Two phosphofructokinase genes have been described previously in *Entamoeba histolytica*. The product of the larger of the two genes codes for a 60-kDa protein that has been described previously as a pyrophosphate (PPi)-dependent enzyme, and the product of the second, coding for a 48-kDa protein, has been previously reported to be a PPi-dependent enzyme with extremely low specific activity. Here it is found that the 48-kDa protein is not a PPi-dependent enzyme but a highly active ATP-requiring enzyme \( k_{cat} = 250 \text{ s}^{-1} \) that binds the cosubstrate fructose 6-phosphate (Fru-6-P) with relatively low affinity. This enzyme exists in concentration- and ATP-dependent tetrameric active and dimeric inactive states. Activation is achieved in the presence of nucleoside triphosphates, ADP, and PPi, but not by AMP, P, or the second substrate Fru-6-P. Activation by ATP is facilitated by conditions of molecular crowding. Divalent cations are not required, and no phosphoryl transfer occurs during activation. Kinetics of the activated enzyme show cooperativity with Fru-6-P \( \text{Fru-6-P}_{0.5} = 3.8 \text{ mM} \) and inhibition by high ATP and phosphoenolpyruvate.

The enzyme is active without prior activation in extracts of *E. histolytica*. The level of mRNA, the amount of enzyme protein, and the enzyme activity of the 48-kDa enzyme are about one-tenth that of the 60-kDa enzyme in extracts of *E. histolytica* trophozoites.

*Entamoeba histolytica* along with a number of other parasitic protists utilizes an unusual form of phosphofructo-1-kinase (PFK) in a central step in carbohydrate metabolism. This form of PFK employs inorganic pyrophosphate (PPi) as a phosphoryl donor. Two genes for PPi-PFK have been described in *E. histolytica* (1–3) with a sequence identity between the two proteins of 17%. The sequence of the larger gene, which codes for a 60-kDa protein, has greater identity to the more phylogenetically advanced plant PPi-PFKs than it does to bacterial PPi-PFKs. The cDNA of this gene has been expressed in *Escherichia coli* and was found to have kinetic properties that were identical to those of the cloned enzyme isolated from *E. histolytica* (3).

The pH dependence and apparent substrate affinities of the cloned enzyme were identical to those of the PPi-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 (3).

The smaller gene, which codes for a 48-kDa protein, has been expressed in *E. coli* as a fusion protein that was found to have a much lower specific activity than that of the larger enzyme (1). Whereas the 60-kDa PFK has been purified from the amoeba, no information concerning the expression of the 48-kDa protein is available. The 48-kDa PFK described in the earlier studies is clearly an expressed product in *E. histolytica* because it was cloned from a cDNA library (1). It may have been present in extracts of the organism but did not copurify with the 60-kDa product or with the activity of PPi-PFK (3). Furthermore, if a second activity had been present which represented at least 10% of the total PFK activity, it would have been detected in native gel electrophoresis.

The problem in attributing a significant role to the 48-kDa protein in phosphorylation of fructose 6-phosphate (Fru-6-P) is its extremely low specific activity with PPi as a phosphoryl donor. The specific activity of the 60-kDa enzyme is about 2,000–3,000 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 for PPi-PFK. One possibility is that the smaller PFK has a yet to be determined catalytic activity. Another possibility is that the 48-kDa protein represents a regulatory protein as one observes in the multisubunit structure of plant PPi-PFKs (4). In the instance of the plant enzymes, the catalytic and regulatory subunits copurify. This was shown to be unlikely regarding the two *E. histolytica* PFKs in the earlier study (3) because no 48-kDa protein was present in the partially purified fractions of the 60-kDa enzyme from *E. histolytica*.

In the current work, we compare expression of the two forms of *E. histolytica* PFK in extracts of trophozoites. The 48-kDa PFK has been purified to homogeneity from both native and recombinant sources and has been found to have no detectable activity with PPi, as a phosphoryl donor. On the other hand, the enzyme has high activity with ATP as a phosphoryl donor, but only after prior activation with ATP.

**EXPERIMENTAL PROCEDURES**

*Expression Constructs—* Two oligonucleotide primers designed on the basis of the sequence at the 5′- and 3′-ends of the 48-kDa PFK gene and containing additional nucleotides at the 5′-ends to generate NdeI and *Bam*HI restriction sites were used to amplify by polymerase chain reaction (PCR) a fragment containing the 48-kDa PFK gene from a genomic clone (2). The PCR fragment was then cloned into the pCR-Script <sup>®</sup> SK (+) plasmid using the PCR-Script<sup>®</sup> cloning kit as directed by the manufacturer (Stratagene, La Jolla, CA). The plasmid construct was digested with NdeI and *Bam*HI to isolate the fragment containing...
bacteria were plated onto LB medium agar plates with 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 2% (v/v) glucose at 37 °C. Freshly harvested cells were then frozen at −80°C until needed. E. coli BL2 (DE3)[pAPlacIQ] E. coli (a gift from Dr. Bruchhaus), and the plasmid construct containing the 48-kDa PFK gene was digested with NdeI and EcoRI and cloned into the complementary sites of the pALTER-Ex1 plasmid (Promega). The E. histolytica 60-kDa PFK gene cloned into the pALTER-Ex1 has been described previously (3).

