Biochimica et Biophysica Acta, 657 (1981) 138—152

BBA 69173

PARTIAL CHARACTERIZATION AND INACTIVATION OF MEMBRANE-BOUND PHOSPHOFRUCTOKINASE FROM *TETRAHYMENA PYRIFORMIS*

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(Received June 2nd, 1980)

Key words: Phosphofructokinase; Solubilization; (Tetrahymena membrane)

Summary

In *Tetrahymena pyriformis*, 6-phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is membrane-bound. Enzyme activity is solubilized by treatment of membranes with Triton X-100 or by high ionic strength in the presence of a chelator. The solubilized enzyme has an approximate molecular weight of 300 000. Both the membrane-bound enzyme and the solubilized enzyme exhibit maximum activity over a wide pH range. At low pH, the membrane-bound form of the enzyme is irreversibly inactivated, whereas the solubilized enzyme is not. The membrane-bound enzyme is inactivated by incubation with Mg^{2+}, ATP, fluoride and a soluble factor that is heat labile, nondialysable, $(NH_4)_2SO_4$ precipitable and sensitive to trypsin. The solubilized enzyme is not inactivated under similar conditions.

Introduction

6-Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is a soluble enzyme in most systems studied. A key enzyme of glycolysis, soluble 6-phosphofructokinase is subject to metabolic regulation by a variety of mechanisms. The enzyme is allosterically regulated by numerous effectors such as nucleotides and glycolytic intermediates [1]. Activity of rat liver 6-phosphofructokinase is regulated by fatty acids which bind to the enzyme and irreversibly inactivate it [2]. The inhibition of 6-phosphofructokinase from chicken liver and rabbit muscle by ATP, 3-phosphoglycerate or

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citrate is enhanced by fructose diphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) which induces conformational changes in the 6-phosphofructokinase molecule [3]. Phosphofructokinase from rat liver is protected against fructose diphosphatase inactivation [4] and thermal and lysosomal inactivation [5] by a small, inducible peptide factor.

Dissociation and reassociation of subunits of soluble 6-phosphofructokinase may also contribute to the regulation of the soluble enzyme in some systems. The active, tetrameric form of rabbit muscle 6-phosphofructokinase is stabilized by two activators of the enzyme, fructose 6-phosphate and fructose 1,6-diphosphate [6]. In the presence of citrate, an inhibitor of 6-phosphofructokinase, the rabbit muscle enzyme dissociates to the less active monomeric or dimeric form [6].

Soluble 6-phosphofructokinase from rabbit muscle is classified as a hysteretic enzyme since it undergoes slow conformational changes in response to a rapid change of such factors as pH or concentration of effectors [7]. The enzyme from rabbit liver exhibits similar properties [8]. It is active in the presence of positive effectors. Upon removal of the effectors, activity is initially retained, but is slowly lost as the enzyme is converted to a less active form. Such conformation changes may well contribute to the overall activity of the soluble enzyme [9].

Recent evidence indicates that soluble 6-phosphofructokinase from mouse muscle [10,11], rabbit muscle [12], swine kidney [13] and rat liver [14] is subject to covalent modification. The rat liver enzyme is activated by phosphorylation and inactivated by a phosphatase-catalysed reaction [14].

Unlike the cytoplasmic location of 6-phosphofructokinase in many systems, the enzyme is instead bound to membrane fractions in nervous tissue [15,16] and erythrocytes [17], as well as in the ciliated protozoan, *Tetrahymena pyriformis* [18]. That the supporting membrane contributes to allosteric properties of the membrane-bound enzyme is evident by the finding that membrane-bound erythrocyte 6-phosphofructokinase, unlike soluble erythrocyte 6-phosphofructokinase, is not inhibited by ATP or 2,3-diphosphoglycerate [17].

In the present study it was observed that 6-phosphofructokinase of *T. pyriformis* is a peripheral enzyme of the membrane, has a molecular weight of approx. 300 000 and exhibits maximal activity over a broad pH range. The membrane-bound enzyme is inactivated in a system containing Mg^{2+}, ATP, fluoride and a soluble protein. The finding that the solubilized enzyme is not inactivated under similar incubation conditions suggests a role of the membrane in the regulation of 6-phosphofructokinase activity in this organism.

