Pyrophosphate-dependent phosphofructokinase from the amoeba *Naegleria fowleri*, an AMP-sensitive enzyme

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PP_i-dependent phosphofructokinase (PP_i-PFK) was detected in extracts of the amoeba *Naegleria fowleri*, with a specific activity of about 15–30 nmol/min per mg of protein, which was increased about 2-fold by 0.5 mM AMP. PP_i-PFK was inactivated upon gel filtration and could be re-activated by incubation at 30 °C in the presence of AMP. *N. fowleri* PP_i-PFK was purified more than 1100-fold to near homogeneity with a yield of about 25%. The pure enzyme had a specific activity of 65 μmol/min per mg of protein, and SDS/PAGE analysis showed a single band, of 51 kDa. Size-exclusion chromatography revealed the existence of two forms: a large one (~ 180 kDa), presumably a tetramer, which was active, and a smaller one (~ 45 kDa), presumably the monomer, which was inactive, but could be re-activated and converted into the large form by incubation at 30 °C in the presence of 0.5 mM AMP. Re-activation was also observed at 30 °C in the absence of AMP, particularly at higher enzyme concentration or in the presence of poly(ethylene glycol). Inactivation of the tetrameric enzyme was promoted by 0.25 M potassium thiocyanate. The enzyme displayed K_m values of 10 and 15 μM for fructose 6-phosphate and PP_i, respectively, in the forward reaction, and of 35 and 590 μM for fructose 1,6-bisphosphate and P_i in the backward reaction. The activity was dependent on the presence of Mg^{2+}. AMP increased $V_{max.}$ about 2-fold without changing the affinity for the substrates; its half-maximal effect was observed at 2 μM.

INTRODUCTION

It is generally assumed that PP_i is rapidly hydrolysed in the cell by inorganic pyrophosphatase, which maintains its concentration at a low level and hence favours biosynthetic reactions. Evidence is growing that this rule does not apply to all organisms, and particularly not to some protists. The amoeba *Entamoeba histolytica* (Reeves et al., 1974), the flagellates *Trichomonas vaginalis*, *Tritrichomonas foetus* (Mertens et al., 1989) and *Giardia lamblia* (Mertens, 1990), the ciliate *Isotricha prostoma* (Mertens et al., 1989) and the intracellular parasite *Toxoplasmia gondii* (Peng and Mansour, 1992) all use PP_i instead of ATP as substrate for one or several glycolytic reactions. Furthermore, these species, or at least those that were examined so far, are devoid of inorganic pyrophosphatase (see Mertens, 1991).

These differences in metabolism prompted us to re-examine the glycolytic status in other protists. In the present paper we report the identification and the purification of a novel type of pyrophosphate-dependent phosphofructokinase (PP_i-PFK), which we found in *Naegleria fowleri*. The *Naegleria* genus includes several species of free-living uninucleate amoebae that live in an aquatic environment (ponds, lakes, sewage). *N. fowleri* is occasionally pathogenic to a variety of mammals, including humans, causing primary amoebic meningoencephalitis after intranasal inoculation (for reviews, see John, 1982; Marciano-Cabral, 1988; Visvesvara and Stehr-Green, 1990). *Naegleria* PP_i-PFK was found to be regulated by AMP, a property that distinguishes it from all of its counterparts, which are either unregulated or stimulated by fructose 2,6-bisphosphate.

EXPERIMENTAL

Organisms

N. fowleri (KUL strain), *N. gruberi* (B G-6 strain) and *Acanthamoeba castellanii* (NEFF strain) were grown axenically at 30 °C in serum/casein/glucose/yeast extract medium (De Jonckheere, 1977). The cells were harvested by centrifugation at late-exponential phase and kept as frozen pellets at −80 °C until further use.

Materials

Biochemicals and auxiliary enzymes were from Boehringer (Mannheim, Germany). Dithiothreitol was from Janssen Chimica (Beerse, Belgium). Blue Trisacryl was from IBF Biotechnics (Villeneuve-la-Garenne, France). Mono Q and Sephacryl S-300 HR were from Pharmacia (Uppsala, Sweden). Other chemicals were from Merck (Darmstadt, Germany) or from Sigma (St. Louis, MO, U.S.A.).

Enzyme assays

PP_i-PFK activity was measured spectrophotometrically at 340 nm and at 30 °C in 1 ml of a mixture containing 1 mM fructose 6-phosphate, 0.5 mM PP_i, 5 mM $MgCl_2$, 0.14 mM NADH, 0.5 mM AMP, 0.5 unit of aldolase, 0.5 unit of triosephosphate isomerase, 1 unit of glycerol-3-phosphate dehydrogenase and 50 mM Hepes, pH 7.0. In the gluconeogenic direction, the activity was monitored by the formation of fructose 6-

Abbreviations used: ATP-PFK, ATP:fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.11); PP_i-PFK, PP_i:fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90).