Enzyme Preparation—The recombinant 48-kDa PFK PPF, PPF-6P, was then purified as described previously as described previously (1). The enzyme preparation was homogeneous on the basis of 10% SDS-PAGE. The enzyme was then diluted 10-fold in a standard dilution buffer (2 mM ATP and 20 mM Fru-6-P in 150 mM KTes (pH 7.2), 3 mM MgCl₂, 1 mM EDTA) unless otherwise indicated, and fixed amounts of the dilution were added to assay cuvettes to start the reaction. The reactions were conducted at standard assay conditions (1 mM ATP and 20 mM Fru-6-P in the aforementioned buffer). The initial velocities were determined to contain less than 0.1% PPi. Nucleoside triphosphate was used in all subsequent analyses.

Assay of the 60-kDa PFK—To measure activity, the 48-kDa PFK was first activated by preincubating at standard activation conditions unless otherwise indicated. The standard activation conditions were 4 μM 48-kDa PFK and 2 mM ATP in 150 mM KTes (pH 7.2), 3 mM MgCl₂, 1 mM EDTA, unless otherwise indicated, and fixed amounts of the dilution were added to assay cuvettes to start the reaction. The reactions were conducted at standard assay conditions (1 mM ATP and 20 mM Fru-6-P) in the aforementioned buffer. The measurement of the decrease of absorbance at 340 nm. The measured rate of the first 60 s of the reaction was recorded. For the determination of kinetic constants, one of the two substrates (a nucleoside triphosphate and Fru-6-P) was kept saturated while the other substrate was varied from 0.1 to 10 K_m. The magnesium ion concentration was kept 4 mM higher than the concentration of nucleoside triphosphates for all assays containing nucleotide to ensure that virtually all of the nucleotides existed as the magnesium complex. All nucleoside triphosphate solutions were determined to contain less than 0.1% PPi. Nucleoside triphosphate decomposition in the assay cuvette to its mono nucleotide monophosphate and pyrophosphate constituents was undetectable.

The initial velocities were determined spectrophotometrically by measuring the decrease of absorbance at 340 nm.

Antibody Preparation and Purification—Antibodies against 60-kDa PFK and histidine-tagged 47-kDa PFK were raised in New Zealand White rabbits. Approximately 200 μg of enzyme with adjuvant was injected at 2, 4, and 8 weeks, and blood was removed 3 days after the last injection. After a 3-week period, sera were collected. Each preparation was purified by passing the polyclonal antibody-containing serum through a column of the respective PFK linked to CNBr-activated Sepharose 4B. In the case of the 48-kDa PFK Sepharose column, the enzyme without the histidine tag was used. The columns were washed extensively with 0.1 M Tris-HCl, 0.3 mM NaCl, pH 8.0, until the absorbance of the flow-through was below 0.01 at 280 nm. The columns were then eluted successively in five steps with buffers of decreasing pH from 7.0 to 2.3 containing 150 mM NaCl. Fractions were neutralized after elution. Specificity of eluted fractions was determined by Western blot analysis using dilutions of the elution fractions as primary antibodies. Antibodies against both 48-kDa PFK and 60-kDa PFK that eluted at pH 5.5 and pH 4.3 had the greatest specificity and were pooled and used in all subsequent analyses.

Northern Blot and Quantitation of the mRNA Level of the Two PFK Genes—E. histolytica total RNA was isolated from an amoebae cell sediment containing 1–2 × 10^6 cells with the Qiagen DNA/RNA isolation kit. Denatured RNA isolated from trypanosomes and RNA markers were then separated on 1.2% agarose gel and transferred to a nylon membrane. The membrane was then air dried and exposed to UV light to visualize the RNA to the membrane. After hybridization, membranes were hybridized with 32P-labeled cDNA probes (1 × 10^6 cpm/ml) prepared by restriction enzyme digestion of the plasmids containing the PFK genes. For quantification of the mRNA level of the two PFK genes, slot blot analysis was performed. A standard was constructed by a series of 2-fold dilutions of the DNA for each of the PFK genes, beginning from 1 ng to 1/64 ng. The DNA standard and 30–50 μg of E. histolytica total RNA were denatured and loaded on a 1.5% agarose gel. The membranes were air dried and UV cross-linked. Northern blot was performed as described above. The content of PFK mRNA within the total RNA was determined by comparing the intensity of the signal from the total RNA with the DNA standard. The ratio of the mRNA level of the two PFK genes was determined.