Materials and Methods

Growth and harvesting of cells. *T. pyriformis*, Phenoset A (ATCC No. 30327: obtained from A. Barnett, University of Maryland), was used in all experiments. The growth medium (pH 7.2) contained 1% protease peptone/ 0.1% yeast extract/0.1% K_2HPO_4/2 μg/ml $FeCl_3$/0.1% glucose (autoclaved separately)/50 units/ml penicillin G/25 μg/ml streptomycin. 1 l cultures were grown in Fernbach-type flasks (capacity 2.5 l) by gentle shaking (approx. 75

rev./min) on a rotating shaker. Larger cultures were grown in 4 or 20 l carboys filled to half-capacity and containing 0.05% (v/v) antifoam B. Air was gently bubbled through these cultures as they mixed slowly on a magnetic stirring motor. All cultures were grown at 30°C. Growth was monitored by counting formalin-immobilized cells on a Levy counting chamber.

Late logarithmic phase cells ($3—5 \cdot 10^5$ cells/ml) were collected by centrifugation at $175 \times g$ for 5 min, in the cold. Cells were washed three times with the indicated buffer and centrifuged as above.

Cell-free extracts and membrane preparation. Washed cells were disrupted by sonication for 60 s using a Bronson sonifier, model LS75, at a setting of 7. Following a $100\,000 \times g$ centrifugation on a Beckman model L ultracentrifuge for 1 h, the membrane fraction was washed once with 20 vol. buffer, centrifuged as above, and resuspended in the same buffer. The supernatant obtained from centrifugation of the crude extract is referred to as the crude supernatant. For enzyme localization studies, cells were gently homogenized in a glass homogenizer and fractionated by discontinuous sucrose gradient sedimentation according to the method of Nozawa and Thompson [19].

Enzyme activities. The reaction mixture for 6-phosphofructokinase contained: 0.1 M Tris-HCl, (pH 7.8)/0.12 mM NADH/1.2 mM ATP/1.2 mM $MgCl_2$/4 mM β-mercaptoethanol/aldolase (EC 4.1.2.13, 0.035 units)/triose phosphate isomerase (EC 5.3.1.1, 3.45 units)/α-glycerol phosphate dehydrogenase (EC 1.1.1.8, 0.3 units) in a total volume of 1 ml. Activity was determined in the absence and presence of fructose 6-phosphate, 0.6 mM, added to the sample cuvette. The change in absorbance at 340 nm was measured continuously on a Cary Model 14 spectrophotometer. Any activity determined in the absence of added substrate was subtracted from activity determined with substrate added. Specific activity is expressed as munits/mg of protein. 1 munit 6-phosphofructokinase activity is that amount of enzyme which catalyses the formation of 1 μmol fructose 1,6-diphosphate per min, under conditions described. Results are expressed as specific activity, percentage of initial specific activity or as munits/vol.

The following enzymes were assayed spectrophotometrically by modifications of published methods: phosphoglucose isomerase (EC 5.3.1.9) [20], hexokinase (EC 2.7.1.1) [21], aldolase [22], lactic dehydrogenase (EC 1.1.1.27) [23], pyruvate kinase (EC 2.7.1.40) [24] and phospho*enol*pyruvate carboxylase (EC 4.1.1.31) [25]. Fructose diphosphatase was assayed at 30°C using 100 mM triethanolamine-HCl buffer (pH 7.1) and 100 mM glycine-NaOH buffer (pH 9.6) [26]. ATPase (EC 3.6.1.3) was measured colorimetrically by determining the amount of inorganic phosphate released from ATP [27].

Protein determination. Protein was determined by the method of Lowry et al. [28] using bovine serum albumin as standard. In samples containing Triton X-100, the Triton was first removed by Bio-Beads SM-2 using the batch procedure of Holloway [29].

Solubilization of 6-phosphofructokinase. Washed membrane pellets from cells harvested in 0.2 M potassium phosphate buffer containing 0.1 M NaCl (pH 7.2) were resuspended in this buffer containing increasing amounts of Triton X-100 (0—4%, v/v), NaCl (0—4 M) or KCl (0—3 M). The samples were incubated on ice for 30 min with slow stirring on a magnetic stirring motor and

then centrifuged for 1 h at 100 000 × *g*. Phosphofructokinase was measured in the supernatant and in the resuspended pellet fraction. Phosphofructokinase levels are expressed as the percentage of initial activity. Similarly, washed pellets from cells harvested in 67 mM potassium phosphate buffer were exposed to EDTA (0—2 mM) or ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) (0—3 mM). These samples were incubated for 60 min at 30°C with gentle stirring, centrifuged and assayed as above.