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phosphate, in the presence of 5 mM P_i, 0.5 mM fructose 1,6-bisphosphate, 5 mM $MgCl_2$, 0.28 mM $NADP^+$, 0.5 unit of glucosephosphate isomerase, 2 unit of glucose-6-phosphate dehydrogenase and 50 mM Hepes, pH 7.0. Except where otherwise indicated, the preincubations were carried out at 30 °C in a mixture containing 0.01% Triton X-100, 50 mM Tris/HCl, pH 7.5, and 0.5 mM AMP. ATP-dependent phosphofructokinase (ATP-PFK) was measured by the production of fructose 1,6-bisphosphate in the same assay mixture as used for PP_i-PFK, except that 2 mM ATP was substituted for PP_i. 6-Phosphofructo-2-kinase was assayed by the formation of fructose 2,6-bisphosphate as described by Larondelle et al. (1986). One unit of PP_i-PFK is the amount that catalyses the formation of 1 μmol of fructose 1,6-bisphosphate/min under standard assay conditions after preincubation with AMP.

Enzyme purification

Except for f.p.l.c. steps which were performed at 20–25 °C, the purification was carried out at 0–4 °C. About 2 ml of frozen pellets harvested from six Roux bottles were homogenized in a Potter–Elvehjem apparatus with 10 vol. of ice-cold Buffer A, containing 20 mM Tris/HCl, pH 7.5, 10 μg/ml leupeptin, 5 mM $MgCl_2$ and 0.01% Triton X-100 (which was added because it stabilized the enzyme at later steps in the purification). The homogenate was centrifuged at 20000 *g* for 10 min, and the resulting extract was applied to a Blue Trisacryl column (15 ml) equilibrated with Buffer A. The column was washed with 30 ml of Buffer A and a linear NaCl gradient (0–2 M; 200 ml) was applied at a rate of 0.5 ml/min. Two peaks of PP_i-PFK were eluted, one at about 75 mM, and the other, the activity of which was revealed after preincubation with AMP, at about 300 mM NaCl. The most active fractions of the second peak were used as such or after storage at −80 °C to investigate the properties of the enzyme, or further purified as follows.

After a 60 min preincubation at 30 °C with 0.5 mM AMP, the active fractions were combined, diluted 10-fold with 0.01% Triton X-100/5 mM $MgCl_2$/leupeptin (10 μg/ml)/0.5 mM AMP, and re-applied to the Blue Trisacryl column equilibrated in Buffer A supplemented with 0.5 mM AMP (Buffer B). The column was washed with 30 ml of Buffer B, and a linear NaCl gradient (0–0.5 M in 80 ml buffer B) was applied at a rate of 0.5 ml/min. A major peak of PP_i-PFK activity was eluted at low (50 mM) NaCl concentration, and about 5% of the activity was recovered as a second peak at about 250 mM NaCl (see the Results section).

The most active fractions were combined, diluted 2-fold in 20 mM Tris/HCl (pH 7.5)/0.05 mM AMP/0.01% Triton X-100/5 mM $MgCl_2$ (Buffer C), and applied to a Mono Q column (1 ml) equilibrated in Buffer C and connected to a f.p.l.c. system (Pharmacia). The column was washed with 10 ml of Buffer C, and a NaCl gradient (0–0.25 M in 15 ml of buffer C) was applied, at a rate of 0.5 ml/min. PP_i-PFK was eluted as a single peak at about 180 mM NaCl.

Other methods

PAGE in the presence of 10% SDS (Laemmli, 1970) and silver staining of the gels (Wray et al., 1981) were performed as indicated. Gel-permeation chromatography was performed at 0–4 °C on a Sephacryl S-300 HR column (1.5 cm × 70 cm): 0.4 ml samples were applied, and the flow rate was 0.4 ml/min. Unless otherwise noted, the protein concentration was measured as described by Bradford (1976) with bovine γ-globulin as a standard. Fructose 2,6-bisphosphate was assayed in heated alkaline extracts as described by Van Schaftingen et al. (1982).