Western Blot Analysis—Optimal dilution of the affinity-purified anti-
tisera for Western blot was determined by dot blot. E. coli cell extract and protein molecular weight markers were used as negative controls. When quantitation was required, a series of dilutions of known amounts of the two PKFs ranging from 1 to 100 ng was run in adjacent lanes of the gel. Negative control and protein samples were separated by 10% SDS-PAGE, then transferred onto nitrocellulose membranes in 25 mM Tris, 200 mM glycine, 20% methanol at 24 V. Washing and detection were performed by following the instructions of Amersham Pharmacia Biotech ECL Western blotting protocols using goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase.

**E. histolytica Cultivation—** Entamoeba histolytica trophozoites (strain HM-1: IMSS) were grown axenically in TYI-S-33 medium (7) at 35 °C. Routine cultures were maintained in 15-ml borosilicate glass tubes and transferred every 3 or 4 days. To obtain sufficient cells for 48-kDa PKF purification, trophozoites were cultured in 600-ml Nunclon triple flasks (Fisher Scientific).

### 48-kDa PKF Purification from E. histolytica—

**Molecular Sizing**—Molecular mass determinations were carried out on a fast performance liquid chromatography system fitted with a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech). A standard curve was constructed by using a mixture containing 200 µg each of cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), glycerol-3-phosphate dehydrogenase (70 kDa), and alcohol dehydrogenase (150 kDa) in a medium of 20 mM Tris-HCl, 1 mM EDTA, and 14 mM β-mercaptoethanol, pH 7.2. The standard mixture, E. coli PKF (142 kDa), and E. histolytica PKFs were chromatographed individually using a Superdex 200 column pre-equilibrated with the buffered medium plus or minus additions as indicated.

**Other Methods**—Gel electrophoresis of proteins was carried out using a 10% polyacrylamide support according to the system of Laemmli (8). Protein concentrations were determined by Bradford’s dye binding assay with bovine serum albumin as the standard (9). All chemicals and enzymes were purchased from Sigma.

### RESULTS

**Purification of the 48-kDa PKF**—In an attempt to repeat the findings of Bruchhaus et al. (1), who were able to detect very low PPi-PFK activity with a recombinant 48-kDa PKF protein bearing a histidine tag, the 48-kDa PKF was prepared as described in their report. A homogeneous protein with a mass of the predicted 50 kDa as indicated by SDS-PAGE was purified successfully (not shown); however, no PPi-PFK activity under the conditions described previously could be detected at any point during the purification. Because the relatively high concentrations of imidazole used to elute the enzyme from the nickel column (400 mM) may have denatured the enzyme, the CD spectrum of the preparation was compared with that of the homogeneous 60-kDa PPi-PFK. The spectra were nearly identical, suggesting that the global structure of the 48-kDa protein was maintained. All attempts at dialyzing the eluted protein into a lower salt buffer resulted in an irreversible precipitation. Several other methods of elution from the nickel column were attempted, including various concentrations of imidazole and gradients of imidazole and EDTA. However, all of these methods also failed to produce an enzyme with detectable PPi-PFK activity. The yield of the 48-kDa PKF fusion protein using this method, however, was sufficient to raise monoclonal antibodies that were used as a means of detection of the native protein expressed without the histidine tag during its subsequent purification.

Conventional PKF purification procedures were attempted to isolate the 48-kDa PKF without the N-terminal histidine tag using the antibody to follow the 48-kDa protein at each step of the procedure. The recombinant enzyme did not bind to phosphocellulose, which is commonly used for the purification of PPi-PFKs (3, 10), under a variety of conditions. No PPi-PFK activity was detected at any point in the purification process or in cell extracts using the assay conditions described by Bruchhaus et al. (1). The inability to duplicate previously reported activity measurements and these results suggested that the 48-kDa PKF gene does not utilize PPi to phosphorylate Fructose-6-P and thus prompted the trial of alternative purification methods.

Because this laboratory commonly uses both N-6-aminohexylcarboxymethyl-ATP-Sepharose and Blue Sepharose for the purification of various ATP-dependent PKFs (11–13), these media were tried with the recombinant 48-kDa PKF. It was found that the protein bound to both ATP-Sepharose and Blue Sepharose. The 48-kDa PKF was eluted from both types of medium by employing 1 mM ATP in the eluting buffer. Subsequent Mono Q anion exchange chromatography of the eluate from either procedure yielded homogeneous enzyme with a size by SDS-PAGE equivalent to the calculated 47.6-kDa mass (not shown). The recombinant enzyme purified by ATP-Sepharose chromatography was identified by the crude antibodies that were raised against the purified, histidine-tagged recombinant 48-kDa PKF.

