

# Cloning and expression of the gene for the active PP<sub>i</sub>-dependent phosphofructokinase of *Entamoeba histolytica*

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Pyrophosphate-dependent phosphofructokinase (PP<sub>i</sub>-PFK) from *Entamoeba histolytica* (HM-1) was purified from trophozoites. Oligonucleotide probes based on partial amino acid sequence were used to clone and sequence the gene and the cDNA of the enzyme. The molecular mass of the subunit was greater than, and the derived sequence significantly different from, that of the product of the PP<sub>i</sub>-PFK gene previously cloned from *E. histolytica* [Huang, Albach, Chang, Tripathi and Kemp (1995) *Biochim. Biophys. Acta* **1260**, 215–217; Bruchhaus, Jacobs, Denart and Tannich (1996) *Biochem. J.* **316**, 57–63]. The sequence identity between the two proteins was 17%. The sequence bore greater identity with the more phylogenetically advanced plant PP<sub>i</sub>-

PFKs than with bacterial PP<sub>i</sub>-PFKs. The cloned cDNA was expressed and the protein purified. The kinetic properties were identical with those of the enzyme isolated from the organism. Furthermore, the specific activity was more than three orders of magnitude higher than that described for the product of the previously cloned *E. histolytica* PFK gene [Bruchhaus et al. (1996)]. The pH-dependence and apparent substrate affinities of the cloned enzyme were identical with those of the PP<sub>i</sub>-PFK in trophozoite extracts, indicating that the product of the cloned gene accounts for most if not all of the PFK activity in *E. histolytica* trophozoites.

## INTRODUCTION

Phosphofructo-1-kinase (PFK) from *Entamoeba histolytica*, as well as a number of other parasitic protozoa, utilizes PP<sub>i</sub> as the phosphoryl donor instead of ATP. The amitochondrial parasitic protists, such as *Entamoeba*, rely on glucose metabolism as a major means of energy production. The PP<sub>i</sub>-PFKs of these organisms in particular represent attractive targets for the development of therapeutic agents that will interfere with vital energy metabolism in the parasite without affecting the host. PP<sub>i</sub>-PFK represents a very critical metabolic step for three reasons. First, there is the obvious reason alluded to above, that is, it is a key step in energy production from carbohydrate. Second, and less obvious, the enzyme acts as a key gluconeogenic enzyme because of its reversibility and because *E. histolytica* does not have a fructose bisphosphatase. Finally, the organism appears to lack a neutral pyrophosphatase. A number of important biosynthetic enzymes generate PP<sub>i</sub>, which must be hydrolysed to provide the phosphate that is used for the resynthesis of ATP. The PP<sub>i</sub>-dependent enzyme was first identified, partially purified and characterized in 1974 by Reeves' laboratory [1].

More recently a genomic sequence for a PP<sub>i</sub>-PFK from *E. histolytica* was reported [2,3]. Furthermore Bruchhaus et al. [3] cloned the cDNA for this gene and found that it coded for a protein with a mass of 47.6 kDa. However, when expressed, the gene product was found to have a specific activity that was several orders of magnitude lower than that of the partially purified native enzyme [3]. This raised the question as to whether this gene coded for the protein responsible for PP<sub>i</sub>-PFK activity in extracts of *E. histolytica*.

In the current study we purify to near-homogeneity the PP<sub>i</sub>-PFK of *E. histolytica*. Its protein sequence differs substantially

from that based on the previously cloned PP<sub>i</sub>-PFK gene. The sequences for the gene and cDNA for this PP<sub>i</sub>-PFK were determined. The properties of the expressed gene were shown to be identical with those of the enzyme in extracts of axenically grown *E. histolytica*.

