Pyrophosphate-dependent phosphofructokinase of *Entamoeba histolytica*: molecular cloning, recombinant expression and inhibition by pyrophosphate analogues

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By using oligonucleotide primers derived from regions highly conserved in prokaryotic and eukaryotic phosphofructokinase sequences, a genomic DNA fragment was amplified and used to isolate cDNA and genomic clones coding for PP_i-dependent phosphofructokinase (PP_i-PFK) of *Entamoeba histolytica*. The open reading frame consists of 1308 bp and the corresponding protein has a calculated molecular mass of 47.6 kDa. The N-terminal half of the protein shows 27–35% identity with PP_i-PFKs or ATP-dependent phosphofructokinases (ATP-PFKs) of various eukaryotic and prokaryotic organisms. The amino acid residues that form the active site of the PP_i-PFK from *Propionibacterium freudenreichii* and the allosteric ATP-PFK from *Escherichia coli* are conserved within the amoeba sequence. The PP_i-PFK was recombinantly expressed by using a prokaryotic expression system. The purified recombinant protein was found to be enzymically active. The K_m values for PP_i and fructose 6-phosphate of the native and the recombinant PP_i-PFKs were nearly identical. Various bisphosphonates (synthetic pyrophosphate analogues) were tested for their ability to inhibit PP_i-PFK activity or amoebic growth. All bisphosphonates tested were competitive inhibitors for amoeba PP_i-PFK activity. The best inhibitors were CGP 48048 and zoledronate, with K_i values of 50 μM. All bisphosphonates inhibited amoebic growth. One of them (risedronate) was inhibitory at a concentration of 10 μM. Bisphosphonates are therefore potential therapeutic agents for the treatment of amoebiasis.

INTRODUCTION

Entamoeba histolytica is a protozoan parasite that infects about 50 million people each year and may cause potentially life-threatening diseases such as haemorrhagic colitis and/or extra-intestinal abscesses.

En. histolytica is an ancestral protozoan organism that evolved shortly after the divergence of prokaryotes and eukaryotes [1,2]. It possesses some ancient metabolic pathways such as glycolysis that differ substantially from those of higher eukaryotes. Parasitic protozoans without mitochondria, such as *En. histolytica* and some prokaryotes, possess enzymes within their glycolytic pathway that are dependent on pyrophosphate [3]. One example of PP_i-dependent enzymes in *En. histolytica* is phosphofructokinase (PFK; EC 2.7.1.90). In most eukaryotes an ATP-dependent PFK (ATP-PFK) is present, which is a key regulatory enzyme of glycolysis. It catalyses the formation of fructose 1,6-bisphosphate (Fru 1,6-P_2) from fructose 6-phosphate (Fru 6-*P*). An exception to this rule was first described by Reeves et al. [4,5], who showed that *En. histolytica* contains a pyrophosphate-dependent PFK (PP_i-PFK). Subsequently, PP_i-PFK activity was detected in other protozoans, including the ciliates *Isotricha prostoma* and *Trimyema compressum*, the flagellates *Tritrichomonas foetus*, *Trichomonas vaginalis*, *Giardia lamblia*, *Toxoplasma gondii* and *Naegleria fowleri*, and the photosynthetic protist *Euglena gracilis* [3,6–10]. PP_i-PFKs are also present in higher plants and prokaryotes [11–13]. Two types of PP_i-PFK are known. The activity of type I, which is a homopolymer with a subunit molecular mass of 40–50 kDa, is independent of fructose 2,6-bisphosphate (Fru 2,6-P_2). Type I PP_i-PFK, which does not coexist with ATP-PFK, was found in protists and prokaryotes. Type II PP_i-PFK is stimulated by Fru 2,6-P_2, coexists with ATP-PFK and has a molecular mass larger than that of type I PP_i-PFK. It is found exclusively in photosynthetic organisms, higher plants and *Eu. gracilis* [10].

The critical role of PFK in the energy metabolism of *En. histolytica*, together with its difference from the human host PFK, makes it an attractive target for antiamoebic agents [14,15].

Here we report on the cloning, sequencing and recombinant expression of *En. histolytica* PP_i-PFK. Furthermore pyrophosphate analogues were tested for their ability to inhibit either amoebic PP_i-PFK or growth of *En. histolytica*.

MATERIALS AND METHODS

Isolation and sequencing of cDNA and genomic clones

Two oligonucleotide primers PFK-S24 (5′-CCT AAA ACA/T ATT GAT AAT GAT ATT) and oligo(dT)18 (5′-GAG AGA GAA TTC TTT TTT TTT TTT TTT TTT) were synthesized on an Applied Biosystems DNA synthesizer. The sequence of the PFK-S24 was deduced from a conserved domain (Pro-Lys-Thr-Ile-Asp-Asn-Asp-Ile) which is found in ATP- and PP_i-PFKs of different organisms (see Figure 3) [16–18]. The two primers and total DNA obtained from λZAP cDNA library of the *En. histolytica* isolate HM-1:IMSS [19] were used for DNA amplification by PCR under standard conditions [20], consisting of 30 cycles each with 1 min of denaturation at 94 °C, 1 min of annealing at 42 °C and 2 min of extension at 72 °C.

The amplified DNA was used to screen a cDNA and a genomic library (kindly provided by Dr. J. Samuelson of the

Abbreviations used: ATP-PFK, ATP-dependent phosphofructokinase; Fru 1,6-P_2, fructose 1,6-bisphosphate; Fru 2,6-P_2, fructose 2,6-bisphosphate; Fru 6-*P*, fructose 6-phosphate; PP_i-PFK, pyrophosphate-dependent phosphofructokinase.

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The nucleotide sequence reported will appear in the EMBL Data Library under the accession number X82173.