For the isolation of specific 48-kDa PKF antibodies, the ATP-Sepharose-purified enzyme was linked to CNBr-activated Sepharose as described under “Experimental Procedures.”

**Catalytic Properties of the 48-kDa PKF Activation**—The affinity chromatography isolation procedure indicated that the 48-kDa PKF interacts with ATP. This observation suggested a reexamination of the activity in the presence of ATP. In such experiments it was observed that when assays with relatively high concentrations of enzyme were allowed to proceed for 30 or more min, a very gradual increase in ATP-dependent activity was observed, suggesting activation in the assay cuvette. This led to preincubation assays of the enzyme with various components of the assay mixture. The testing of the assay components led to the significant finding that ATP-dependent PKF activity can only be detected when relatively high concentrations of enzyme and ATP are preincubated together before adding the enzyme to the assay mixture (details discussed below). Addition of the same amount of enzyme to the assay mixture without prior incubation with ATP resulted in no activity even when ATP concentrations in the assay mixture were high. In such cases no activity is detected because the enzyme concentration in the reaction mixture is too low to become activated. Consistent with this hypothesis, when the enzyme is preincubated with ATP at too low an enzyme concentration, no activity results when adding an equivalent amount of enzyme as above to the assay mixture.

The dependence of the activation process on the concentrations of enzyme and ATP is shown in Fig. 1. The enzyme and ATP concentrations in the preincubation mixtures that result in half-maximal activity are 0.72 µM and 0.21 mM, respectively. The time course of activation was measured at saturating concentrations of both ATP and enzyme. Maximal activity is attained after 5 min of preincubation as shown in Fig. 2A. To determine whether the temperature of the preincubation had any effect on the resultant rate of the enzyme, the preincubation mixtures were incubated at various temperatures before the resultant activity was measured. The temperature optimum for the preincubation is 30 °C (Fig. 2B). Based on these results, preincubations for all standard kinetic assays were subsequently conducted using 4 µM enzyme and 2 mM ATP in 150 mM KTes (pH 7.2), 3 mM MgCl2, 1 mM EDTA at 30 °C and lasted for at least 30 min.

The enzyme concentration dependence of the activation process was investigated further using polyethylene glycols. PEGs have been shown to have an associative effect on macromolec-
ular solutes in aqueous solution without specifically interacting with them (14). Aggregating systems have been shown to be shifted to higher degrees of association by increasing PEG concentration (15). Inclusion of PEG in preincubation mixtures allowed the 48-kDa PFK to be activated at preincubation enzyme concentrations that were too low to become activated in the absence of PEG (Fig. 3). The activation process was enhanced by increasing concentration and size of PEG in the preincubations, with the enhancement effect peaking at 20% PEG. PEG apparently encourages native self-association of the 48-kDa PFK into the activated state by increasing the local protein concentration in solution.

The 48-kDa PFK does not require the MgATP complex for activation because it is activated maximally without Mg$^{2+}$ in the preincubation buffer. Maximal activation of the 48-kDa PFK can also achieved when it is incubated with other nucleotide triphosphates (Table I). GTP and ITP as well as the pyrimidines UTP and CTP are all equally as effective as ATP at activating the enzyme for measuring ATP activity in the resultant assay mixture. The nonhydrolyzable ATP analog AMP-PNP and ADP also can activate the enzyme, both being at least 60% as effective as ATP. Incubation with AMP, the cosubstrate Fru-6-P, and the product orthophosphate results in no activation at all. Interestingly, PP$_i$, despite lacking the nucleotide moiety entirely, is quite capable of activating the 48-kDa PFK to achieve ATP-dependent activity, being 75% as effective as ATP in a 30-min preincubation.

Inactivation—Once activated, the enzyme spontaneously inactivates by simple dilution. This inactivation can be seen during the PFK assay, where one observes a decrease in the rate about 100 s after the start of the reaction which is the result of the dilution of activated enzyme from the concentrated preincubation mixture into the assay. The inactivation proceeds as a first order reaction. To characterize the dilution effect, the enzyme was activated by preincubation at the optimal conditions and subsequently diluted in 150 mM KTes (pH 7.2), 3 mM MgCl$_2$, 1 mM EDTA, with or without 2 mM ATP.