## MATERIALS AND METHODS

### Preparation of native enzyme

The enzyme was purified by a modification of the procedure previously published for the preparation of *Naegleria* PFK [4]. Trophozoites of *E. histolytica* (HM-1) were maintained axenically in TY1-S-33 medium [5] in glass tubes or in Nuclon Triple Flasks. Amoebae [(3–4) × 10<sup>8</sup>] from 3-day cultures were harvested by chilling followed by centrifugation at 500 *g* for 5 min. Cells were washed twice with 10 vol. of 5.7 mM phosphate buffer, pH 7.2, containing 137 mM NaCl and 2.7 mM KCl, and suspended in approx. 2 vol. of ice-cold buffer consisting of 20 mM Pipes, pH 7.0, 0.1 mM EDTA, 1 mM dithiothreitol (extraction buffer) plus 1 mM PMSF. The cells were then lysed with a sonic dismembrator (Fisher Scientific). The suspended cells were sonicated at a setting of 4 for three 10 s bursts, with chilling on ice between each sonication. Polyethyleneimine (10% aqueous solution) was added to the extract to a concentration of 0.1% to precipitate the nucleic acids. After centrifugation, the supernatant was loaded on to a 120 ml Sephadex G-25 column equilibrated with extraction buffer for desalting. The active fractions were pooled and loaded on to a 300 ml column of Whatman P11 pre-equilibrated with extraction buffer. The column was washed with the extraction buffer until the A<sub>280</sub> was below 0.02. The enzyme was eluted with the same buffer containing 0.075 mM fructose

Abbreviations used: ATP-PFK, ATP-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11); PP<sub>i</sub>-PFK, PP<sub>i</sub>-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90); RACE, rapid amplification of cDNA ends.

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The nucleotide sequence of *Entamoeba histolytica* PP<sub>i</sub>-dependent phosphofructokinase has been deposited with EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF013986.

1,6-bisphosphate. Active fractions were pooled, concentrated to a volume of approx. 10 ml and applied to a Pharmacia Mono Q HR 5.5 column pre-equilibrated with 20 mM Tris/HCl (pH 7.2)/0.1 mM EDTA. The enzyme was eluted with a linear gradient of NaCl from 0 to 500 mM in the same buffer; it was eluted at a salt concentration of approx. 100 mM.

### Protein chemistry

The purified preparation of PFK was precipitated with 10% trichloroacetic acid and collected by centrifugation. The sediment was washed twice with ice-cold 95% acetone, dissolved in 5% NH<sub>4</sub>OH and lyophilized. Thiol groups were modified by reaction with *n*-isopropyl iodoacetamide as described by Krutzsch and Inman [6]. The protein was digested in 0.1 M Tris/HCl, pH 8.0 plus 10% acetonitrile and 1% RTX-100 with trypsin or endoproteinase LysC as suggested by Fernandez et al. [7]. The digest was lyophilized, and the dry sediment dissolved in 0.05% trifluoroacetic acid. Peptides were resolved on a wide-pore C<sub>4</sub> reverse-phase HPLC column on an Applied Biosystems 130A separation system using a linear gradient from 0.05% trifluoroacetic acid to 80% acetonitrile containing 0.042% trifluoroacetic acid. Several well-separated peptides were subjected to Edman degradation on an Applied Biosystems 470A gas-phase protein sequencer with on-line identification of phenylthiohydantoin derivatives using protocols suggested by Tempst and Riviere [8].

### Molecular sizing

The mass of the native enzyme was determined on a Pharmacia FPLC system fitted with a Superose 12 column pre-equilibrated with 50 mM Tris/HCl (pH 7.2)/1 mM EDTA. A standard curve was constructed by using a mixture of standards: cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa) and  $\beta$ -amylase of sweet potato (200 kDa).

### Library screening

Because introns have not been described in *E. histolytica*, it was appropriate to choose a genomic library for cloning. Degenerate primers were designed according to the peptide sequence derived from the purified native enzyme. Potential degeneracy was reduced by considering the codon usage of *E. histolytica*, which has a very high A + U content in the third codon position. The PCR product was used to screen an *E. histolytica*  $\lambda$  ZAPII genomic library, kindly provided by Dr. J. Samuelson of the Harvard School of Public Health, Cambridge, MA, U.S.A. Positive clones were sequenced using the Thermo sequenase radiolabelled terminator cycle sequencing kit (Amersham).