Harvard School of Public Health, Boston, MA, U.S.A.) of the *En. histolytica* isolate HM-1:IMSS constructed in the λZAP phage vector. Hybridizing phages were isolated and the plasmids released in accordance with the manufacturer's recommendations (Stratagene, Heidelberg, Germany). Selected inserts were sequenced on both strands by the dideoxy-chain termination method.

Standard DNA and RNA technologies

Total *En. histolytica* RNA and DNA were isolated from trophozoites of the isolate HM:1-IMSS as previously described [19]. Southern blot and Northern blot analyses were performed according to published procedures [21], with 10 μg of DNA and 20 μg of RNA. Blots were hybridized with the radiolabelled probe by using the Prime-a-Gene Labeling system (Promega, Madison, WI, U.S.A.). For primer extension experiments the oligonucleotide primer PFK-AS20/1 (5′-TGAAGTTCCTAATA TACTTCC) was used. The nucleotide sequence of the extension product was determined by using the procedure described by Frohman et al. [22]. Briefly, after reverse transcription of poly(A)$^+$ RNA with the primer PFK-AS20/1, the complementary DNA was tailed in a terminal deoxynucleotidyl transferase reaction with dGTP. Using an oligo(dC) primer and a second oligonucleotide primer (PFK-AS20/2; 5′-TTAATGAAGGAAGTG-GTGCG), the tailed cDNA was amplified by polymerase chain reaction, subcloned into the plasmid vector pBS (Stratagene) and sequenced.

Recombinant expression and purification of *En. histolytica* PP_i-PFK

The complete coding region of the *En. histolytica* PP_i-PFK gene was amplified by polymerase chain reaction by using the two oligonucleotide primers PFK-S30 (5′ CAA CAA CAT ATG TCA GTT AAA AGA AGA GAC) and PFK-AS31 (5′ ATT GGA TCC ATT TAC TTT GGC TTT TCA ATA G). The primers contain *Nde*I and *Bam*HI restriction sites, allowing rapid cloning of the amplified DNA in a predicted orientation into the prokaryotic expression plasmid pJC45 [23]. pJC45 is a derivative of pJC40 and encodes a N-terminal histidine tail of 10 residues, which is added to the gene product [23]. Recombinant plasmids are transformed into Bl21(DE3)[pAPlacIQ] and the bacteria are plated on to Luria broth agar plates [100 μg/ml ampicillin, 50 μg/ml kanamycin, 2% (w/v) glucose]. Freshly transformed single colonies were inoculated into Luria broth medium [100 μg/ml ampicillin, 50 μg/ml kanamycin, 2% (w/v) glucose] and grown at 37 °C until D_{600} reached 0.2. Subsequently isopropyl-β-D-thiogalactoside was added (final concentration 1 mM) and incubation was continued for an additional 3 h. Purification of recombinant PP_i-PFK was performed by using Ni-NTA-Resin following the manufacturer's recommendations for native purification of cystoplasmic proteins (Qiagen GmbH, Hilden, Germany).

Protein concentrations were determined by the method Bradford [24], with BSA as standard. Aliquots of fractions were analysed by SDS/PAGE (12% gel).

Purification of *En. histolytica* PP_i-PFK

Protein purification was performed from trophozoite lysates of the *En. histolytica* isolate HM-1:IMSS cultured axenically in TYI-S-33 medium [25]. Approx. 3×10^8 cells at late-exponential growth phase were harvested by chilling on ice for 10 min and centrifuged at 430 ***g*** at 4 °C for 5 min. The resulting pellet was washed twice in PBS, freeze–thawed five times in solid CO_2/ethanol, and sedimented by centrifugation at 150000 ***g*** at 4 °C for 40 min. The 150000 ***g*** supernatant (104 mg total protein) was passed over a column of phosphocellulose (Whatman P-11) previously equilibrated with 7 mM Pipes, 0.01 mM EDTA, pH 6.7 (column buffer). The column was washed with 10 column volumes of the column buffer, and the enzyme was eluted with column buffer plus 0.5 mM Fru 1,6-P_2. The recovery of the enzyme was approx. 65%. All purification procedures were performed on ice at 4 °C.

For inhibition studies the PP_i-PFK was purified by passing the 150000 ***g*** supernatant in two batches of 5.5 ml, containing 170 mg of protein each, over a Hi/Load Superdex 200 HR 16/60 FPLC column (Pharmacia, Uppsala, Sweden) with 20 mM imidazole buffer, pH 7.0. Fractions containing the most of the PFK activity were pooled (35 ml, 55 mg) and applied to a Mono Q HR 5/5 anion-exchange column (Pharmacia). The column was equilibrated with 20 mM imidazole buffer, pH 7.0. Absorbed proteins were eluted by washing the column with the same buffer (40 ml), followed by a linear NaCl gradient from 0 to 500 mM (30 ml). The PFK activity was eluted by 100 mM NaCl. The fractions containing the highest activity were pooled (16 ml, 4 mg). Samples of active fractions were analysed by SDS/PAGE (12% gel) under reducing conditions.

Enzyme assays

The standard assay for PP_i-PFK contained 50 mM imidazole/HCl, pH 7.0, 2.5 mM $MgCl_2$, 1 mM sodium pyrophosphate, 1.4 mM Fru 6-*P*, 0.2 mM NADH, 50 μg of aldolase, 13 μg of glycerol-3-phosphate dehydrogenase–triosephosphate isomerase, and water to a final volume of 400 μl. The reaction was started by addition of sodium PP_i. The assay was done at 25 °C. The rate of change in absorbance was determined at 340 nm (ϵ 6.22×10^3 $M^{-1} \cdot cm^{-1}$).

One unit of activity is defined as the amount of enzyme that produces 1 μmol of fructose biphosphate per min under the conditions of the standard enzyme.

For the determination of K_m values for Fru 6-*P* and PP_i of both native and recombinant expressed PP_i-PFK, the standard PFK assay was used except that the concentration of Fru 6-*P* and PP_i varied between 25 and 150 μM.