RESULTS

Presence of a PP_i-dependent phosphofructokinase in *Naegleria*

In the presence of 2 mM fructose 6-phosphate, extracts of *N. fowleri* catalysed PP_i-dependent formation of fructose 1,6-bisphosphate, at a rate of about 25 nmol/min per mg of protein, and a P_i-dependent formation of fructose 6-phosphate from fructose 1,6-bisphosphate at about the same rate, indicating the presence of PP_i-dependent phosphofructokinase (PP_i-PFK). PP_i-PFK activity was not affected by fructose 2,6-bisphosphate, a potent stimulator of several ATP- or PP_i-dependent phosphofructokinases (Van Schaftingen, 1987), whereas 0.5 mM AMP, known to stimulate mammalian and yeast ATP-dependent PFK, stimulated the enzyme 2-fold. No significant ATP-dependent activity could be detected in the same extracts, even in the presence of 1 μM fructose 2,6-bisphosphate.

Figure 1(a) shows that the PP_i-PFK activity progressively decreased upon incubation of the extract at 30 °C in the absence of AMP and to a much lesser extent in the presence of 0.5 mM AMP. Almost 80% of the activity was lost after gel filtration of the extract on Sephadex G-50 (Figure 1b). Subsequent incubation at 30 °C in the presence of 0.5 mM AMP allowed the progressive re-activation of the enzyme, whereas complete inactivation was observed in the absence of AMP. Re-activation was much slower at 0 °C (results not shown).

The enzyme was purified to study its kinetic properties and to understand the mechanism of the AMP effect.

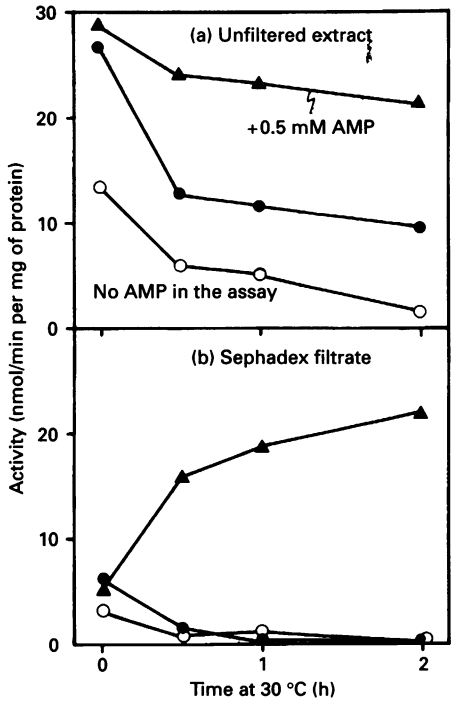

Figure 1 Effect of preincubation of an extract at 30 °C in the absence or in the presence of AMP on PP_i-PFK activity

In (**a**) a cell extract was incubated at 30 °C at a protein concentration of 0.44 mg/ml with (▲) or without (○, ●) 0.5 mM AMP. The activity was measured in the absence (○) or in the presence (●, ▲) of 0.5 mM AMP. In (**b**), the extract was gel-filtered on a Sephadex G-50 column (PD 10, Pharmacia) equilibrated with extraction buffer, and the resulting filtrate was incubated at the same protein concentration as above. Symbols are the same as in (**a**).

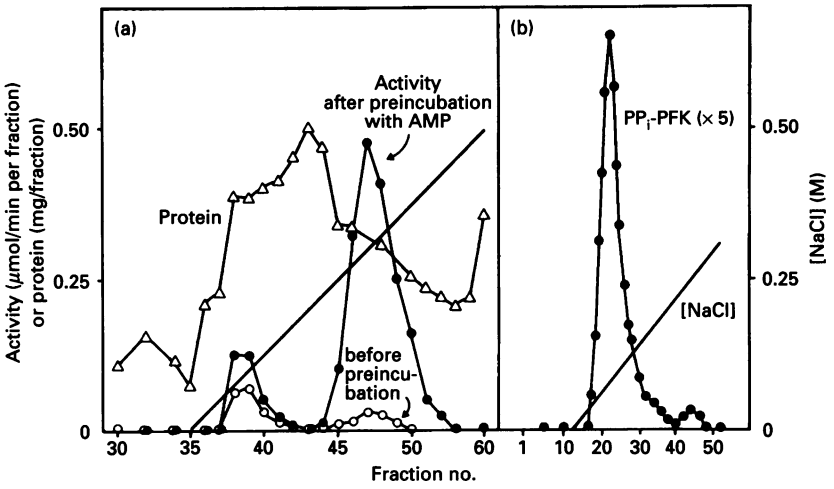

Figure 2 Purification of PP$_i$-PFK by two successive steps on Blue Trisacryl

(**a**) A 29 ml portion of an extract was loaded on a Blue Trisacryl column, which was washed with 30 ml of equilibration buffer and developed with a salt gradient; 2.6 ml fractions were collected. PP$_i$-PFK activity was measured before (○) or after (●) preincubation with 0.5 mM AMP. (**b**) Fractions 46–49 of the first column were pooled, incubated for 60 min at 30 °C in the presence of 0.5 mM AMP, diluted 10-fold and loaded on to a Blue Trisacryl column, which was processed as above except that 0.2 mM AMP was present in all buffers; 1.1 ml fractions were collected.