Membrane pellets collected in 0.2 M potassium phosphate buffer containing 0.1 M NaCl (pH 7.2) were washed at pH 7.2 or 7.4 (0.1 M potassium phosphate buffer), or at pH 7.6, 7.8, 8.0 or 8.2 (0.1 M Tris-HCl buffer). Following centrifugation for 1 h at 100 000 × *g*, the washed pellets were resuspended at pH 7.2 and assayed for 6-phosphofructokinase activity.

Determination of molecular weight. The approximate molecular weight of 6-phosphofructokinase solubilized by treating washed membranes with 3 M NaCl and 0.6 mM EDTA for 60 min, was determined by using a Bio-Gel A 0.5 M, 200—400 mesh (0.9 × 30 cm) column, equilibrated with 0.2 M potassium phosphate buffer containing 0.1 M NaCl and 2 mM β-mercaptoethanol (pH 7.2). In determining the molecular weight of the Triton-solubilized enzyme, 0.5% Triton was added to the above equilibration buffer and the sample, in buffer-containing 0.5% Triton, applied to the column. The molecular weight was also determined on a Triton-solubilized sample from which the Triton was removed by use of Bio-Beads. After removal of Triton, the sample was passed through the column equilibrated with buffer, as above, containing no Triton. The molecular weight of 6-phosphofructokinase was estimated from a calibration curve using ribonuclease A, aldolase, ovalbumin, hexokinase, catalase and phosphorylase *a*.

In vitro inactivation of 6-phosphofructokinase. Cells were routinely washed and sonicated in 100 mM Tris-HCl buffer (pH 7.8) or 67 mM potassium phosphate buffer (pH 7.2). Washed membrane pellets were resuspended in the same buffer and incubated with 20 mM NaF or KF/10 mM $MgCl_2$/10 mM ATP/crude supernatant. The final volume of the inactivation mixture was between 0.1 and 0.8 ml. Samples were incubated in a water bath at 30°C. At set time intervals, aliquots were withdrawn from the inactivation mixture and 6-phosphofructokinase activity was measured. Modifications of the inactivation mixture are described in the text.

Reactivation attempts. Cells were harvested and broken in 100 mM Tris-HCl buffer (pH 7.8). Following inactivation of membrane-bound 6-phosphofructokinase, 0.016 units alkaline phosphatase (EC 3.1.3.1) were added directly to the inactivation mixture as well as to the pellet fraction obtained following centrifugation of an inactivation mixture. The samples were incubated at 30°C for 2 h during which time 6-phosphofructokinase was assayed at 15 min intervals. The same experiments were carried out using 0.5 mg snake venom phosphodiesterase (EC 3.1.4.1). In other experiments, following inactivation, the inactivation mixture was dialyzed against 100 mM sodium acetate buffer, pH 5.0. 0.06 mg acid phosphatase (EC 3.1.3.2) was added to the dialyzed mixture and 6-phosphofructokinase activity was monitored for 2 h during incubation at 30°C. Also, membranes collected following inactivation were washed twice in sodium acetate buffer (pH 5.0) and incubated as above with acid phosphatase.

Characterization of the soluble factor. Crude supernatant as well as the supernatant of an inactivation mixture were concentrated through a PM 10 membrane at 4°C under a nitrogen atmosphere of 40 lbs./inch^2 using an Amicon filter.

The crude supernatant, 0.1 mg protein, was incubated on ice with 0.003 mg trypsin prepared in 0.001 M HCl. After 20 min, 0.8 mg tosyllysinechloroketone (TLCK) were added to the sample and incubation continued for 10 min at which time 20 mM NaF, 10 mM $MgCl_2$, 10 mM ATP and a membrane pellet were added. The activity of 6-phosphofructokinase was determined at set time intervals. A control containing 0.8 mg TLCK was also incubated and examined for 6-phosphofructokinase activity after addition of NaF, $MgCl_2$, ATP and a membrane pellet as above.

Chemicals. Protease peptone and yeast extract were from Difco. Bio-Beads SM-2 and Bio-Gel A 0.5 M, 200—400 mesh, were obtained from Bio-Rad Laboratories. Catalase, phosphorylase *a*, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, creatine phosphokinase and phosphocreatine were purchased from Boehringer-Mannheim. RNAase A, aldolase, Blue Dextran 2000 and ovalbumin were from the Pharmacia calibration kit. Other chemicals and suppliers were: Triton X-100, B grade, Calbiochem; Tris, Ultrapure, Bethesda Research Laboratories, Rockville, MD; snake venom phosphodiesterase, trypsin, Worthington Biochemical Corporation; Ado*P*[CH_2]*PP*, Miles and $(NH_4)_2SO_4$, ultra pure, Schwarz Mann. Acid phosphatase and alkaline phosphatase were from Sigma as were TLCK, Ado*PP*[NH]*P*, and all other chemicals.