The K_i values of PP_i-PFK were calculated from double-reciprocal plots in which the concentration of pyrophosphate varied between 20 and 100 μM and the analogue inhibitor was held at some fixed value.

Chemicals used for inhibition studies

The chemicals used were: 1-hydroxyethylidene-1,1-bisphosphonate (etidronate), dichlormethylene bisphosphonate (clodronate), 3-amino-1-hydroxypropylene-1,1-bisphosphonate (pamidronate), 4-amino-1-hydroxybutylidene-1,1-bisphosphonate (alendronate), 2-(3-pyridinyl)-1-hydroxyethylidene-1,1-bisphosphonate (risedronate), 2-(imidazol-1-yl)-1-hydroxyethylidene-1,1-bisphosphonate (zoledronate; CGP 42446), 3-[*N*-(2-phenylthioethyl)-*N*-methylamino]-1-hydroxypropylidene-1,1-bisphosphonate (CGP 48084), EDTA (Sigma). All bisphosphonates were a gift from Ciba-Geigy AG (Basel, Switzerland).

Growth inhibition experiments

En. histolytica trophozoites of the isolate HM-1:IMSS were cultured axenically in TYI-S-33 medium. Inoculae for experimental tubes and their controls were taken from stocks still in the exponential growth phase. The amoeba were cultured in 24-well plates under anaerobic conditions. Various concentrations

of the bisphosphonates and EDTA were added to the amoebae, maintaining sterility. The inocula were 1000, 2000 and 4000 amoebae. In each growth test the amoebae were cultured over 72 h in the presence of 500, 250, 50 or 10 μM concentrations of the different substances. Serial transfers were made after 72 h to a new tube with the same drug at the same concentration. The amoebae were cultivated for another 72 h. Each experiment was done on three different occasions and each time in duplicate. Amoebae were counted daily.

RESULTS

Primary structure of *En. histolytica* PP_i-PFK

To determine the primary structure of the *En. histolytica* PP_i-PFK, the corresponding cDNA and genomic sequences were analysed. Isolation of these sequences was achieved with an oligonucleotide probe deduced from a conserved domain (positions 207–214 of the *En. histolytica* sequence; Pro-Lys-Thr-Ile-Asp-Asn-Asp-Ile) (Figure 1) that is found in ATP- and PP_i-PFKs of different organisms (see the Materials and methods section). Three different cDNA clones were sequenced. The longest cDNA clone revealed an open reading frame of 1305 bp but lacked an in-frame initiation codon. Primer extension analysis and sequencing of the primer extension product revealed that the initiation ATG and 10 nucleotides were missing from the transcribed product. The full coding sequence revealed an open reading frame of 1308 bp, encoding a protein with a calculated molecular mass of 47580 Da. The cDNA sequence completely matched the genomic sequence, implying a lack of introns for the *En. histolytica* PFK gene (Figure 1).

The sequence motif (ATTCA or ATCA), which is known to be the transcriptional start site in most *En. histolytica* genes, is not present in the PFK sequence [26]. Primer extension analysis mapped the respective transcriptional start site to an adenine residue 10 nucleotides upstream of the translation initiation site. The sequence motif is therefore ACAA. A second consensus sequence at the 5′ region of amoebic genes is the TATA box-like motif TATTTAAA, which is usually found about 30 nucleotides upstream of the start of transcription [26]. This motif is not present. Instead the sequence TATTATAA is found 39 bp upstream of the transcriptional start site (Figure 1).

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-270    ATTTAGAAATAATACAAACCAACTTGTTAAGTTTTATTCCTTAATTCTCTTTTTTAATTAACAATGAAATAAAATGAAGGTGTATTATTT

-180    CTCTTTTCATTGAGCAAATGAGAGATTTTCATACTAATGAAATTAAAGTTTAATGTATGAAAGTAAGATAGAATGAGAATAGAAACTATA

                                                                                              *
 -90    ACACAAAATCATTTTTTTAACAAAAAAAATTATAATAAAATTATTATAAAGAAGTTATTTTGATTGACAAAAACTAAAATAACAACAAAAC

   1    ATGTCAGTTAAAAGAAGAGACCATATATTAATTCCTAAAAACCCAGACGCACCACTTCCTTCATTAAAAATAGAAGAAGTTGGAGAATGT
   1     M  S  V  K  R  R  D  H  I  L  I  P  K  N  P  D  A  P  L  P  S  L  K  I  E  E  V  G  E  C

  91    ACTATTGACAATATTTATGCATCACCAGAACCATTTGTTAATGGTATGACAATGAAATTATCAGCAGTCAAAAATCATGGAATAGAAAGA
  31     T  I  D  N  I  Y  A  S  P  E  P  F  V  N  G  M  T  M  K  L  S  A  V  K  N  H  G  I  E  R

 181    GATAGTGGAGAAGTAGAACTAGCAGGACCAATGGAAAAGATATTTTACAATCCTGAAACAACAAAAGTTGCCATTGTAACATGTGGAGGG
  61     D  S  G  E  V  E  L  A  G  P  M  E  K  I  F  Y  N  P  E  T  T  K  V  A  I  V  T  C  G  G

 271    TTATGTCCAGGATTAAATAATGTTATCAGGGGGTTAGTATTGAATTTATATAATGCTTACCATGTAAACAATATTTTTGGACTAAGATGG
  91     L  C  P  G  L  N  N  V  I  R  G  L  V  L  N  L  Y  N  A  Y  H  V  N  N  I  F  G  L  R  W

 361    GGATATGAAGGACTTGTTCCAGAATTGTCTGAAGTACAAAGACTAACACCAGAAATAGTTAGTGATATTCATCAAAAAGGAGGAAGTATA
 121     G  Y  E  G  L  V  P  E  L  S  E  V  Q  R  L  T  P  E  I  V  S  D  I  H  Q  K  G  G  S  I