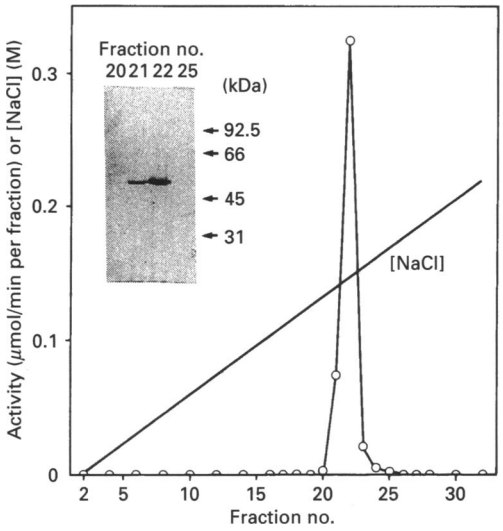

Figure 3 Chromatography on Mono Q

Fractions 19–24 from the second Blue Trisacryl column were loaded on the column; 0.5 ml fractions were collected. Inset: 100 μl portions of the indicated fractions were submitted to SDS/PAGE. The gel was silver-stained. Molecular-mass markers (kDa) were phosphorylase *b*, BSA, ovalbumin and carbonic anhydrase.

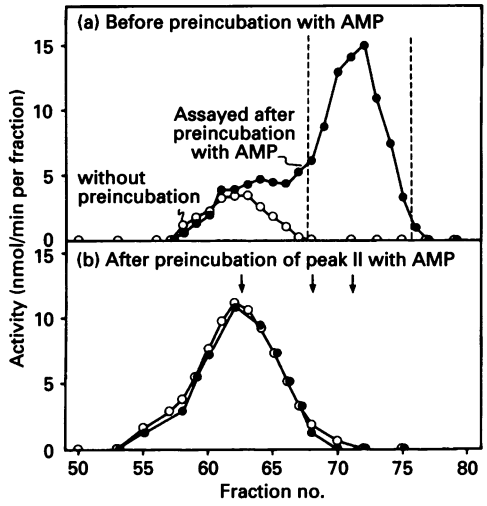

Figure 4 Effect of preincubation in the presence of AMP on the elution profile of PP$_i$-PFK from a Sephacryl S-300 column

In (**a**) partially purified PP$_i$-PFK (2.1 mg of protein in 0.4 ml) was chromatographed on a Sephacryl S-300 column (1.5 cm × 70 cm) equilibrated with a buffer containing 50 mM Tris/HCl, pH 7.5, 200 mM NaCl and 0.01% Triton X-100. The flow rate was 0.4 ml/min and 1 ml fractions were collected. The activity was measured in the presence of 0.5 mM AMP either as such or after a 2 h preincubation at 30 °C in the presence of 0.5 mM AMP. In (**b**), after preincubation with AMP, fractions 67–75 of the first column were pooled, concentrated to 0.6 ml and chromatographed as above except that the equilibration buffer also contained 0.5 mM AMP. Markers (arrows; left to right) were aldolase (168 kDa), BSA (68 kDa) and chicken ovalbumin (44 kDa).

Table 1 Purification of *N. fowleri* PP$_i$-PFK

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
Crude extract	41	2.28	0.058	100	1
Blue Trisacryl (I)	1.2	1.47	1.23	62	21
Blue Trisacryl (II)	0.012*	0.59	48	25	827
Mono Q	0.0095*	0.62	65	26	1124

* Estimated on silver-stained SDS/PAGE.

Purification of PP$_i$-PFK

Figure 2 illustrates the elution profile of a Blue Trisacryl column on which a crude extract was applied. The PP$_i$-PFK activity was measured in the fractions either without further treatment or after a 60 min preincubation at 30 °C and in the presence of 0.5 mM AMP. Two peaks of activity were eluted, one at about

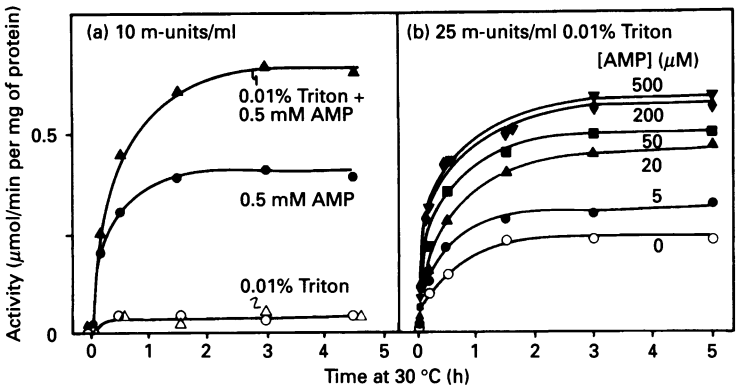

Figure 5 Effect of the concentration of enzyme, the concentration of AMP and the presence of Triton X-100 on the activation of PP_i-PFK at 30 °C