**Fig. 1.** Enzyme- and ATP-dependent activation of 48-kDa PFK. After activation, the activity was measured under standard assay conditions. The data were fitted using the Michaelis-Menten model to estimate the preincubation enzyme concentration that achieves half the maximal rate. A, enzyme concentration dependence of activation. The enzyme was incubated at concentrations from 0.1 to 28.5 µM in KTes (pH 7.2) assay buffer containing 10 mM ATP in 20-µl volumes for 30 min at 30 °C. Fixed amounts of enzyme from the preincubations were then diluted 10-fold in standard dilution buffer, and identical volumes were taken from each dilution and added to assay cuvettes. B, ATP dependence of activation. The enzyme was incubated in KTes (pH 7.2) assay buffer at fixed concentrations of 4 µM in separate tubes containing increasing ATP concentrations from 0.1 to 4 mM. Incubations were carried out in 20-µl volumes at 30 °C. Identical amounts of enzyme were taken after 30 min of incubation from each tube and were assayed under standard assay conditions. Increasing the incubation time an extra 90 min did not increase the activation of the enzyme.

**Fig. 2.** Time and temperature dependence of PFK activation. A, time dependence. The enzyme was first prepared at 4 µM in assay buffer at 30 °C. ATP was added to a final concentration of 2 mM, and aliquots of the activated enzyme were subsequently added to assay cuvettes using the standard dilution method at time points from 1 s to 2 h after the addition of ATP. The reactions were then measured under standard assay conditions. B, temperature dependence. Fixed concentrations of enzyme at 4 µM were activated at various temperatures in assay buffer containing 2 mM ATP in 100-µl volumes for 30 min.
Aliquots were then taken from each dilution mixture at increasing time points and added to assay mixtures to measure the activity (Fig. 4). The first order inactivation rate constant without additions was 0.09 min⁻¹, and it decreased by nearly half to 0.05 min⁻¹ when the enzyme was diluted in buffer containing 2 mM ATP and all other preincubation and dilution conditions were identical. Diluting in buffer containing both substrates at concentrations that produce maximal PFK activity (2 mM ATP and 20 mM Fru-6-P) substantially decreases the rate of inactivation (not shown). This experiment is complicated by the fact that the reaction is proceeding under these conditions. The rate of inactivation measured at early time before significant reaction has taken place gave a rate constant of 0.016 min⁻¹. As a result of these experiments, all kinetic assays were performed by diluting the activated enzyme into assay buffer containing 2 mM ATP and 20 mM Fru-6-P when dilution was necessary.

**Aggregation State**—The above experiments on activation and inactivation suggested that the state of polymerization of the molecule was the determinant of activity. To determine the aggregation state of the native 48-kDa PFK as well as the activated enzyme, a size exclusion chromatography experiment was performed. The polymerization state was determined for the native enzyme, the enzyme activated with ATP, and the enzyme incubated with the cosubstrate Fru-6-P alone, which does not activate the enzyme. For the enzyme-ATP experiment, the concentrations of ATP and enzyme which were found to activate the enzyme maximally were used in the preincubation mixture. Inactivation by dilution. The enzyme was first activated under standard activation conditions. Aliquots of enzyme were then each diluted 10-fold in assay buffer (150 mM KTes (pH 7.2), 3 mM MgCl₂, 1 mM EDTA) or assay buffer with 2 mM ATP. At time points from 0 to 120 min, identical amounts of enzyme were assayed under standard conditions. The rate constants (k) were calculated as the negative slope of the first order plot of the natural log of the rate against time. Only time points within the first 20 min of the ATP-buffer diluted mixture were used because the reaction reaches an equilibrium after that time. The reversible first order reaction with ATP was fitted using the first order exponential decay equation v - vₚₐₜ = (v_max - vₚₐₜ)e⁻ᵏᵗ.

**Kinetic Properties**—The 48-kDa PFK was found to be a highly active ATP-utilizing enzyme with a Kcat value of 250 s⁻¹, which is almost three times the maximum activity of the ATP-dependent activity of E. coli ATP-PFK (16) and about three-fourths the maximum activity of the PP₁-PFK activity of E. histolytica 60-kDa enzyme. No PFK activity (0.01% level of detectability) was observed when PP₁ (at 2 mM) was used as a phosphoryl donor in the assay. Also, ATP activity was not inhibited by this concentration of PP. To determine if the 48-kDa PFK could phosphorylate other sugars using ATP as a phosphoryl donor, the production of ADP was measured when the enzyme was incubated with other sugar compounds. Fru-1-phosphate, glucose, glucose 1-phosphate, glucose 6-phosphate, mannose, and ribose 5-phosphate could not substitute for Fru-6-P in the kinase assay.