 451    TTAGGAACTTCAAGAGGGGCACAAAGTCCAGAGGTTATGGCACAATTTCTTATAGACAATAACTTTAATATTTTATTTACACTAGGAGGA
 151     L  G  T  S  R  G  A  Q  S  P  E  V  M  A  Q  F  L  I  D  N  N  F  N  I  L  F  T  L  G  G
                                                                                    PFK-S24
 541    GATGGTACATTAAGAGGAGCAAATGCAATCAATAAAGAATTAAGAAGAAGAAAAGTTCCAATTACCGTTGTTGGTATTCCTAAAACAATT
 181     D  G  T  L  R  G  A  N  A  I  N  K  E  L  R  R  R  K  V  P  I  T  V  V  G  I  P  K  T  I

 631    GATAATGATATTTGTTATACTGATTCTACATTTGGATTTCAAACAGCTGTTGGACTTTCACAAGAAGCTATTAATGCTGTTCATAGTGAA
 211     D  N  D  I  C  Y  T  D  S  T  F  G  F  Q  T  A  V  G  L  S  Q  E  A  I  N  A  V  H  S  E

 721    GCAAAATCAGCAAAGAATGGGATTGGAATAGTTAGATTAATGGGTAGAGATGCTGGATTTATTGCATTATATGCATCATTAGCTAATGGT
 241     A  K  S  A  K  N  G  I  G  I  V  R  L  M  G  R  D  A  G  F  I  A  L  Y  A  S  L  A  N  G

 811    GATGCTAATTTAGTATTAATTCCAGAGATTGATATTCCAATTACACAAATCTGTGAATTTGTTGGTAAAAGAATAATGTCAAAAGGACAT
 271     D  A  N  L  V  L  I  P  E  I  D  I  P  I  T  Q  I  C  E  F  V  G  K  R  I  M  S  K  G  H

 901    GTTGTTATTGTAGTAGCAGAAGGTGCATTACAAAATCAAAAACCTAAAGATCTTGATTTAGGAACAGATAAATCAGGAAATATTCTTCAT
 301     V  V  I  V  V  A  E  G  A  L  Q  N  Q  K  P  K  D  L  D  L  G  T  D  K  S  G  N  I  L  H

 991    TGGGATTCAATCAATTATTTGAGAGATTCTATTACAAAGTATTTAAAGTCTATTGGAATTGAAGAACATACAATTAAATTTGTTGATCCT
 331     W  D  S  I  N  Y  L  R  D  S  I  T  K  Y  L  K  S  I  G  I  E  E  H  T  I  K  F  V  D  P

1081    TCATATATGATTAGATCAGCTCCTTGTAGTGCTGCTGATGCCCATTTTTGTATGTGTTTAGCTAATGCAGCAGTTCATGTAGCTATGGCA
 361     S  Y  M  I  R  S  A  P  C  S  A  A  D  A  H  F  C  M  C  L  A  N  A  A  V  H  V  A  M  A

1171    GGAAAAACAGGATTAGTTATTTGTCATCACCATAATAATTTTGTTTCAGTACCAATTGATAGAACTAGTTATTATATTAAACGAGTTAAT
 391     G  K  T  G  L  V  I  C  H  H  H  N  N  F  V  S  V  P  I  D  R  T  S  Y  Y  I  K  R  V  N

1261    ACCGATGGACCATTATATACTATGATGACAGCTATTGAAAAGCCAAAGTAAATCAATAAAATTACATTAATAAATTTTTGTCTAAACTTCT
 421     T  D  G  P  L  Y  T  M  M  T  A  I  E  K  P  K  *

1351    TATTTATGAATCTTAAAATCTTATTATTTCATTAATTAAAATAAAAAAAAATTACAAAAGAAGAAAATTATAAATTATTATTTAAAAATTA

1441    GTTTTGTTTGTACACAAATTCTTCATCTTCATCATAACCAAAAGATAAGTTGAGGTAAAATTTTCATTAATGTTGGTTTATCTATTCCACA

1531    AATACTAAGAATATCATCAAATTGAATAACATCTTTACATTCAGCAATTAATTCATTAGTTAAATATCTCGTACTTTTCTAGCAATATCAT
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Figure 1 Nucleotide sequence and deduced amino acid sequence of the gene encoding the PP-PFK_1 of *En. histolytica*

The stop codon is marked by an asterisk. The proposed TATA-box consensus motif as well as the motif ACAA at the start of transcription are underlined. The transcription initiation site determined by primer extension is printed in bold and also marked by an asterisk. The poly(A) tail, as determined by analysis of the cDNA sequence, starts at the underlined position and is printed in bold. The primer sequence for genomic amplification is marked PFK-S24 and underlined.

