by SDS-PAGE gave small amounts of a product exhibiting 70 kDa. Again, the presence of ATP or Mg^{2+} or different heating protocols prior to the crosslinking had no effect on its result (Fig. 1b). This indicated that the enzyme is a dimer of two identical subunits.

Sequence similarity

The deduced amino acid sequence of *frk* showed similarity to other Frks of the ribokinase family of archaea and bacteria. High similarity was noticed to the putative ATP-fructokinases of *Pyrococcus abyssi* (PAB0482, 58% identity) and *P. furiosus* (PF1458, 54% identity). The latter is a second and putative Frk in addition to the one encoded in the 16-kb transposon insertion (DiRuggiero et al. 2000) described in this publication. Low similarity was observed to Frks from the Gram-negative bacterium *Rhizobium leguminosarum* biovar *trifolii* (34% identity; Fennington and Hughes 1996), the ATP-dependent

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FrK-T.litoralis     -----MIYAIGEILIDFIAKEE------GKLKDVREFEKHPGGAPANVVVGLRRLGAKSA
FrK-P.abyssi        -MVLVMICSIGELLVDVIATEE------GDLKDVRLFEKHPGGAPANVAVGIARLGFDSC
FrKII-P.furiosus    -----MITAFGEVLIDFIAAEE------GDLENVKIFEKHPGGAPANVAVGVSRLGIPSF
FrK-Rhizobium       -----MILCCGEALIDMLPRDT------TLGE--KGFAPYAGGAIFNTAIALGRLGIPTA
PFK-A.pernix        ---MSKIVSLGIHVLDVLGRHVNRFPPGGELDIINEIRLTVAGTAAGMSVDLAKLGGDVY
RK-E.coli           MQNAGSLVVLGSINADHILNLQS-FPTPGETVTGNHYQVAFGGKGANQAVAAGRSGANIA
                          :   *    * :                .     .*   .  :   : *

FrK-T.litoralis     LISKVGDDPFGEFLIEELKKERVETKYIIKDTNKHTGIVFVQLIGAKPEFILYDGVAYFN
FrK-P.abyssi        LVSKVGDDPFGEFLVESLRRENVKTDGIVKDEEKHTGIVFVQLTGVSPSFILYDGVAYFN
FrKII-P.furiosus    LVSKVGDDPFGRFLIRRLKEEGVNTEGVLIDNEKHTGVVFVQLKGAKPSFILYDEVAYFN
FrK-Rhizobium       FFTGIADDMMGEILLETLKASNVDYSPCAITPRPST-IAFVKLVNGQATYAFYDEGTAGR
PFK-A.pernix        AMGAVGLDESGNFIIDTLRRYGVNTDHIVRKSEVQTSCSMLPIRPNGERPVLHVLGANAT
RK-E.coli           FIACTGDDSIGESVRQQLATDNIDITPVSVIKGESTGVALIFVNGEGENVIGIHAGANAA
                     .   . *  *. :   *    :.           *   :: :             :

FrK-T.litoralis     LRKEEIQWDFMRDAELLHFGSVLFAREPSRSTVFEVLRAVKGKVP***ISYDVNIRL***DLWRGR
FrK-P.abyssi        LRREDINWELINSSRIVHFGSVILARSPSRETVLDVIKRVK--TI***VSFDVNLRL***DLWKNR
FrKII-P.furiosus    IKLSEVPWEILESSKIVHFGSVLLAREPSRGTMIKVIETLKEKSL***VSFDVNIRK***DLWRSE
FrK-Rhizobium       MITTADLPDLGDDCEALHFGAISLIPSPCGETYEALLDREAASRV***ISLDPNIRP***GFIKDK
PFK-A.pernix        YRLEDIHWDVIASADILHFGGTYLLPGLDGPPTATILKFAK-----EHGVTTTLDLLVNA
RK-E.coli           LSPALVEAQRERIANASALLMQLESPLESVMAAAKIAHQNKTIVALNPAPARELPDELLA
                           :    .    :           .   :          .