### Amplification of the 5' end by rapid amplification of cDNA ends (RACE)

*E. histolytica* RNA was isolated with a Qiagen DNA/RNA isolation kit. Reverse transcription was performed by using a gene-specific primer. A single-stranded anchor oligonucleotide was ligated to the 3' end of the cDNA by T<sub>4</sub> RNA ligase. After anchor ligation, the cDNA was used as template for PCR amplification, using a nested gene-specific primer and a primer complementary to the anchor. The PCR product was then cloned into the pCR-Script cloning vector (Stratagene) and sequenced.

### Amplification of the 3' end by PCR

A gene-specific primer and a vector primer of the SK<sup>-</sup> was used to PCR-amplify the 3' end of the gene by using the *E. histolytica*  $\lambda$  ZAPII cDNA library, kindly provided by Dr. E. Tannich of the

Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany. The PCR product was cloned and sequenced.

### Expression of *E. histolytica* PP<sub>1</sub>-PFK gene

Two primers, 60K-S1 (TCGCATATGTCTCTTTCTGCTCTC-CACA) and 60K-A1 (CTAGTCTAGACA A TAAGTTCACC-CAACAATCA), designed on the basis of the gene sequence of the 5' and 3' end respectively and adding nucleotides to generate *Nde*I and *Xba*I restriction sites, were used to PCR-amplify the PFK gene from total amoebal DNA, which was isolated with a Qiagen DNA/RNA isolation kit. The PCR product was digested with *Nde*I and *Xba*I and cloned into the complementary sites of the pAlter-Ex1 expression vector. The recombinant plasmid was transformed into JM109 *Escherichia coli* host which was subsequently grown on Luria-Bertani medium at 37 °C. Expression was induced by adding 0.4 mM isopropyl thiogalactoside to the bacterial culture after it reached an A<sub>600</sub> of 0.8. The incubation was continued for 4 h.

### Purification of recombinant enzyme

The procedure was similar to that for the native enzyme with the following exception. After nucleic acid precipitation, solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to the supernatant solution to a saturation of 65% to precipitate the protein. The sediment was then suspended in the Pipes buffer described above and loaded on to the Sephadex G-25 column.

### Kinetic assays

Enzyme activity was assayed spectrophotometrically at wavelength 340 nm. The assay solution contained 20 mM Tes, pH 7.2, 3 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2 mM NADH, 1 mM sodium pyrophosphate, 1 mM fructose 6-phosphate, 2–6 units each of aldolase, triose phosphate isomerase and glycerol-3-phosphate dehydrogenase. The auxiliary enzymes were dialysed against 50 mM Tes (potassium salt; pH 7.2)/1 mM EDTA before use. The reaction temperature was 30 °C. One unit of enzyme activity was defined as the amount of enzyme producing 1  $\mu$ mol of fructose 1,6-bisphosphate/min under the standard assay conditions. Apparent *K<sub>m</sub>* and *k<sub>cat</sub>* were measured by fixing one substrate at 1.5 mM and varying the other substrate concentration. The *k<sub>cat</sub>* values were based on a subunit mass of 60 kDa. Kinetic parameters were calculated by using the GraFit graphical analysis program. Standard errors of intercepts and slopes were all less than 10%. The pH-dependent assay was carried out using a 25 mM Pipes/25 mM Tes buffer at different pH values with 1.5 mM PP<sub>1</sub> and 1.5 mM fructose 6-phosphate. Precautions were taken to ensure that the auxiliary enzymes in the assay were not rate-limiting at the extremes of pH examined.

### Non-denaturing PAGE

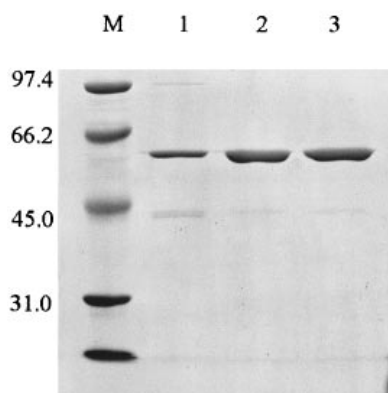
Crude extract from *E. histolytica* was electrophoresed in a 10% non-denaturing polyacrylamide gel at 4 °C. After electrophoresis, enzyme activities on the gel were located by a modification of the method described previously for ATP-PFK [9]. The staining solution contained 50 mM Pipes, pH 6.8, 10 mM NaHAsO<sub>4</sub>, 2 mM EDTA, 1 mM fructose 6-phosphate, 1 mM sodium pyrophosphate, 4 mM MgCl<sub>2</sub>, 1 mM NAD<sup>+</sup>, 0.24 mg/ml glyceraldehyde-3-phosphate dehydrogenase, 0.1 mg/ml aldolase, 7  $\mu$ g/ml triose phosphate isomerase, 0.24 mg/ml phenazine methosulphate and 0.4 mg/ml Nitro Blue Tetrazolium.