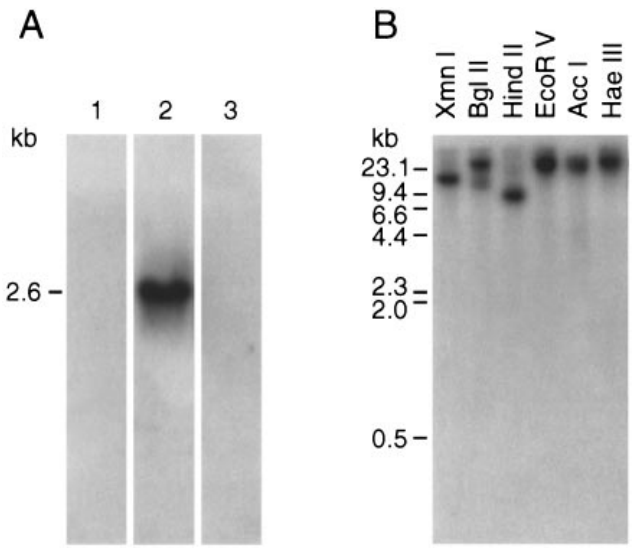

Figure 2 Northern and Southern blot analyses

(**A**) Northern blot. Total RNA (20 μg) from *En. histolytica* was submitted to electrophoresis, blotted and sequentially hybridized under low stringency with the genomic DNA segments of the 5′ untranscribed region (positions −60 to −208; lane 1), the transcribed region (positions 4–1287; lane 2) and the 3′ untranscribed region (positions 1338–1497; lane 3) of the PP_i-PFK gene of *En. histolytica*. (**B**) Southern blot. *En. histolytica* genomic DNA (10 μg) was digested to completion with the restriction enzymes as indicated, submitted to electrophoresis, blotted and hybridized under low stringency with the cDNA probe encoding the PP_i-PFK.

Southern blot analysis showed that the PP_i-PFK is encoded by a single-copy gene (Figure 2). Northern blot analysis demonstrated that the PP_i-PFK is encoded by a single transcript of about 2.6 kb, in contrast with the results determined by analyses of the cDNA, genomic sequences and primer extension (Figure 2). Therefore Northern blot studies with the untranscribed regions upstream (positions −60 to 208 of the nucleotide sequence; Figure 1) and downstream (positions 1338–1497 of the nucleotide sequence; Figure 1) of the PP_i-PFK gene were performed. The Northern blot analysis gave no hybridization signals, indicating that no transcripts for these genomic regions are present (Figure 2). It might be that the analysed genomic clone is a hybrid clone consisting of two unrelated sequences generated during construction of the library. To test the hybrid-clone hypothesis we synthesized two oligonucleotide primers (PFK-S20, 5′-CAATGAAATAAAATGAAGGT; PFK-AS21, 5′-GGATTTCAAACAGCTGTTGGA) derived from the untranslated 5′ region (positions −209 to −190 bp) and from the translated region (positions 664–684 bp). By using these primers and total *En. histolytica* DNA a fragment was amplified by PCR. Sequencing of the 893 bp amplified fragment confirmed its identity with the genomic sequence. Further investigations are necessary to explain the existence of the 2.6 kb PP_i-PFK transcript detected by Northern blot analysis.

Comparison of the deduced amino acid sequence of the N-terminal section from the *En. histolytica* PP_i-PFK (positions 82–263) revealed 35% identity with PP_i-PFK of *Naegleria fowleri* and 27% identity with PP_i-PFKs of *Ricinus communis* (α subunit), potato (β subunit) and *Propionibacterium freudenreichii*. With the human and *Schistosoma mansoni* ATP-PFK, 28% and 33% identity were found. The prokaryotic allosteric ATP-PFK of *Bacillus stearothermophilus*, *Spiroplasma citri* and *Escherichia coli* showed 33%, 28% and 32% identity [16–18,27–31]. Identities among the carboxyl halves were less than 18% when compared with other eukaryotic and prokaryotic PFKs.

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Eh   KVAIV-TCGGLCPGLNNVIRGLVLNLYNAYHVNNIFGLRWGYEGL  125
Nf   PTLGVLVGGGPAPGINGVI-GAVTIEA----INN------GYRVL   51
Pf   KKVALLTAGGFAPCLSSAIELIKRYTEVSPETTLI-GYRYGYEGL   47
Pb   LKIGVVLSGGQAPGGHNVISGIFDYLQTHCKGSTMYGFRGGPAGV   86
Rc   LKIGLVLSGGQAPGGHNVISGIFDYLQDRAKGSILYGFRGGPAGI  125
Sm   ECIAVLTSGGDAQGMNAAVRAVVRMGIYCGCRVF-FIRE-GYQGL   60
Bs   KRIGVLTSGGDSPGMNAAIRSVVRKAIYHGVEVYGVYH--GYAGL   44
Sc   KKIGILTSGGDSQGMNAAIAGVIKTAHAKGLETY-SIIRDGYLGL   46
Ec   KKIGVLTSGGDAPGMNAAIRGVVRSALTEGLEVM--GIYDGYLGL   45
          A                                   A

Eh   GGSILGTS              RGAQSP                    160
Nf   GGSILKTS              RANPTK                     96
Pf   GGSPIGNSRVKLTNVKDLVARGLVAS                       94
Pb   GGFDMICSG             RDKIET                    120
Rc   GGFDMICSG             RDKIET                    159
Sm   GGTKIGSA              RCMDFR                     95
Bs   GGTILYTA              RCPEFK                     77
Sc   GGTVIGSAS             RLPEFK                     80
Ec   GGTFLGSA              RFPEFR                     78
                           AA   A

Eh   LIDNNFNILFTLGGDGTLR-GA                           187
Nf   LQKFNVSLLVTIGGDDTAF-SS                          128
Pf   LIADGVDVLHTIGGDDTNT-TA                          127
Pb   AKKLDLDGLVVIGGDDSNTN-A                          151
Rc   AGKLDLNGLVVIGGDDSNTN-A                          190
Sm   LVKNQITNLVVIGGDGSLT-GA                          127
Bs   LKKHGIEGLVVIGGDGSYQ-GA                          109
Sc   LKKQEIAALVVIGGDGSYQSGA                          113
Ec   LKKRGIDALVVIGGDGSYM-GA                          110
                    AAA A A

                  *******
Eh   ITVVGIPKTIDNDICYTDS--TFGFQTA                     226
Nf   IHVVTLPKTIDNDLPLPYGIPTFGYETA                     166
Pf   LTVVGLPKTIDNDIVPIRQSL--GAWTA                     166
Pb   TRVIGCPKTIDGDLKSKEVPTSFGFDTA                     192
Rc   TRVIGCPKTIDGDLKCKEVPTSFGFDTA                     231
Sm   LNIVGLVGSIDNDFCGTDM--TIGSADS                     182
Bs   FPCVGVPGTIDNDIPGTDF--TIGFDTA                     142
Sc   INCIALPGTIDNDITSSDY--TIGFDTA                     146
Ec   FPCIGLPGTIDNDIKGTDY--TIGFFTA                     143
                  F F F