FrK-T.litoralis     EKEMLKDIEEALKLA***DIVKIGDGEL***EYLNKNG------IALEDFNFALVAITRGAEG-ST
FrK-P.abyssi        GEDMLRTIEEAIKLA***DIVKASEEEV***DYLEDNG------IEVEGK--LITAITMGSKG-AK
FrKII-P.furiosus    -EELVETLKRVLSNV***DILKLSDEEF***NLLKEYG------LEPRGK--LITAITYGAEG-CR
FrK-Rhizobium       PSHMAR-IKRMAAKS***DIVKFSDEDL***DWFGLQGDHDALAAHWLNHGAKLVVITKGAEG-AS
PFK-A.pernix        QPDLLAKLEPALPYIDYFMPGLDEARAICGLEKRADVIAFFLNRGVGHTVFKMGAEGSSI
RK-E.coli           LVDIITPNETEAEKLTGIRVENDEDAAKAAQV--------LHEKGIRTVLITLGSRG-VW
                      .:    :        .    :                          . :. *:.*
                                          XXX
FrK-T.litoralis     IIHKDIRVDVPSFKVEPVDTTGAGDAFMAALLASLFYMGKLDILEFSKEELKELGSFAN-
FrK-P.abyssi        LNG----IQVPGYKVNPVDTTGAGDAFTAALLVGIMKVR--EITNE---NLEKIGKFAN-
FrKII-P.furiosus    IESDEASIFVPAYKVSPVDTTGAGDAFVSALLVGLLALDKQKLEES---DMFLLGKFAN-
FrK-Rhizobium       GYTKDRKVTVPSERVTVVDTVGAGDTFDAGILASLKMDNLLTKRQVASLDEQALRNGPDP
PFK-A.pernix        ASLEMAEILIPAYKAQVVDSTGCGDSYCAAFIMGLTKGWDLATSGRFASAAASLVVSGLG
RK-E.coli           ASVNGEGQRVPGFRVQAVDTIAAGDTFNGALITALLEEKPLPEAIRFAHAAAAIAVTRKG
                           :*. :.  **: ..**:: ..:: .:                  :

FrK-T.litoralis     ----------LVAALSTTKRGAWSVPSLEEVLKHRKFSFLP-----
FrK-P.abyssi        ----------LVAALSTLKRGAWSVPKVEELLKYKEAREILRL---
FrKII-P.furiosus    ----------LVAGLSTQKRGAWSVPRREELEKYEEGRKIFSLITL
FrK-Rhizobium       RRQSRRRHRLPRRRQSTLGARDWSLRLEQDSDPHPPDDTFSP----
PFK-A.pernix        -----------SDAGIIDFEHTLKVMNTAETLPIAQET--------
RK-E.coli           --------------------AQPSVPWREEIDAFLDRQR-------
                                        .:    :
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Fig. 2 Sequence alignment. Multiple alignment of the amino acid sequences of Frks from *Thermococcus litoralis*, *Pyrococcus abyssi* (putative, PAB0482), *P. furiosus* (putative, PF1458, the "second" Frk) and *Rhizobium leguminosarum*, phosphofructokinase from *Aeropyrum pernix*, and ribokinase from *Escherichia coli*. The *asterisks* indicate identical amino acids in thermococcal Frk and any of the other sugar kinases. The proposed sequence motifs involved in ATP-binding and substrate recognition are *shaded* and *boldface*/*italics*, respectively. *XXX* indicates the conserved sequence AGD at positions 253, 254, and 255 in the crystal structure of *E. coli* ribokinase (Sigrell et al. 1998). *Frk* Fructokinase, *FrkII* fructokinase II, *PFK* phosphofructokinase, *RK* ribokinase