## RESULTS

### Purification of native enzyme and gene cloning

Purification of the native enzyme by substrate elution from the Whatman P11 phosphocellulose column step revealed four proteins in fractions containing activity. SDS/PAGE of the purified enzyme is shown in Figure 1. Approx. 90% of the protein was about 60 kDa in mass. Two proteins with masses close to 42 kDa were seen, and the fourth protein had a mass close to 100 kDa. No protein was observed that had a mass similar to the 47.6 kDa gene product described by Bruchhaus et al. [3]. The 60 kDa enzyme was purified to near-homogeneity by ion-exchange FPLC on a Mono Q column. The purified enzyme had a specific activity of 316 units/mg. The purification data are reported in Table 1.

Trypsin digestion of the purified protein produced 11 peptides which could be easily resolved in high yield, suggesting that they came from the major 60 kDa component of the preparation. Attempts to match these peptides to the sequence of the *E. histolytica* PFK described previously [3] were unsuccessful. On the other hand, many of the peptides were similar to but not identical with peptide sequences from the  $\beta$ -subunit of potato PFK. Degenerate oligonucleotide primers with sequences of GAAAT(T/A)GAAGG(A/T)AA(A/G)CC(A/T)TTT (sense primer) and (A/G)G(T/C)TTAGT(T/A)GGTTGATCACA (antisense primer) corresponding to the peptide sequences EIEGKPF and CDQPTKT respectively were used for PCR with the *E. histolytica* genomic DNA as template. The translated sequence of the 150 pb PCR product was very similar to the C-



**Figure 1** SDS/PAGE of PP<sub>i</sub>-PFK from *E. histolytica*

The acrylamide was 10% and staining was performed with Coomassie Blue. Lane M, molecular-mass standards (kDa); lane 1, native PP<sub>i</sub>-PFK after P11 column chromatography; lane 2, native PP<sub>i</sub>-PFK after Mono Q column chromatography; lane 3, recombinant PP<sub>i</sub>-PFK after Mono Q column chromatography.

**Table 1** Summary of *E. histolytica* native PP<sub>i</sub>-PFK purification

Purification was carried out as described in the Materials and methods section.

	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Recovery (%)
Crude extract	23	230	117	1.96	100
P11 column	10	100	0.351	285	43
Mono Q column	1.5	55	0.174	316	24

terminal portion of the potato PFK. A second round of PCR was performed by using an exact-match antisense primer based on the first PCR product and a degenerate sense primer, AATAC(A/C/T)GGAGGATTTGATTTAGT(A/T)GG (Figure 2), based on a peptide that was expected to be closer to the N-terminus of the enzyme. The 1.1 kb PCR product was labelled by random hexamer labelling and used to screen the genomic library. Three positive clones were isolated and sequenced. Unfortunately, all three clones were truncated at about 360 bp from the 5' end of the gene, which results from the presence of an *EcoRI* site that had not been blocked during the original construction of the genomic library. The remainder of the coding sequence was determined not from the genomic sequence, but from mRNA. The upstream sequence from the truncated site was obtained by RACE PCR using RNA isolated from the organism and utilizing a primer for cDNA synthesis based on a sequence 172 bases downstream from the truncation site (GACACAATCTGAACCATGTGCAGC) (Figure 2). The 5' terminus was amplified with the linker primer and a nested primer 124 bases downstream from the truncation site (TAATGCTGTAATATGTTTAAAAGC) (Figure 2). The 3' untranslated region of the cDNA library was determined from the cDNA library.