Eh    GIGIVRLMGRDAGFIAL                               263
Nf    RYFIVVAMGRQAGHLAL                               202
Pf    ELIIHEIMGRNCGYLAA                               203
Pb    YYHFVRLMGRAASHITL                               229
Rc    YYHFVRLMGRAASHITL                               268
Sm    RCFILEVMGRHCGYLAL                               219
Bs    RTYVIEVMGRHAGDIAL                               178
Sc    RCSIVEVMGHACGDIAL                               183
Ec    RISVVEVMGRYCGDLTL                               179
      F      FFF

Eh   KFVDP----SYMIRS                                 366
Nf   KIAFTEKNLGYELRC                                 326
Pf   KTMVQKS--GYFSRS                                 327
Pb   KGQSHFF--GYEGRC                                 390
Rc   KGQSHFF--GYEGRC                                 429
Sm   AEAVLALMDADRDPN                                 334
Bs   ARAVELLLEGKGGRC                                 283
Sc   QFAVEQIIAGVGGLA                                 292
Ec   AYAIDLLLAGYGGRC                                 284
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Figure 3 Alignment of homologous domains of the PFK sequences from *En. histolytica* (Eh), *N. fowleri* (Nf) [26], *P. freudenreichii* (Pf) [17], potato (Pb) [32], *R. communis* (Rc) [28], *Sch. mansoni* (Sm) [27], *B. stearothermophilus* (Bs) [19], *Sp. citri* (Sc) [18] and *E. coli* (Ec) [19]

Residues identical with the *En. histolytica* sequence are printed in bold. The ATP and Fru 6-*P*/Fru 1,6-P_2 binding sites of *E. coli* PFK are marked by characters (A/F) below the respective sequences [30]. Residues essential for the function of PP_i-PFK found in *P. freudenreichii* are underlined. The conserved region that was used for the design of the oligonucleotide primer for PCR is marked by asterisks.

The amino acids that are involved in binding of Fru 6-*P*/Fru 1,6-P_2 and the amino acids building the catalytic ATP-binding site were identified in *E. coli* [32]. Out of 11 amino acids that bind Fru 6-*P*/Fru 1,6-P_2 in the *E. coli* PFK, 6 are conserved in the *En. histolytica* PFK (Thr-209, Asp-211, Asp-213, Met-254, Gly-255

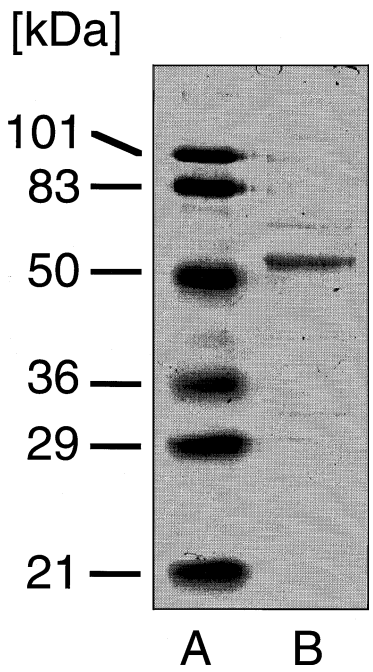

Figure 4 SDS/PAGE of the recombinant expressed PP_i-PFK from *En. histolytica*

Lane A, standards; lane B, PP_i-PFK expressed in *E. coli*.

and Arg-256). Four residues that bind Fru 6-*P*/Fru 1,6-P_2 in the *E. coli* PFK are in the carboxyl half. Owing to the low degree of identity it was not possible to identify these amino acids in the *En. histolytica* PFK. Out of 10 residues that form the ATP-binding site of *E. coli* PFK, 7 are found in *En. histolytica* PP_i-PFK and one represents a conservative substitution (Gly-89, Tyr-122, Arg-155, Asp-181, Gly-182, Thr-183 and Gly-186). The conservation of the ATP-binding site in the PP_i-PFK of *En. histolytica* suggests that PP_i binds to the same site in *En. histolytica* PFK as ATP in the *E. coli* PFK (Figure 3). By using site-directed mutagenesis the amino acid residues that are essential for the catalysis of PP_i-PFK in *P. freudenreichii* were identified [33]. These residues were also found in the *En. histolytica* PFK sequence (Figure 3). In contrast with other eukaryotic and prokaryotic PFKs, the *En. histolytica* enzyme has an additional 60–80 amino acid residues extending the N-terminus. It has the same length as the PP_i-PFK of *Ricinus communis*, but no similarity within this region could be observed.

Purification of the native and the recombinant-expressed *En. histolytica* PP_i-PFK

The native PP_i-PFK was purified on a column of phosphocellulose. The trophozoite extract that was passed over the column contained 104 mg of total protein and had a total activity of 25 units (specific activity 0.24 unit per mg of protein). The active pool of the phosphocellulose column had a total activity of 16 units and contained 0.46 mg of total protein (specific activity 35 units per mg of protein). The purification resulted in a final recovery of approx. 65% and a 146-fold purification. SDS/PAGE analysis of the active fractions after use of the phosphocellulose column revealed a partial purification of the PP_i-PFK but it was not possible to attribute the enzyme activity to a distinct protein band. Further purification of the enzyme led to a rapid loss of enzymic activity.