Similar to many other ATP-PFKs, the 48-kDa PFK shows cooperative kinetics with respect to Fru-6-P, with a Hill con-
Assays were performed at pH 7.2 as described under “Experimental Procedures.” Apparent $K_m$ values for ATP, GTP, and ITP were determined at 20 mM Fru-6-P. Apparent $K_m$ values for UTP and CTP were determined at 60 mM Fru-6-P. Fru-6-P values were determined at 1 mM ATP, 1 mM GTP, 1 mM ITP, 7 mM UTP, and 7 mM CTP.

| Phosphoryl donor | Fru-6-P $K_m$ (mM) | ATP $n_H$ | $k_{cat}/K_m$ ATP (mM s$^{-1}$) | GTP $n_H$ | $k_{cat}/K_m$ GTP (mM s$^{-1}$) | UTP $n_H$ | $k_{cat}/K_m$ UTP (mM s$^{-1}$) | CTP $n_H$ | $k_{cat}/K_m$ CTP (mM s$^{-1}$) |
|-----------------|------------------|-------|-------------------|-------|-------------------|-------|-------------------|-------|-------------------|
| ATP             | 0.12             | 3.8   | 2.3               | 250   | 2,200             |
| GTP             | 0.067            | 4.1   | 2.3               | 240   | 3,600             |
| ITP             | 0.136            | 8.3   | 1.6               | 230   | 1,720             |
| UTP             | 1.93             | 16.4  | 3.0               | 168   | 87                |
| CTP             | 3.6              | 16.5  | 3.4               | 194   | 54                |

**Table II: Kinetic parameters of E. histolytica ATP-PFK**

Assays were performed at pH 7.2 as described under “Experimental Procedures.” Apparent $K_m$ values for ATP, GTP, and ITP were determined at 20 mM Fru-6-P. Apparent $K_m$ values for UTP and CTP were determined at 60 mM Fru-6-P. Fru-6-P values were determined at 1 mM ATP, 1 mM GTP, 1 mM ITP, 7 mM UTP, and 7 mM CTP.

**FIG. 5. Substrate dependence of E. histolytica ATP-PFK.** Assays were performed at pH 7.2 as described under “Experimental Procedures.” A, Fru-6-P concentration dependence. B, ATP concentration dependence.

**Fig. 5.** Substrate dependence of E. histolytica ATP-PFK. Assays were performed at pH 7.2 as described under “Experimental Procedures.” A, Fru-6-P concentration dependence. B, ATP concentration dependence.

**mRNA Levels for 60-kDa and 48-kDa PFKs in E. histolytica Trophozoites**

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coupled assay. In contrast, E. coli and all known mammalian PFKs have alkaline pH optima. Also unusual was the proficiency of the enzyme in using other nucleotides as substrates relative to ATP (Table II). The apparent affinity and the activity at low concentrations of substrate were even higher for GTP than for ATP. The 48-kDa PFK still showed cooperativity with Fru-6-P with each of the nucleotides as cosubstrates, and the apparent affinity for Fru-6-P remained relatively high with each of the nucleotides tested. In comparison, the E. coli ATP-PFK has $k_{cat}/K_m$ values for GTP and ITP which are an order of magnitude lower than the value for ATP (16).

Although the 48-kDa PFK did not require Mg$^{2+}$ for activation, it was required for catalytic activity, similar to other PFKs. Substituting Mn$^{2+}$ in the assay resulted in only 16% of the observed activity with Mg$^{2+}$, whereas no activity was detectable when substituting with Ca$^{2+}$ and Zn$^{2+}$.

The 48-kDa PFK appears to be inhibited by ATP at high ATP concentrations. This inhibition was evident at low concentrations of the cosubstrate Fru-6-P and disappeared at saturating Fru-6-P (Fig. 5B). ATP inhibition has been demonstrated in other ATP-PFKs. Mammalian PFK has a separate ATP inhibitory site (17), whereas E. coli PFK displays mechanism-based, nonallosteric inhibition by ATP (18). The mechanism of ATP inhibition in the 48-kDa PFK remains to be elucidated. Cooperativity in the interaction with Fru-6-P increased at a higher ATP concentration (Fig. 5A). The mechanism of the cooperative interaction appears to be allosteric, but the mechanism needs to be resolved. It may be related to association/dissociation behavior, but the failure of PEG to eliminate cooperativity argues against this interpretation. PEP, which is known to inhibit other PFKs (12, 19), is an inhibitor of the 48-kDa PFK (Fig. 6). PEP decreased the apparent Fru-6-P affinity substantially, although it had a limited effect on cooperativity ($n$) and no effect on ATP binding (Table III). The steady-state PEP concentration in E. histolytica is not known.