The open reading frame was 1638 bp long, encoding a protein of 546 amino acids. The calculated mass was 60575 Da. The untranslated region at both ends was short; only 17 nucleotides were detected upstream and 20 nucleotides were observed between the termination codon and the poly(A) tail. Within this short downstream region there was no obvious poly(A)-recognition site. It is interesting that, in the gene sequence 23 nucleotides downstream from the poly(A) site, there is a series of three ATAAA sequences within a sequence of 28 bases. The genomic sequence of the 3' terminus and the downstream sequence containing the AT-rich region are shown in Figure 2.

The entire coding sequence was PCR-amplified from genomic DNA as described in the Materials and methods section. The entire coding sequence was sequenced and revealed no differences from the sequence derived from the cDNA or the RACE cloning. The PCR product was used for expression of the recombinant enzyme.

### Enzyme expression and purification of the recombinant enzyme

The recombinant enzyme was purified as described in the Materials and methods section. The purified enzyme showed a single band on SDS/PAGE (Figure 1). Figure 1 also indicates that the mass of the monomer of the recombinant PFK was the same as that of the native PFK isolated from the cultured organism. The specific activity of the purified recombinant enzyme was 310 units/mg, again very similar to that of the enzyme purified from trophozoites. Approx. 250 units of activity could be isolated per litre of culture.

### Aggregation state

The molecular mass of the PFK in non-denaturing conditions was about 100 kDa, as determined by Superose 12 column chromatography. This suggests that the PFK exists as a dimer of two identical subunits.

### Identity of the PFK expressed in *E. histolytica* trophozoites

As indicated above, the enzyme isolated from the organism as well as the recombinant enzyme that was cloned had much higher activity than the recombinant enzyme previously cloned by Bruchhaus et al. [3]. The question of the identity of the