The PP_i-PFK from *En. histolytica* was recombinantly expressed by using the pJC45 plasmid vector, which encodes an N-terminal histidine tail. The purification of the protein was performed by metal chelate chromatography over Ni-NTA resin. The molecular mass of the purified PP_i-PFK was approx. 50 kDa under reducing conditions (Figure 4). This is in agreement with the calculated molecular mass derived from the amino acid sequence plus the histidine tail.

The recombinantly expressed protein is enzymically active. It was found to contain 0.136 unit per mg of protein. Therefore the purified native enzyme has an approx. 260-fold higher specific activity than the purified recombinant-expressed enzyme. No enzymic activity was detected for either protein if ATP instead of PP_i was used as substrate.

We calculated the K_m values of the native and the recombinant-expressed PP_i-PFKs for each substrate of the forward reaction by linear plots in double-reciprocal co-ordinates. The calculated K_m for PP_i was 15 μM for the native PP_i-PFK, and 18 μM for the recombinant-expressed enzyme. For Fru 6-*P* the kinetic studies revealed apparent K_m values of 40 μM for the native enzyme and 45 μM for the recombinant-expressed PP_i-PFK. Because of the similar K_m values it is highly probable that the native protein corresponds to the isolated gene of the PP_i-PFK in *En. histolytica*.

Table 1 Bisphosphonates as inhibitors of amoebic PPi-PFK and as inhibitors of amoebic growth

Abbreviation used: n.d., not determined.

Bisphosphonate	K_i* (μM)	Inhibitory concentration† (μM)
Risedronate	300	35
CGP 48048	50	38
Zoledronate	50	41
Pamidronate	n.d.	46
Alendronate	400	270
Etidronate	850	560
Clodronate	1200	850

* Values are the average of two determinations, which differed by less than 5%.
† Bisphosphonate concentration inhibiting growth by 50% after 72 h of incubation.

Enzyme inhibition by bisphosphonates

For inhibition studies the PP_i-PFK was partly purified by two successive chromatographic steps (gel filtration through Superdex 200 and anion exchange on MonoQ), resulting in a final recovery of 48% of PP_i-PFK activity and a 41-fold purification (specific activity 9 units per mg of protein). Although this enzyme preparation was not homogeneous, it was free from interfering enzyme activities and could be used for inhibition studies.

Bisphosphonates were used to inhibit amoebic PP_i-PFK. Bisphosphonates are compounds characterized by a P–C–P bond. They are therefore analogues of pyrophosphate, with a carbon instead of an oxygen atom. Table 1 indicates the bisphosphonates tested and the K_i values for the amoebic PP_i-PFK. All bisphosphonates tested were competitive inhibitors of PP_i. The most effective inhibitors were CGP 48084 and zoledronate, each with a K_i of 50 μM. The bisphosphonates tested were not able to inhibit ATP-PFK from rabbit liver.

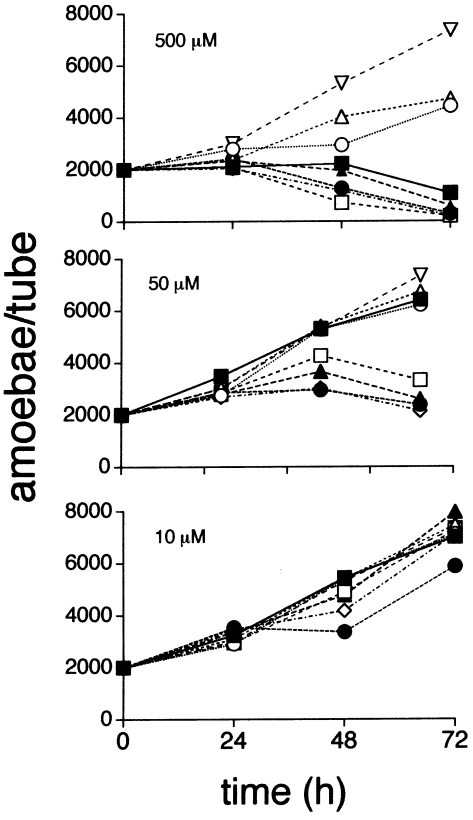

Figure 5 Growth curves of *En. histolytica* in TY-1 medium alone (▽) and in the presence of different bisphosphonates (500, 50 or 10 μM)

Each point represents the average of the results derived from two culture tubes. The following bisphosphonates were tested: ■, alendronate; ○, etidronate; △, clodronate; ●, risedronate; □, pamidronate; ◇, CGP 48084; and ▲, zoledronate.

Amoebic growth inhibition by bisphosphonates and EDTA

The amoebae (inocula 1000, 2000 and 4000 cells) were cultivated for 72 h in the presence of 500, 250, 50 or 10 μM concentrations of the different bisphosphonates. All bisphosphonates tested inhibited amoebic growth. Five of the seven tested bisphosphonates were inhibitory at a concentration of 500 μM. At concentrations of 50 μM an inhibitory effect was seen for zoledronate, CGP 48084, risedronate and pamidronate (Figure 5 and Table 2). To investigate the effects of a longer exposure of *En. histolytica* to the bisphosphonates, transfers were made after 72 h of cultivation to new tubes with the same drug at the same concentration. After a further 72 h the amoebae were counted. The results indicate that risedronate was the most effective inhibitor of amoebic growth, with an inhibitory effect at a concentration of 10 μM (Table 2). To calculate the analogue concentration inhibiting growth by 50%, the amoebae were cultivated in the presence of various concentrations of each bisphosphonate and harvested after 72 h. The bisphosphonate concentrations are listed in Table 1.

EDTA was also tested as an inhibitor of amoebic growth. After 72 h at concentrations of 5 μM EDTA, all amoebae were killed (Table 2).

The same effect of inhibition was observed for bisphosphonates and EDTA if 0.5 mM $MgCl_2$ and/or 0.5 mM $CaCl_2$ were added to the culture medium of *En. histolytica*.