We investigated many other compounds for their ability to regulate the activity of the 48-kDa PFK and have tentatively found no other effectors. Activity with each potential effector was measured both at half-saturating (2.5 mM) and saturating concentrations (20 mM) of Fru-6-P. The apparent Fru-6-P affinity of the 48-kDa PFK was not affected by the metabolites AMP, ADP, GDP, cAMP, orthophosphate, sodium ion, ammonium ion, phosphocreatine, citrate, fructose 2,6-bisphosphate, 3-phosphoglycerate, and glucose 6-phosphate (all at 1 mM concentrations), metabolites that have been demonstrated to modulate PFK in other organisms. Other compounds that were examined for their ability to regulate the 48-kDa PFK included phosphoglycerate, lactate, calcium ion, and calmodulin. No effects were seen.

**mRNA Levels for 60-kDa and 48-kDa PFKs in E. histolytica Trophozoites**

Total RNA isolated from trophozoites was used for Northern blots to determine the expression of the two PFKs.
interact between the two PFKs was motivated by the observation of a multisubunit structure in plant PPI-PFKs (4). In the instance of the plant enzymes, catalytic and regulatory subunits copurify. No copurification was observed, nor was coprecipitation of the two enzymes from trophozoite extracts seen when either specific antibody was used. Furthermore, assays of purified 60-kDa PPI-PFK were not influenced by the presence of an equal amount of purified 48-kDa ATP-PFK, nor was there any effect when the two enzymes were preincubated together. Similarly, no effect of 48-kDa PFK was seen when the reverse experiments were performed. Thus any direct interactions between the two proteins are very unlikely.

A reinvestigation of trophozoite extracts showed that ATP-PFK activity could be detected without prior activation. ATP-dependent PFK activity in amoebae is about 11 fold lower than PPI-dependent activity (0.43 unit of ATP activity versus 4.1 units of PPI activity in 100 μl of trophozoite extract), corresponding to the relative amounts of the two PFKs enzymes detected by Western analysis. To ensure that the measured ATP-PFK activity was not an artifact of the 60-kDa PFK catalyzing PPi, produced in other metabolic pathways, amoebal extracts were dialyzed exhaustively to eliminate all small metabolites. Also, ATP-PFK activity was readily measured at high Fru-6-P concentrations (20 mM) and was totally undetectable at 1.5 mM Fru-6-P, which is a saturating concentration of the sugar phosphate for the 60-kDa PFK. This indicated that the ATP-PFK activity detectable only at the higher Fru-6-P concentration was not measuring the 60-kDa PFK catalyzing contaminating PPi, because such contamination would have been detectable at 1.5 mM Fru-6-P. In the study that first identified the PPI-PFK enzyme of \textit{E. histolytica}, Reeves et al. (20) also detected ATP-PFK activity in trophozoite homogenates. Those investigators were unable to characterize the \textit{E. histolytica} ATP-PFK activity further because of activity losses during purification. In fact, Reeves later concluded that the observed ATP-PFK activity was an artifact (21). That is clearly not the case as demonstrated here.

An interesting finding was that the amoebal ATP-PFK activity was not increased by preincubation of amoebal extracts with 2 mM ATP even after eliminating the small metabolites by dialysis. In contrast, the trace of ATP-PFK activity in bacterial extracts containing recombinantly expressed \textit{E. histolytica} 48-kDa PFK was increased dramatically after incubation with 2 mM ATP (data not shown).

**DISCUSSION**

The two PFKs of \textit{E. histolytica} display distinct phosphoryl donor specificities. The 60-kDa PFK is a PPI-dependent enzyme and is responsible for all detectable PPI-PFK activity in trophozoite extracts (3). The 48-kDa PFK, contrary to previous reports, demonstrates no detectable PPi-PFK activity when produced recombinantly. The 48-kDa PFK is in fact a highly active ATP-utilizing PFK that is also able to use other nucleotides efficiently for catalysis. However, the apparent \(K_m\) value for Fru-6-P of the 48-kDa PFK is more than 20-fold greater than previously measured intracellular Fru-6-P concentrations (0.16 ± 0.06 mM) in amoebae (20), indicating that without a positive effector this enzyme may have limited physiological activity unless one invokes compartmentalization. Considering that the 60-kDa PFK has been shown to account for the glycolytic flux in amoebal extracts (21) and that mRNA, protein, and activity levels all indicate that the 60-kDa PFK is present in trophozoites in 10-fold greater amounts than the 48-kDa enzyme, the significance of the ATP-PFK in the glycolysis of trophozoites remains in question. However, \textit{E. histolytica} has a complex life cycle, and the ATP-PFK may have functions in other stages of that cycle.
The findings in this study as well as results from earlier studies from this laboratory (3) argue against the possibility of the two PFKs of *E. histolytica* associating or affecting each other in some regulatory manner. The two PFKs do not copurify when isolating either protein, a protein 47 kDa in size was not seen in the active PFK fractions during native PPi-PFK purification, immunoprecipitation of the trophozoite cell extract with antibodies against the 60-kDa PFK did not precipitate a protein close to 47 kDa in mass, and the activity of either the 60-kDa PFK or 48-kDa PFK was unaffected by the presence of its PFK counterpart in the assay mixture or in preincubations (data not shown).