In (**a**) PP_i-PFK was incubated in a volume of 200 μl at 30 °C and at a final concentration of 10 m-units/ml in the presence of the indicated concentrations of AMP and of Triton X-100. In (**b**) the enzyme was incubated at a concentration of 25 m-units/ml with 0.01% Triton X-100 and the indicated concentrations of AMP. All assays were carried out in the presence of 0.5 mM AMP.

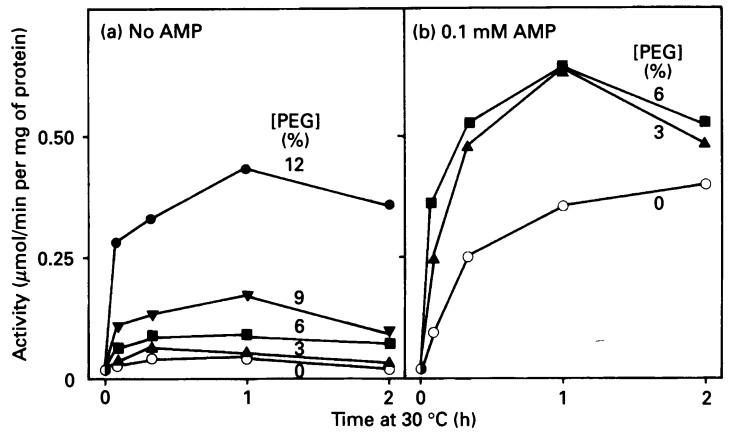

Figure 6 Effect of poly(ethylene glycol) on the activation of PP_i-PFK in the absence (a) or in the presence (b) of 0.5 mM AMP

PP_i-PFK was incubated at 30 °C and at a concentration of 10 m-units/ml in the presence of the indicated concentrations of AMP and of poly(ethylene glycol) (PEG). Triton X-100 was omitted from the incubation medium.

50 mM NaCl and the second at about 300 mM. The two peaks differed markedly in the effect of preincubation in the presence of AMP, the first being activated only 2-fold and the second close to 20-fold. After incubation with AMP, the fractions corresponding to the second peak (fractions 46–49 in Figure 2a) were rechromatographed on Blue Trisacryl, this time in the presence of 0.5 mM AMP. The major peak of activity was now eluted at 50 mM NaCl rather than 300 mM (Figure 2b), the second peak representing only a few per cent of the total activity recovered.

As expected, the AMP-dependent shift in the elution profile of the PP_i-PFK peak was a very efficient purification step. SDS/PAGE analysis showed that a major 51 kDa polypeptide co-eluted with PP_i-PFK activity. Additional bands were still present in the preparation (results not shown). These contaminants were removed by chromatography on Mono Q. As shown in Figure 3, PP_i-PFK co-eluted again with the 51 kDa polypeptide and was apparently homogeneous. Protein was below the detection level of the Bradford method, and was therefore estimated by comparing the intensity of the spots on silver-stained gels with protein standards. This method allowed us to estimate that the enzyme was purified more than 1100-fold over the crude extracts with a yield of approx. 25% (Table 1).

Effect of AMP on the molecular mass

Figure 4(a) illustrates the elution profile of PP_i-PFK from a Sephacryl S-300 column on which a pool of the fractions corresponding to the second peak of the first Blue Trisacryl column was loaded. Assay of the fractions without preincubation with AMP indicated the presence of one single peak of activity, corresponding to a protein with an apparent molecular mass of ~ 180 kDa. Preincubation of the fractions with 0.5 mM AMP revealed the presence of a second major peak of enzyme with an apparent molecular mass of 46 kDa. When the second peak was rechromatographed on the same column after activation by AMP, a single peak of ~ 180 kDa was observed (Figure 4b). This time, as expected, preincubation of the fractions at 30 °C with 0.5 mM AMP did not lead to further activation.