Table 2 Growth of amoebae in the presence of bisphosphonates and EDTA

The inoculum was 2000 amoebae. Each culture was harvested after 72 h of incubation.

	First transfer		Second transfer	
Inhibitor	50 μM	10 μM	50 μM	10 μM
Control	7330	7330	20500	20500
Zoledronate	2580	7960	1500	4470
Risedronate	2370	5870	0	1150
CGP 48048	2130	7115	1320	4450
Pamidronate	3310	7240	14040	17000
Alendronate	6400	7000	17560	21100
Clodronate	6710	7450	28160	23000
Etidronate	6210	7100	20000	19980
EDTA	0*	0	0*	0

* The concentration of EDTA used was 5 μM.

DISCUSSION

Entamoeba histolytica possesses a PP_i-dependent phosphofructokinase different from the ATP-dependent enzyme of its human host [4,5,14]. Because of the critical role in energy metabolism and its differences from the host ATP-PFK, this enzyme is considered to be a good target for chemotherapy of amoebiasis.

In an attempt to analyse the *En. histolytica* PP_i-PFK in more detail, we partly purified the enzyme from amoeba extracts, and isolated and sequenced the corresponding gene. The DNA-derived protein has a calculated molecular mass of 47.6 kDa. Southern blot analysis indicated that the enzyme is encoded by a single copy gene. Recently, Huang et al. [34] also reported on the isolation of a gene coding for a PP_i-PFK in *En. histolytica* but with a calculated molecular mass of 41.5 kDa. The difference in the molecular mass is due to substantial differences in length and primary sequence of the C- and N-terminal parts of the two proteins. The gene described here contains an additional thymidine residue, resulting in a reading frame shift and consequently leading to an extension of the protein-coding region at the 3′ end. In addition, the translation initiation ATG was found to be located 135 nucleotides further upstream of the sequence of Huang et al. [34]. Several reasons can be put forward to confirm the validity of our gene sequence: (i) primer extension mapped the start of transcription 10 nucleotides upstream of start of translation, which is in agreement with the fact that *En. histolytica* mRNAs contain short untranslated 5′ sequences; (ii) all three isolated cDNA clones extend the previously published sequence, and in addition all of them contain the extra thymidine residue found in the genomic sequence; (iii) recombinant expression of the gene revealed an active PP_i-PFK with K_m values for PP_i and Fru 6-*P* rather similar to those of the native *En. histolytica* enzyme.

The N-terminal half of the *En. histolytica* PP_i-PFK showed substantial similarity to various prokaryotic and eukaryotic ATP-PFKs and PP_i-PFKs with amino acid identities between 27% and 35%, whereas no overt similarity was found for the C-terminal half [16–18,27–31]. All of the regions known to be conserved between the different ATP- and PP_i-PFKs were found within the *En. histolytica* enzyme. From studies of the allosteric ATP-PFK of *E. coli* it is known that these regions are involved in binding of Fru 6-*P*/Fru 1,6-P_2 and ATP [32]. Furthermore all of the amino acid residues that have been shown to be functionally important in the PP_i-PFK of *P. freudenreichii* are present within the amoeba sequence [33]. In addition our comparison of the various PFKs revealed conservation of two lysine residues

(positions 208 and 356 of the *En. histolytica* sequence) found exclusively in PP_i-PFKs. These residues might therefore help to distinguish between PP_i- and ATP-PFKs on the basis of the primary sequence.

The similarity between PP_i-PFKs and ATP-PFKs from various species strongly suggests that both types of PFK have a common ancestor. The occurrence of PP_i-PFKs in protozoans, prokaryotes and higher plants appears to correlate better with metabolic characteristics than with phylogenetic relationships, because the use of a PP_i-PFK can improve the ATP yield of glycolysis.

From earlier studies it is known that only PP_i and not ATP functions as phosphoryl donor for the PP_i-PFK of *En. histolytica* [5]. Here we have confirmed this result by using the native as well as the recombinantly expressed enzyme. Therefore bisphosphonates, synthetic pyrophosphate analogues, were tested for use as inhibitors of amoebic PP_i-PFK activity or as inhibitors of amoebic growth. In contrast with pyrophosphate, which contains a P-O-P bond, bisphosphonates are characterized by a P-C-P bond and are therefore resistant to chemical and enzymic hydrolysis. Bisphosphonates have been used successfully for the treatment of human diseases that are characterized by an increased bone turnover, such as Paget's disease, osteoporosis or metastatic bone disease. These compounds are well tolerated and only minor side effects have been reported [35–37]. Eubank and Reeves [14] tested six different bisphosphonates: all were found to be competitive inhibitors for the *En. histolytica* PP_i-PFK. Among these substances only 1-hydroxynonane was inhibitory at low concentrations of at least 1 mM, but only when culture medium supplemented with the drug was renewed after 72 h (second transfer). In the present study we identified four different bisphosphonates that are all competitive inhibitors of the amoeba enzyme and are fully inhibitory at concentrations of 50 μM. After a second transfer one of these substances (risedronate) was found to be inhibitory at a concentration of 10 μM and killed all cells at a concentration of 50 μM.

Besides the PFK, other *En. histolytica* enzymes are known that use pyrophosphate instead of ATP, such as PEP carboxytransphosphorylase (EC 4.1.1.38), pyruvate, phosphate dikinase (EC 2.7.9.1) and PP_i-acetate kinase (EC 2.7.2.1) [3,38,39]. The inhibitory effect of bisphosphonates could therefore be the result of synergistic inhibition of different *En. histolytica* enzymes.

However, the low concentration of bisphosphonates necessary to inhibit amoebic growth suggest that these compounds are indeed potential agents for the treatment of amoebiasis. A major problem today is the lack of effective drugs that are able to eliminate the parasite from the gut. In this respect, bisphosphonates would have another advantage: these drugs have a poor oral bioavailability because they are poorly resorbed from the intestine.

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