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1   TGAATATCTTAATTTTTATGTCCTCTTCTGCTCTCCACAAATATAGACTTCAATATAAACCTGTTTTACCAAACACATTGCTGATATA
1   M S L S A L H K Y R L O Y K P V L P K H I A D I
90  GATAATATTACTATTGAAGAAGGTGCTAAAACCCCAATCTGCTGTTAACCCAAAAGAAGCTTTCAGAATTATTTAAACATACTTATGGACTT
25  D N I T I E E G A K T Q S A V N Q K E L S E L F K H T Y G L
180 CCAATTTGTAATATGTTGCAGGTAAAAATGCTGATATTATAGAGTTATTAGATGTGGTTTTATCTTTTCAGGTGGACCTGCTGCAGGA
55  P I C N I V A G K N A D I H R V I R C G F I L S G G P A A G
270 GGACATAATGTAGTTGCTGGTCTTTTTGATGGTTAATGAAAGGAAATAAGAAAATAAACTTTATGGTTTCAGATGTGGTGGCTGGAGGA
85  G H N V V A G L F D G L M K G N K E N K L Y G F R C G A G G
360 ATTCTTTCAAATGATTATATTGAAATTACTGCAGAACTTGTGATAAACATCGTAAATACAGGTGGATTGATCTTGTGGTTCTGGAAGA
115 I L S N D Y I E I T A E L V D K H R N T G G F D L V G S G R
450 ACAAGATTGAAACTGAAGAACAATTTGCTACTGCTTTTTAAACATATTACAGCATTAACCTTAATGCTATGGTTGTAGTTGGTGGTGTAT
145 T K I E T E E Q F A T A F K H I T A L K L N A M V V V G G D
540 GATTCTAATACTAATGCTGCTCTTCTGCTGAATACTTTGCTGCACATGGTTTCAGATTGCTCTTTGTTGGAGTACCAAAAACATATTGAT
175 D S N T N A A L L A E Y F A A H G S D C V F V G V P K T I D
630 GGTGATCTTAAGAATCAATATATTGAAACATCATTGGATTGATACTGCTGTGAAAACATATTCTGAGCTTATTGGAAATATTCAAAGA
205 G D L K N Q Y I E T S F G F D T A C K T Y S E L I G N I Q R
720 GATGCTATTTCATCAAGAAAATATTGGCACTTTTATAAGGTAATGGGAAGATCTGCTTCTCATATTGCTCTTGAAGCTGCTTGAAGACA
235 D A I S S R K Y W H F I K V M G R S A S H I A L E A A L E T
810 CAACCAATATTTGATTTTTCAGAAGAAGTTGAAGATAAGAAGATGACAGTTAGTCAAAATGCTTCAGAAATTCAGATATTGTTATT
265 Q P T Y C I I S E E V E D K K M T V S Q I A S E I A D I V I
900 GAAAGACATAAGAAAGGATTAACTTTGGTGTAGTATTAATCCAGAAGGACTTGTGAAATTTATCCAGAAGTTATTGCACCTATTAA
295 E R H K K G L N F G V V L I P E G L V E F I P E V I A L I K
990 GAACTTAATAATCTTCTTGCACACAAGAAAGAATACTCTAAATTAATGAAATTTCTCAGCTCAAAAAGCTTTTGTGTTGTGAGAATATT
325 E L N N L L A H K K E E Y S K C I T E F S A Q K A F V C E N I
1080 TCTGAAAGTTGCTGCTACTTTCAAGAACCTCCAGATAACATTCGTAAACAACCTTCTTCTTGACAGAGATCCCATGGAATGTAAT
355 S E S C A A T F K N L P D N I R K Q L L L D R D P H G N V N
1170 GTTTCAGCTATTGAAACTGAATCATTGTATCAGGAATTGTTAAAGCTGAAATTTGTTAAGAGAGTATTAAAGTTCCATTTACTCCAGTT
385 V S A I E T E S F V S G I V K A E I V K R G I K V P F T P V
1260 CATCACTTCTTTGGATATGAAGGAAGATGTGCCCTCCCAAGCAATTTTGATTTCTACATATTGTTATGCACTTGGATATACTGCCTTTATT
415 H H F F G Y E G R C A F P S N F D S T Y C Y A L G Y T A F I
1350 TTACTTGCCTTAAAGAAAACAGGACAAATTTGTTGTATTTCAGGACTTCAAAAACCAGCAGAAGAATGGATTTGTGGAGGTGTTCCATTA
445 L L A L K K T G Q I C C I S G L Q K P A E E W I C G G V P L
1440 ACTATTATGATGAATATGGAACAAAGAAATGGTGAATGAAACCAGTTATTAAGAAAGCTTGTGTTGAAATGAAAGAAAACCATTTAA
475 T I M M N M E Q R N G E M K P V I K K A L V E I E G K P F K
1530 TTCTATCAATCCAACGAGCTCAATGGGCATCAGCTGAAGATTTGTATTTCCAGGTGCTATTCAATACTTTGGTCCATCTGAAGTATGT
505 F Y Q S K R A Q W A S A E D F V F P G A I O Y F G P S E V C
1620 GATCAACCAACTAAGACTTCTCTCTTGAACAAAATTAATTTAGACTTCTTTTTGTTTTAAAAAAAAAAAAAAAAAAAA
535 D O P T K T L L L E Q N

gatcaaccaactaagactcttctccttgaacaaaattaatttagacttctttttgtttaattgttttttgttaaagaatatatt
aaaaaattaaattatttaattaaatat

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**Figure 2** Nucleotide and deduced amino acid sequence of *E. histolytica* PP<sub>1</sub>-PFK gene

The underlined amino acid sequences are those that were determined by protein sequencing of peptides. The lower-case letters indicate the genomic sequence for the 3' region of the gene. The solid underlined DNA sequences indicate the positions of the primers used in the initial PCR cloning. The dotted underlined DNA sequences indicate the positions of the primers used for the RACE cloning.

47.6 kDa and 60 kDa preparations with PFK activity found in crude extracts of the organism remains unanswered. To address this question, the properties of the recombinant enzyme, the enzyme purified from trophozoite extracts and the total PFK activity in trophozoite extracts were compared. The three preparations were assayed at various substrate concentrations and their  $K_m$  values were compared (Table 2). Because the data were obtained at a single concentration of the second substrates, the values are apparent  $K_m$ s. The values determined for  $K_m$  and  $k_{cat}$  for the purified and recombinant enzymes were very similar and the apparent  $K_m$  for the activity in the crude extract was not significantly different from those of the other preparations. No co-operativity was observed in the substrate-dependence of the activity.