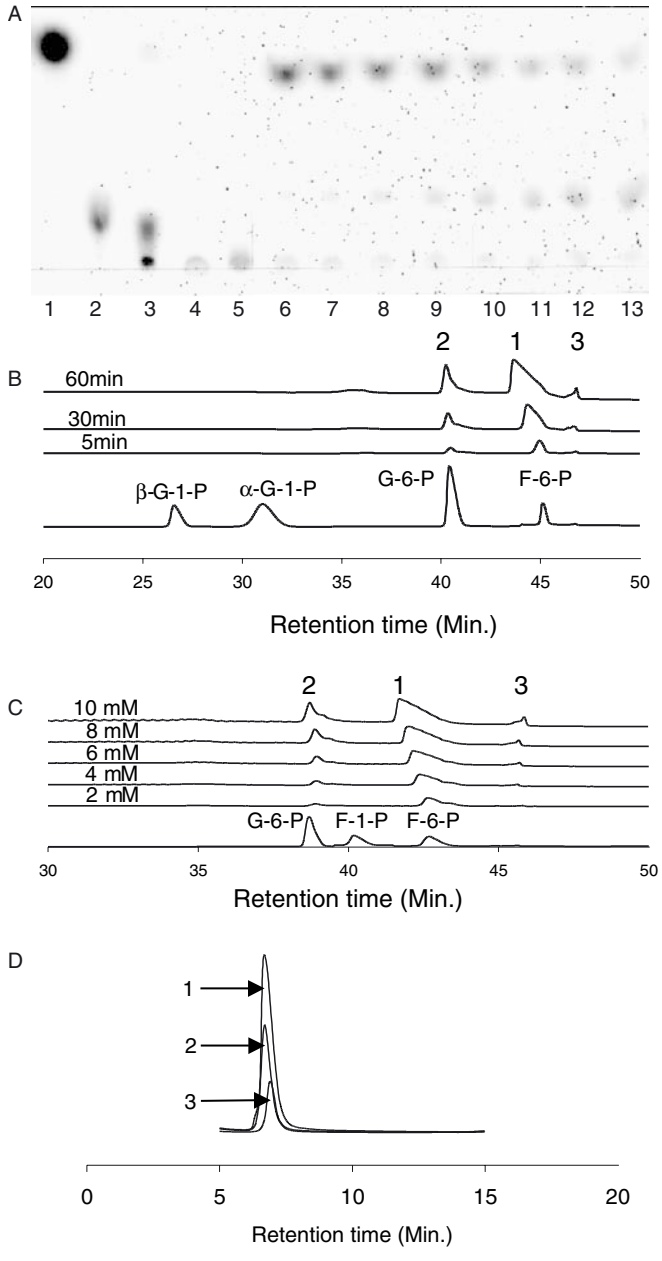

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Fig. 3a–d Analysis of the products of fructose phosphorylation. **a** Thin-layer chromatographic (TLC) analysis of enzymatic fructose phosphorylation. The reaction was performed at 60°C with 30 µg Frk in 200 µl 50 mM Tris-HCl, pH 7.5, 20 mM fructose, 25 mM ATP, and 100 mM $MgCl_2$. Six-microliter aliquots were applied onto TLC plates at different time intervals. Standards: *lane 1* fructose; *lane 2* fructose-6-P; *lane 3* fructose-1-P; *lane 4* ATP; *lane 5* ADP; *lanes 6–13* incubation mixture at 0, 10, 20, 30, 40, 60, 120 min, and after 24 h. **b** HPLC analysis of the phosphorylation reaction mixture after different time intervals. Samples were taken at 0, 5, 30, and 60 min after starting the reaction. *1*, *2*, and *3* indicate fructose-6-P and fructose-2- or -3-P. **c** HPLC analysis of product formation at different initial fructose concentrations. The reaction was performed for 30 min at 60°C at 2, 4, 6, 8, and 10 mM fructose. The reaction mixture contained 10 µg purified enzyme in 200 µl 50 mM Tris-HCl, pH 7.5, 10 mM ATP, and 50 mM $MgCl_2$. *1*, *2*, and *3* indicate fructose-6-P and fructose-2- or -3-P. **d** HPLC analysis of the unkown fructose phosphates after treatment with alkaline phosphatase. The reaction mixture described in (**c**) (10 mM fructose, 30 min incubation time) was treated with alkaline phosphatase and analyzed by HPLC. *1* Reaction mixture after treatment with alkaline phosphatase, *2* standard fructose, *3* reaction mixture without alkaline phosphatase treatment. Note: sugar phosphates are off scale in this analysis

phosphofructokinase of the archeaon *Aeropyrum pernix* (26% identity) (Hansen and Schönheit 2001) and ribokinase of *E. coli* (26% identity). There was no significant similarity to the ATP-dependent Frks of *Lactococcus lactis* (Thompson et al. 1991) and *Zymomonas mobilis* (Zembrzuski et al. 1992), which belong to the ROK (*r*epressor, *O*RF, *k*inase) family. Figure 2 shows the alignment of some of these kinases that belong to the ribokinase family, including their conserved regions that are connected to the ATP-dependent kinase activity.