Effect of different conditions on the activation of PP_i-PFK

Figure 5 illustrates the effect of enzyme concentration, AMP and Triton X-100 on the activation of the enzyme corresponding to the second peak of Figure 2(a), upon preincubation at 30 °C and subsequent assay in the presence of a saturating concentration of

AMP. When preincubated at 30 °C in the absence of AMP at a concentration of 10 m-units/ml, the enzyme became slightly activated. The activation was much larger in the presence of AMP and was clearly time-dependent, being half-maximal after about 10 min. Triton X-100 was devoid of effect when added alone, but reinforced the effect of the nucleotide. As shown in Figure 5(b), an increase in the concentration of enzyme to 25 m-units/ml augmented about 10-fold the AMP-independent activation without changing the degree of activation observed in the presence of this compound. The concentration of AMP required to observe a half-maximal effect was 8 μM. When added to the preincubation medium, poly(ethylene glycol) also caused an increase in the degree of activation of PP_i-PFK (Figure 6). The effect of this compound was synergistic with that of AMP.

When tested at a concentration of 100 μM, IMP caused an activation that was approx. 35% of that induced by 100 μM AMP. For GMP, 2-deoxyAMP and aminoimidazolecarboxamide ribotide, the values were slightly lower (~ 25%), as well as for ADP-Mg or ATP-Mg (~ 15%). Cyclic AMP, XMP, UMP, CMP, P_i, NAD^+ and adenosine produced less than 10% of the activation observed with AMP. Moreover, the activation observed with 100 μM AMP was unaffected by the presence of 1 mM P_i, PP_i, adenosine, ADP or fructose 6-phosphate, 5 mM EDTA or dithiothreitol, 0.5 M NaCl, or 10% formamide (results not shown).

We have also tested the effect of KSCN, a chaotropic agent which is known to cause dissociation of several proteins such as *Escherichia coli* ATP-PFK (Deville-Bonne et al., 1989). Incubation of the AMP-activated enzyme in the presence of 0.25 M KSCN resulted in the progressive inactivation of the enzyme. Figure 7 illustrates that the enzyme could be successively inactivated by incubation at 0 °C in the presence of 0.25 M KSCN and re-activated at 30 °C in the presence of 0.2 mM AMP after a 5-fold dilution to decrease the concentration of KSCN. The whole process of inactivation and re-activation could be repeated without apparent loss of enzyme. KSCN also induced inactivation of PP_i-PFK at 30 °C; in this case, however, the enzyme could not be fully re-activated upon subsequent incubation with AMP, presumably because of partial denaturation of the monomer. Figure 7 also shows that in the absence of AMP and KSCN (○ symbols), the concentrated enzyme underwent partial activation at 30 °C and inactivation at 0 °C. No re-activation was observed at 30 °C when the preparation was more diluted (20 m-units/ml).

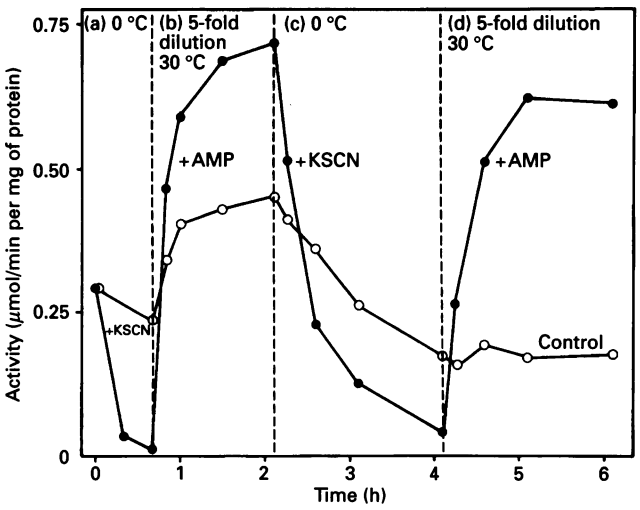

Figure 7 Reversibility of the inactivation induced by KSCN at 0 °C and of the activation induced by AMP at 30 °C

All incubations were carried out in the presence of 0.2 M NaCl, 0.01% Triton X-100 and 20 mM Tris/HCl, pH 7.5, and partially purified PP_i-PFK at an initial concentration of 500 m-units/ml. The temperature and duration of each incubation were as indicated on the graph. When indicated, 0.25 M KSCN and 0.25 mM AMP were also present.

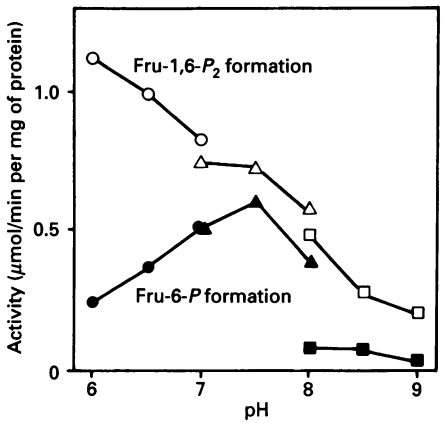

Figure 8 Effect of pH on the activity of PP_i-PFK measured in the forward or the backward direction

PP_i-PFK was activated by preincubation with AMP. Its activity was measured at the indicated pH by the production of fructose 1,6-bisphosphate (Fru-1,6-P_2) or of fructose 6-phosphate (Fru-6-P) in the presence of 0.5 mM AMP. Buffers used were 50 mM Mes (○, ●), Hepes (△, ▲) or Tris (□, ■).