The three preparations also exhibited similar pH-dependence patterns (Figure 3), with the optimum pH between 6.4 and 7.2. This suggested that the recombinant PFK was the same as the

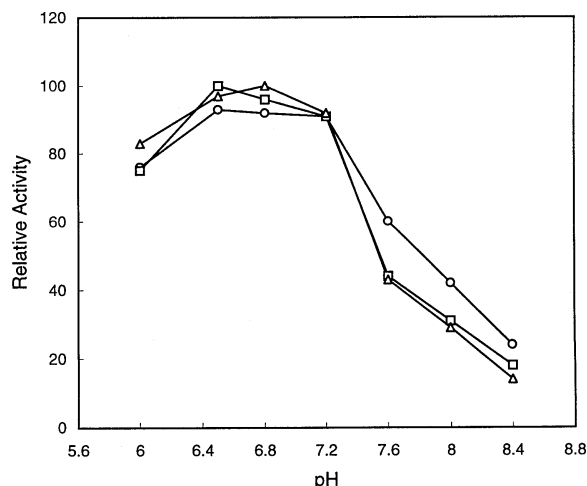
native PFK and that virtually all PFK activity in trophozoites is largely, if not exclusively, associated with the 60 kDa enzyme.

Although the foregoing indicates that most if not all of the PFK activity in trophozoite extracts can be accounted for by the 60 kDa enzyme, there remained the possibility that a small amount was associated with the lower-molecular-mass form previously characterized. Furthermore, the possibility existed that the smaller subunit is associated with the 60 kDa subunit in a manner reminiscent of the heterotetramers seen in plant PP<sub>1</sub>-PFKs [10]. The smaller subunit may be lost during purification. To investigate these possibilities, native PAGE was performed. The results (not shown) indicate a single band of PFK activity. A second activity would have been detected if it represented 10% or more of the total PFK activity. The active band was excised from the gel, denatured and placed on a denaturing polyacrylamide gel. On electrophoresis and staining, a major band at 60 kDa was observed. Although several other less intensely

**Table 2** Kinetic constants of the *E. histolytica* PP<sub>i</sub>-PFK

Assays were performed as described in the Materials and methods section.  $K_m$  and  $k_{cat}$  were measured by fixing one substrate at 1.5 mM and varying the second substrate, F6P, fructose 6-phosphate.

	$K_m$ ( $\mu$ M)		$k_{cat}$ ( $s^{-1}$ )
	PP <sub>i</sub>	F6P	
Crude extract	39	100	—
Native PFK	40	85	344
Recombinant PFK	42	105	325

**Figure 3** pH-dependence of the *E. histolytica* PP<sub>i</sub>-PFK

○, Crude extract from *E. histolytica* trophozoites; △, recombinant PP<sub>i</sub>-PFK; □, native PP<sub>i</sub>-PFK. Assay conditions were as described in the Materials and methods section.

staining bands were also observed, none had a mass in the region of 47 kDa, i.e. the mass of the protein described by Bruchhaus et al. [3].

## DISCUSSION

This laboratory previously reported a partial sequence for *E. histolytica* PP<sub>i</sub>-PFK that was cloned on the basis of conserved sequences in the active site of PP<sub>i</sub>-PFKs [2]. Subsequently, Bruchhaus et al. [3], also using homology cloning with degenerative primers, reported the full sequence and demonstrated the presence of the transcript in a cDNA library. In the present work, the PP<sub>i</sub>-PFK isolated from axenically grown cultures of *E. histolytica* was clearly different from the previously described structures. The enzyme described here has a higher molecular mass, much higher specific activity and a distinctly different amino acid sequence. The data suggest that the 60 kDa enzyme represents at least 90%, if not all, of the PFK activity present in extracts of trophozoites grown under the conditions described here.

The 47.6 kDa PFK described in the earlier study is clearly an expressed product because it was cloned from a cDNA library [3]. It may have been present in the extracts of the organism but did not co-purify with the 60 kDa product. Furthermore, if a second activity had been present that represented at least 10% of

the total PFK activity, it would have been detected by native PAGE. The problem with attributing a significant role to the 47.6 kDa protein in the phosphorylation of fructose 6-phosphate is its extremely low specific activity. The specific activity of the 60 kDa enzyme is about 2000–3000 times higher than that reported for the smaller PFK [3]. Thus, if expressed at the same level in the organism, the smaller PFK would be virtually undetectable under normal assay conditions. A possible explanation is that the smaller PFK does not phosphorylate fructose 6-phosphate as its primary function. It may have another as yet undetermined catalytic activity.