Identification of enzymatic products

To identify the product of fructose phosphorylation, samples of the incubation mixture containing purified enzyme, fructose, Mg^{2+}, and ATP were analyzed by thin-layer chromatography (TLC) (Fig. 3a). While fructose was used up, a compound was formed with similar migration properties on TLC as fructose-6-P, but different from fructose-1-P and fructose-1,6-phosphate. Using HPLC under alkaline conditions, three compounds could be identified as the products of phosphorylation. All increased with time (Fig. 3b) and initial fructose concentration (Fig. 3c). The main peak, (product 1, about 85% of the total phosphorylation products) was eluting at 42.6 min close to the retention time of standard fructose-6-P (42.7 min, Fig. 3c). The two additional peaks (products 2 and 3, about 12 and 3%, respectively) were eluted at 38.7 and 45.8 min. Treatment of the product mixture by alkaline phosphatase yielded only free fructose but no other sugar (Fig. 3d). Thus, the additional phosphorylated products 2 and 3 could only be fructose phosphates. Conceivably, product 3 could be fructose-1,6-diphosphate (retention time 46.9 min), being formed by the phosphorylation of fructose-6-P. Yet, this is excluded as well since fructose-6-P was not a substrate for the enzyme and did not form the slower migrating compound. Taken together, this led us to conclude that products 2 and 3 are fructose-2-P and fructose-3-P.

Enzyme characteristics

The rate of the enzymatic reaction was determined by measuring the formation of fructose-6-P via the formation of NADPH (isomerization to glucose-6-P and its oxidation by $NADP^+$ and glucose-6-P dehydrogenase, assay 1). Using this two-step assay, typical Michaelis–Menten kinetics were observed. At 80°C the apparent K_m and V_{max} values for fructose at saturating ATP

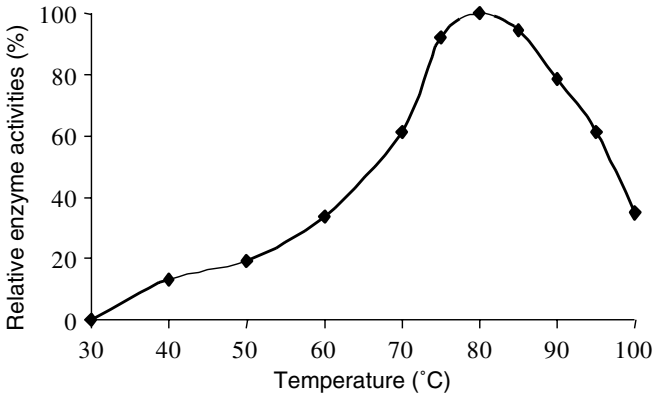

Fig. 4 Effect of temperature on the specific activity of purified Frk. The assay was performed under the conditions of V_{max} for fructose and ATP

concentrations were 2.3 mM and 730 U/mg protein. The apparent K_m and V_{max} values for ATP at saturating fructose concentrations (assay 2) were 0.81 mM and 920 U/mg protein. The difference in the V_{max} values must be due to the formation of fructose-2-P and -3-P. Only the formation of fructose-6-P contributes to the determination of the kinetic constants for fructose in the above assay.

Frk showed highest activity with ATP. Other nucleoside triphosphates can substitute for ATP: ITP (73%), GTP (62%), UTP (16%), and CTP (16%) can act as phosphoryl donors. ADP, GDP, UDP, PEP, polyphosphate, and pyrophosphate were inactive.

The enzyme required divalent cations for its activity. $MgCl_2$was most effective, but could be partially substituted by Mn^{2+} (65.2%) and Co^{2+} (43.7%). Very low enzyme activity was observed with Ni^{2+} (8.7%) and Sr^{2+} (1.0%) and no activity with Ca^{2+} and Zn^{2+}.

Frk specifically catalyzed the phosphorylation of D-fructose to fructose-6-P. Other monosaccharides, such as mannose, glucose, D-fructose-6-P, glucosamine, 2-deoxy-D-glucose, D-galactose, D-fructose-1-P, and D-glucose-6-P, did not serve as substrates.

At 30°C the activity of the enzyme was very low (< 1% of its maximal activity at 80°C). It increased rapidly above 60°C, exhibited an optimum at 80°C, and 11.7% of that value at 100°C (Fig. 4). The thermostability was determined by preincubation at 80°C from 10 min to 4 h. The enzyme did not lose any activity for 4 h at 80°C. It was active at pH values from 6.0 to 9.0 and had optimal activity between pH 7.5 and 8.