Kinetic properties

Kinetic studies were performed on a preparation corresponding to the second peak of the first Blue Trisacryl column, which had been activated by preincubation at 30 °C in the presence of 0.5 mM AMP [or of 10% poly(ethylene glycol) when the effect of AMP was investigated]. The enzyme activity was dependent on the presence of a bivalent cation, which could be either Mg^{2+} or Mn^{2+}. Under standard assay conditions, half-maximal activity was observed at 40 μM $MgCl_2$ (result not shown). The saturation curves for fructose 6-phosphate or for PP_i were hyperbolic. Double-reciprocal plots were linear and allowed us to calculate K_m values of 10 μM and 15 μM for fructose 6-phosphate and PP_i respectively (result not shown). AMP increased $V_{max.}$ about 2-fold, without change in the affinity for the substrates. Its half-maximal effect was observed at 2 μM. In contrast with the effect observed during preincubation, the stimulatory effect of AMP was instantaneous, as indicated by the linearity of the changes in absorbance as a function of time. Fructose 2,6-bisphosphate had no effect on the kinetics (results not shown).

When the enzyme was measured in the gluconeogenic direction, K_m values of 35 and 590 μM were observed for fructose 1,6-bisphosphate and P_i respectively. In this case again, AMP increased $V_{max.}$ without change in the affinity for the substrates. As shown in Figure 8, maximal activity was observed at the lowest pH investigated (pH 6) in the glycolytic direction and at pH 7.5 in the gluconeogenic direction.

The enzyme present in the first peak of the first Blue Trisacryl column displayed similar K_m values for fructose 6-phosphate and PP_i to the enzyme present in the second peak after activation by AMP.

Fructose 2,6-bisphosphate in *N. fowleri*

Alkaline extracts of *N. fowleri* did not contain detectable fructose 2,6-bisphosphate (< 0.1 nmol/mg of protein), although the cells had been incubated in the presence of glucose. 6-Phosphofructo-2-kinase could not be detected in neutral extracts of this organism.

Effects of AMP and of fructose 2,6-bisphosphate on other PP_i-PFKs

An AMP-sensitive PP_i-PFK was also observed in extracts of *Naegleria gruberi*, with a specific activity of about 20 nmol/min per mg of protein. By contrast, extracts of *Acanthamoeba castellanii*, a protist which is often considered as related to *Naegleria* spp., did not display PP_i-PFK activity, but contained ATP-PFK. This enzyme was stimulated about 4-fold by micromolar concentrations of fructose 2,6-bisphosphate, to reach a specific activity of 0.15 unit/mg of protein.

Finally, we also observed that preincubation at 30 °C in the presence of 0.5 mM AMP of PP_i-PFK from potato tubers (partially purified, from Sigma), from *Giardia lamblia* (crude extract) and from *Trichomonas vaginalis* (partially purified enzyme) did not activate these enzymes.

DISCUSSION

A novel type of PP_i-PFK

Our results demonstrate the presence of a PP_i-PFK in *N. fowleri* and in *N. gruberi*. The enzyme of the former species has been purified to apparent homogeneity. The procedure was based on the fact that preincubation with AMP caused a decrease in the affinity of PP_i-PFK for Blue Trisacryl, possibly because of a change in quaternary structure (see below) or because of competition between AMP and the immobilized ligand, Cibacron Blue. SDS/PAGE of the purified enzyme indicated that it consists of one single type of subunit, of 51 kDa.

As mentioned in the Introduction, two different types of PP_i-PFK have been described until now. (1) The 'non-regulated' enzyme, which displays a rather low K_m for fructose 6-phosphate (< 50 μM) and is found in several anaerobic protozoa or in bacteria that are devoid of ATP-PFK and of fructose-1,6-bisphosphatase; the activity of this type of PP_i-PFK is usually quite high in crude extracts (~ 0.5 unit/mg of protein), the size of its subunits is 45–50 kDa (O'Brien et al., 1975; Mertens et al., 1989; Peng and Mansour, 1992; E. Mertens, unpublished work), and its pH optimum in the forward reaction is acidic (Mertens et al., 1989). (2) The fructose 2,6-bisphosphate-dependent enzyme, which is present, together with ATP-PFK and fructose-1,6-bisphosphatase, in the cytosol of plants (Sabularse and Anderson, 1981) and of *Euglena gracilis* (Miyatake et al., 1984; Enomoto et al., 1988). The effect of fructose 2,6-bisphosphate is greatly to increase $V_{max.}$ and the affinity for fructose 6-phosphate, and its pH optimum in the forward reaction is neutral. The plant enzyme is composed of two types of subunit with molecular masses of about 65 and 60 kDa (Yan and Tao, 1984; Kruger and Hammond, 1988), whereas *E. gracilis* PP_i-PFK consists of one single type of subunit of 110 kDa (Enomoto et al., 1988).