Another possible explanation is that the 47.6 kDa protein represents a regulatory protein, as observed in the multi-subunit structure of plant PP<sub>i</sub>-PFKs [10]. In the instance of the plant enzymes, the catalytic and regulatory subunits co-purify. This is unlikely because not only was no 47.6 kDa protein present in the partially purified fractions of the 60 kDa enzyme, native PAGE of the extract did not show co-migration of the two proteins. Thus any direct interactions between the two proteins would have to be very weak if indeed there are any.

Again using the model provided by the regulated PP<sub>i</sub>-PFKs of plants, differential expression of the catalytic and regulatory subunits have been observed under different conditions of growth [11,12]. It is possible that in *E. histolytica* the 47.6 kDa protein or both proteins are simultaneously produced under growth conditions different from those described here for the trophozoites. Expression may be associated with different phases of the growth cycle *in vitro* or the life cycle in nature.

All known PP<sub>i</sub>-PFK sequences, including the 59 kDa PP<sub>i</sub>-PFK, were aligned as described by Alves et al. [13]. A neighbour-joining phylogenetic analysis of the data (not shown) places the 60 kDa protein in a tight cluster with the plant enzymes and *Giardia* PFK. On the other hand, the 47.6 kDa *Entamoeba* enzyme clusters with *Naegleria* PP<sub>i</sub>-PFK and the bacterial PP<sub>i</sub>-PFKs [13]. Sequence identity between the two *Entamoeba* PFKs is only 17%, whereas the identity between the large *Entamoeba* enzyme and the  $\beta$ -subunit of potato PFK is 46%. The 47.6 kDa enzyme has 22% identity with *Propionibacterium* PFK, whereas the 60 kDa enzyme has only 17% identity with the bacterial enzyme. It is obvious that the two *Entamoeba* enzymes are quite distinct; the sequence of one, the larger of the two, may be considered more advanced phylogenetically, whereas the smaller is a more primitive bacterial-like enzyme. Rosenthal et al. [14], on the basis of the similarity of a number of *Entamoeba* gene sequences to those of eubacteria, have suggested that some genes of *Entamoeba* may have been acquired from bacteria by a process of horizontal gene transfer. The 47.6 kDa protein as a second PFK may represent an example of this process.

Although the overall sequence identity among all known PP<sub>i</sub>-PFK genes is quite low, the sequence identity among residues thought to be catalytically relevant is fairly high. On the basis of site-directed mutagenesis studies of the PP<sub>i</sub>-dependent PFK from *Propionibacterium* [15,16], a number of residues were identified as catalytically important. Most of these residues can be readily aligned with identical residues in the 60 kDa enzyme. The two catalytically important Asp residues of *Propionibacterium* PP<sub>i</sub>-PFK are found within the sequence TIDXD (residues 202–206) of the 60 kDa enzyme that is seen in all PP<sub>i</sub>-dependent PFKs. The arginine at position 422 can be aligned in all PP<sub>i</sub>-PFKs, and its homologue in *Propionibacterium* has been found to be crucial to the binding of fructose 6-phosphate. The sequence MGR (position 249–251) is found in all PP<sub>i</sub>-PFKs and in most ATP-dependent PFKs as well. Furthermore, the arginine of that sequence has been recently found by mutagenesis to be important for the activity of *Naegleria* PP<sub>i</sub>-PFK (J. Xu and R. G. Kemp,

unpublished work). One other sequence of interest is GGDD (positions 171–174). This sequence or its variation GGED is found in all known PP<sub>1</sub>-PFKs with one exception, the 47.6 kDa *Entamoeba* enzyme in which the sequence is GGDG. The latter sequence is that found in all ATP-PFKs. It is possible that this variation found in the 47.6 kDa enzyme is responsible for its reported low activity.

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