Fructose binding

To determine the affinity of Frk for fructose in the absence of ATP hydrolysis, we measured binding of ^{14}C-fructose in a test used for periplasmic binding proteins (Richarme and Kepes 1983). In order to form an enzyme–substrate complex, the enzyme was heated up with ^{14}C-fructose at 80°C for 10 min prior to precipitation

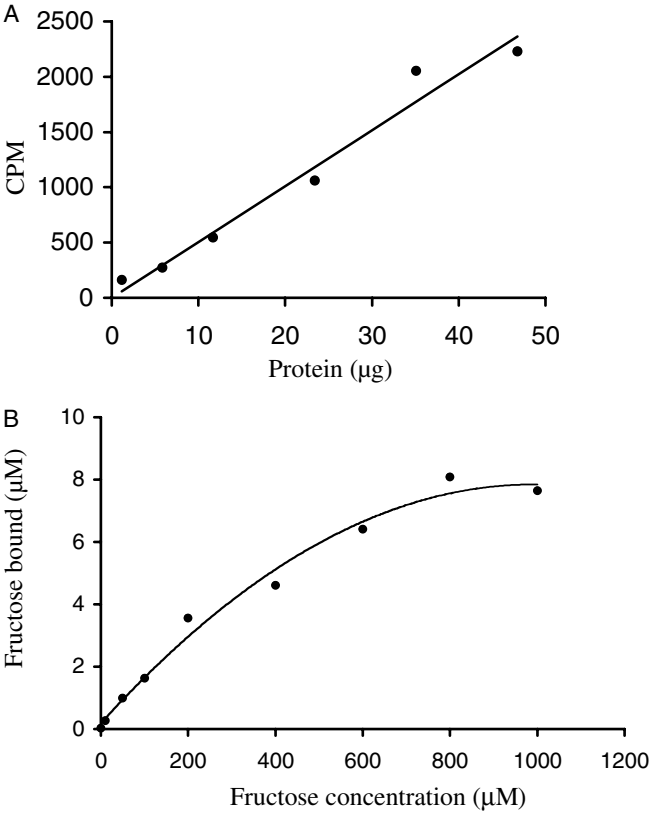

Fig. 5a, b Fructose-binding assay. **a** A test volume of 100 µl 0.14 µM ^{14}C-fructose was incubated with increasing amounts of Frk at 80°C for 10 min. **b** Ten micromolar purified Frk was incubated with 0.14 µM ^{14}C-fructose and increasing amounts of unlabeled fructose. Half-maximal binding occured at 0.25 mM fructose and defined the K_d

with ice-cold saturated ammonium sulfate. Fructose binding was linearly dependent on enzyme concentration (Fig. 5a) and showed Michaelis–Menten kinetics (Fig. 5b). The K_d for fructose was 0.25 mM, about ten times lower than the K_m for fructose phosphorylation. Maximal binding showed a stoichiometry of 0.8 per monomeric protein.

Discussion

In this publication, we report the characterization of Frk, an ATP-dependent Frk, from *Thermococcus litoralis* as well as *Pyrococcus furiosus*. *frk*, the gene encoding this enzyme, is positioned on a 16-kb transposon-like DNA fragment that occurs with identical sequence in both organisms (DiRuggiero et al. 2000). Comparison of the amino acid sequence of Frk showed similarity to those kinases that belong to the ribokinase type (Bork et al. 1993), a large family of prokaryotic and eukaryotic carbohydrate kinases including Frk, ribokinase, 1-phosphofructokinase, archaeal 6-phosphofructokinases, and adenosine kinase. In contrast, the *T. litoralis* Frk did not exhibit significant sequence simi-

Table 1 Comparison of the biochemical properties of fructokinases from different organisms

Parameters	*Thermococcus litoralis*	*Lactococcus lactis*	*Zymomonas mobilis*	*Rhizobium leguminosarum*	*Streptococcus mutans*
Sugar kinase families	Ribokinase family	ROK[a] family	ROK[a] family	Ribokinase family	ROK[a] family
Calculated MW (kDa)	36.22	33.5	32.57	–	31.68
Subunit MW (kDa)					
SDS-PAGE	35	32	28	36.5	–
Gel filtration	58	44	–	–	49
Oligomer structure	Dimer	80% dimer 20% monomer	Dimer	–	Monomer–dimer equilibrium
Assay temperature (°C)	80	–	25	25	25
Substrate specificity	Fructose	Fructose Mannose	Fructose Mannose (trace activity)	Fructose	Fructose Mannose
V_{max} (U/mg protein)					
D-fructose	730	190–200	350	31	20.0 (fructose)
Mannose	–	–	–	–	5.0 (mannose)
ATP	920	–	–	–	–
K_m (mM)					
D-fructose	2.3	0.31	0.7	0.31	0.77
Mannose	–	–		–	1.0
ATP	0.81	0.59	0.45	–	
Phosphoryl group donor	ATP > ITP > GTP > UTP, CTP	dATP > GTP > ITP > UTP	–	–	–
Divalent cation	$Mg^{2+} > Mn^{2+} > Co^{2+} > Ni^{2+} > Sr^{2+}$	$Co^{2+} > Fe^{2+} > Mn^{2+} > Ni^{2+} > Zn^{2+} > Cd^{2+}$	–	$Co^{2+} > Mg^{2+} > Cd^{2+} > Mn^{2+} > Ca^{2+}$	–
Reference	This work	Thompson et al. (1991)	Zembrzuski et al. (1992)	Fennington and Hughes (1996)	Sato et al. (1993)