The enzyme of *N. fowleri* falls in neither of these categories. Like the first type of PP_i-PFK, it is not accompanied by ATP-PFK, displays a low K_m for fructose 6-phosphate, has an acidic optimum pH and has a subunit of about 50 kDa. Like the second type it is allosterically regulated, in this case, by AMP, which however does not change the affinity for the substrates, but only $V_{max.}$.

Reversible activation and inactivation and the role of AMP

We have obtained *N. fowleri* PP_i-PFK both as a 180 kDa active enzyme and as a 45 kDa inactive precursor, and these two forms could be interconverted by various means. Since the subunit molecular mass of the active form, as measured by SDS/PAGE, is 51 kDa, one can assume that the two forms correspond to an active tetramer and the inactive monomer. A partial activation of the monomer occurs spontaneously at 30 °C when the enzyme concentration exceeds 10–20 m-units/ml and is also favoured by the presence of poly(ethylene glycol), which has the property to increase the local concentration of macromolecules by a steric exclusion effect (Ingham, 1990). Since we estimate the intracellular concentration of PP_i-PFK to be around 5–10 units/ml, it seems most likely that the enzyme is essentially in the tetrameric form *in vivo*.

However, at the low concentration of enzyme used in most of our experiments, a nearly complete activation occurred only in the presence of 0.5 mM AMP. The latter is a ligand of the tetramer, as indicated by the fact that it increases $V_{max.}$ about 2-fold. Due to this binding, AMP therefore has two different actions on the enzyme: a stimulation, which is instantaneous, and an activation, which requires 1–2 h at 30 °C and corresponds to polymerization. Triton X-100, which is inactive by itself, amplifies the activating effect of AMP, but the mechanism of its action is not known. The equilibrium between the two forms of the enzyme is also affected by temperature, since, at a concentration of about 100 m-units/ml, activation occurred at 30 °C, whereas inactivation was observed when the same preparation was left at 0 °C (Figure 7). KSCN, which is known to cause the dissociation of several oligometric proteins such as *E. coli* ATP-PFK (Deville-Bonne et al., 1989), markedly accelerated this inactivation in the cold.

Role of PP_i-PFK in *N. fowleri*

It is difficult to evaluate the physiological significance of *Naegleria* PP_i-PFK and of its regulation by AMP, because of the limited knowledge on the metabolism of these species. Axenic cultivation of these protozoa is possible in chemically defined media that contain glucose (Nerad et al., 1983; Fulton et al., 1984). Glucose can be replaced by glutamic acid, which, however, sustains slower growth and poorer yields (Nerad et al., 1983). The cells contain mitochondria and actively respire (Weik and John, 1977, 1979). Glucose is only slightly utilized, amino acids or protein hydrolysates being the major source of energy and of carbon (Weik and John, 1977). A marginal utilization of carbohydrates by *Naegleria* is not surprising, considering that they feed mainly on bacteria in their natural habitat.

Several glycolytic enzymes have been identified in *N. fowleri* (Pernin et al., 1985), which indicates that glucose can be glycolysed in these cells. By analogy with the situation observed in mammalian cells, it is likely that anoxia causes an increase in the concentration of AMP, which, in turn, stimulates glycolysis at the phosphofructokinase level. Note that in plants and in *E. gracilis*, this role is taken over by fructose 2,6-bisphosphate, which stimulates PP_i-PFK (Mertens, 1991). Furthermore, the protists that have a non-regulated PP_i-PFK are obligate fermenters. This is in agreement with the concept that reutilization of PP_i is particularly beneficial on fermentative metabolism (Wood et al., 1977; Mertens, 1991).

In contrast with *Naegleria* spp., *Acanthamoeba castellanii* relies on a classical ATP-PFK stimulated by fructose 2,6-bisphosphate. In relation with that, it should be noted that both kinds of amoebae are rather distant from each other in phylogenic terms.

rRNA sequence comparisons suggest that *Acanthamoeba* is more closely related to animals and plants than to *Naegleria*, which is a relatively early branch in eukaryotic evolution (Clark, 1990).

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