*E. histolytica* 48-kDa PFK is an unusual ATP-utilizing PFK that is only active after incubation at high enzyme concentrations with ATP. This activation appears to be the result of a change in the state of aggregation of the enzyme upon binding ATP rather than a catalytic event such as ATP hydrolysis or the formation of a phosphoenzyme complex. The activation can be reversed by simply diluting the enzyme-ATP incubation, and the enzyme-ATP preincubation mixture eventually reaches an equilibrium. These results indicate that activation does not involve a permanent alteration in either the enzyme or the ATP molecule. The enzyme can also be activated by other nucleotide triphosphates, AMP-PNP, ADP, and even PPi, indicating that a specific ATP modification is not involved in activation. These observations suggest that the nucleoside moiety is not essential for activation and that the last two phosphoryl groups of ATP are the most critical features. Closer analysis reveals PPi to be a better activator than ADP and AMP-PNP, both of which deviate from ATP in the terminal polyphosphate region. This polyphosphate moiety is completely absent in AMP and orthophosphate, and incubation with these compounds does not activate the enzyme at all. The Michaelis constant value for ATP derived from the ATP-dependent activation assay \( K_m = 0.21 \text{ mM} \) is similar to that observed from the substrate dependent assay \( K_m = 0.12 \text{ mM} \), which is consistent with ATP binding at the same site for both activation and catalysis. However, our results show that the adenosine moiety seems to be of little relevance in activation, whereas PPi activates the enzyme to near maximum levels. Although PPi can activate the enzyme, it is not a substrate, nor can it inhibit ATP activity. These results introduce the possibility that the 48-kDa PFK may bind PPi, for activation at a site other than the substrate binding site. It is also possible that the activators may produce their effects by chelating some unknown inhibitor; however, this situation is unlikely because all activation assays were performed in the presence of 1 mM EDTA. Although the enzyme cannot be activated by the cosubstrate Fru-6-P alone, the sugar phosphate does provide some protection against inactivation when present with ATP.

The dependence of the activation process on protein concentration suggests that the reversible activity loss is associated with association-dissociation behavior of the protein. This was supported by experiments with molecular crowding with PEG which showed that crowding increased the rate of activation. Finally, molecular sizing experiments show that the inactive PFK exists as a dimer that associates into an active tetramer upon incubation with ATP.

The 48-kDa PFK in dialyzed amoebal extracts was found in an activated state. This information introduces many new possibilities for the native activation state of the enzyme. Although it is possible that ATP was not eliminated from the trophozoite extracts by dialysis, it is more likely that another activator exists in the amoeba which is either too large or too tightly associated to be removed by dialysis. Also, the 48-kDa PFK may be activated in trophozoites by some other means such as subcellular localization. Whatever the mechanism of native activation, it is likely that in *vivo* the 48-kDa PFK displays substantially different kinetic features.

The two PFKs of *E. histolytica* have a low sequence identity of about 17%, although there are many identical residues in the presumed active site. Phylogenetic studies of the sequences of PFKs place the two *E. histolytica* PFKs in a large group of proteins, most of which have been described as PPi-PFKs that are distinct from the typical ATP-PFKs such as those found in *E. coli* as well as all mesozoaons. The 60-kDa enzyme falls into a monophyletic subgroup that contains a number of other well characterized PPi-PFKs including those of plants (22, 23). On the other hand, the 48-kDa PFK sequence from *E. histolytica* falls into a monophyletic subgroup within the PPi-PFK group that also contains *Treponema pallidum* and *Borrelia burgdorferi* and the peroxisomal ATP-PFK of *Trypanosoma brucei* (22, 23). Of the other three members of the group, the *T. pallidum* gene product has not been characterized and preliminary studies of the *B. burgdorferi* product have not found either ATP- or PPi-PFK activity (24). The *T. brucei* ATP-PFK is a homotetramer with a subunit mass of 50 kDa and is not regulated by the metabolites that modulate the activity of ATP-PFKs in other organisms (25). The members of this group of four proteins have a common sequence in the presumed region where the phosphoryl transfer reaction takes place. The two sequences are GGDG and PGTDND, which may be contrasted to GGDG and PTIDND of almost all well characterized PPi-PFKs and GGDG and PTIDND in ATP-PFKs of *E. coli* and all mesozoaons. Recently we have shown that mutation of the second Asp in the GGDG sequence of the *E. histolytica* 60-kDa PPi-PFK to Gly changes the specificity to that of an ATP-PFK (26). The last residue in the GGDG sequence would appear to be a particularly important determinant of the phosphor donor specificity of all PFKs.

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