[a] *ROK* Repressor, ORF, Kinase. Data from ExPASy enzyme nomenclature database: http://us.expasy.org/enzyme/

larity to the ADP-dependent glucokinase from the same organism (Ito et al. 2001), even though the crystal structures of the latter enzyme as well as of that of ribokinase from *Escherichia coli* (Sigrell et al. 1998) reveal a fold similar to the one in the proteins of the ATP-dependent ribokinase family to which the Frk of *T. litoralis* belongs.

Conserved domains in Frk were recognized in the C-terminal section and are indicated in Fig. 2. The shaded sequence has been associated with ATP-binding by others (Aulkemeyer et al. 1991; Fennington and Hughes 1996). Based on the crystal structure of *E. coli* ribokinase (Sigrell et al. 1998), Asp 255 is conserved in a number of kinases as the catalytic residue; Ala253 and Gly254 (indicated by XXX in Fig. 2) are conserved to form the anion pocket. The other two motifs, shown in boldface/italics in Fig. 2, are conserved in four Frks, but not in ribokinase and phosphofructokinase. These two motifs were suggested to represent the fructose recognition site (Fennington and Hughes 1996).

The enzyme showed substrate specificity only for fructose and was inactive towards a range of other sugars tested. Some Frks are known to have limited substrate specificity towards mannose, for example, the Frks from *Lactococcus lactis* (Thompson et al. 1991), *Zymomonas mobilis* (trace activity on mannose, Scopes et al. 1985; Zembrzuski et al. 1992), and *E. coli* (Sebastian and Asensio 1972; Table 1). The K_m of the *T. litoralis* enzyme for ATP at saturating fructose concentrations was 0.81 mM. This is in keeping with other ATP-dependent sugar kinases, for instance, a phosphofructokinase from *Aeropyrum pernix*, which has a K_m of 0.68 mM (Hansen and Schönheit 2001). Other sugar kinases of hyperthermophilic archaea are ADP-dependent (Ronimus and Morgan 2003), which has been explained by the instability of ATP at high temperatures. Thus, the high affinity of galactokinase of *P. furiosus* for ATP (K_m 8 μM, V_{max} 41.9 units/mg protein) was interpreted by the low availability of ATP (Verhees et al. 2002). The apparent discrepancy to the low affinity of *T. litoralis* Frk and other ATP-dependent kinases from hyperthermophiles that also have a low affinity for ATP may possibly be explained by the rather high V_{max} of these enzymes, thus ensuring efficient phosphorylation even at low ATP concentrations.

The K_m for fructose phosphorylation (2.3 mM) was about ten times higher than the K_d (0.25 mM) for fructose binding in the absence of ATP. The reason for this discrepancy is unclear at present. If one does not evoke conformational changes upon binding ATP, it may simply be due to the high turnover of the enzyme, which limits the access of substrate under phosphorylation conditions.

Since *T. litoralis* appears to be unable to grow on fructose, the role of the cytoplasmic Frk must be connected to the metabolism of sucrose. The latter has been reported to be a carbon source for *T. litoralis* (Rinker and Kelly 1996). Since phosphotransferase-type trans-

porters are absent in archaea, this implies that sucrose is taken up by the cell without phosphorylation and cleaved internally to free fructose. Sucrose is no substrate for the maltose/trehalose transporter even though TrmB, the negative regulator of the corresponding operon, does recognize this sugar. This implies that it is the putative operon encoding sucrose degradation that is controlled by TrmB. In contrast, *frk*—located adjacent to the gene cluster encoding the maltose/trehalose ABC transporter—appears not to be regulated by TrmB as judged by Western blotting of the enzyme or Northern blotting of the cognate RNA after growth in the presence of different possible inducers.

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