Results

Localization of 6-phosphofructokinase

Following sonication of cells for 60 s and centrifugation of the extract for 1 h at 100 000 × *g*, 6-phosphofructokinase activity was found primarily in the pellet fraction; little or no activity could be detected in the supernatant fraction.

Table I shows the cellular distribution of 6-phosphofructokinase and other glycolytic enzymes as determined in cell fractions obtained by dicontinuous sucrose gradient sedimentation [19]. Pyruvate kinase, aldolase, hexokinase and phosphoglucose isomerase were found primarily in the microsomal supernatant indicating the soluble nature of these enzymes. However, 6-phosphofructokinase, phospho*enol*pyruvate carboxylase and lactic dehydrogenase activities were mainly membrane-bound. Pyruvate kinase [30] and aldolase [31] have been reported to be soluble enzymes in *T. pyriformis* whereas hexokinase [31] is partially membrane-bound in another strain of this organism. Phosphofructokinase [18], phospho*enol*pyruvate carboxylase [30] and lactic dehydrogenase [31,32] are presumably mitochondrial enzymes in *Tetrahymena*.

Solubilization of 6-phosphofructokinase activity

Fig. 1 shows that incubation of membrane pellets with Triton X-100 (8.5 μmol Triton X-100 per 1 mg membrane protein) solubilizes approx. 95% of membrane-bound 6-phosphofructokinase. Solubilization of the membrane proteins by this detergent results in a 200% recovery of 6-phosphofructokinase activity.

TABLE I

DISTRIBUTION OF ENZYME ACTIVITY IN EXTRACTS OF *T. PYRIFORMIS*

Cells were gently homogenized. Crude extracts were layered onto discontinuous sucrose gradients.

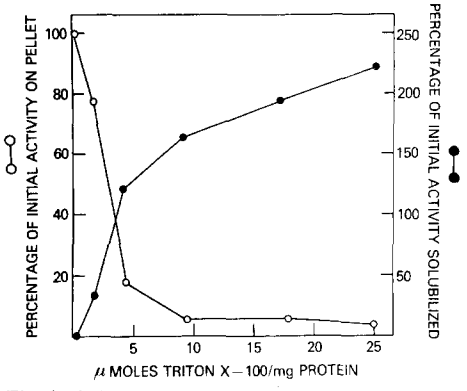
Enzyme	Percent of total activity in:			
	Mitochondria	Pellicle	Microsome pellet	Microsome supernatant
Expt. 1				
Pyruvate kinase	1.2	0	0	98.8
Aldolase	0	0	0	100.0
Phosphoglucose isomerase	5.6	4.1	5.8	87.2
Lactic dehydrogenase	49.0	51.0	0	0
6-Phosphofructokinase	30.0	40.0	30.0	0
Expt. 2				
Pyruvate kinase	2.4	0	1.2	96.4
Aldolase	0	0	0.6	99.4
Phosphoglucose isomerase	2.9	1.7	4.3	91.1
Hexokinase	0	0	0	100.0
Phospho*enol*pyruvate carboxylase	11.7	88.3	0	0
6-Phosphofructokinase	6.9	86.1	7.0	0

Incubation of membrane pellets in 0.2 M potassium phosphate buffer containing high concentrations of either NaCl or KCl also resulted in the release of membrane-bound activity. Fig. 2A shows that incubation of pellets in 1 M NaCl, in the presence of EDTA, for 30 min on ice resulted in the release of approx. 25% of the membrane-bound enzyme. Increasing the concentration of salt failed to further decrease the enzyme activity on the membrane. As the salt concentration in the incubation buffer increased the total enzyme activity found in the supernatant increased; recovery of over 100% of the initial activity was obtained in the presence of 3 M NaCl. Since the membrane-bound activity was not reduced at the higher salt concentrations, the high recovery of 6-phosphofructokinase activity at these concentrations indicates an activation of the enzyme under these conditions.

Absence of EDTA in the incubation mixture, containing increasing concentrations of salt, resulted in a greater loss of membrane-bound activity but a lesser recovery of soluble activity, (Fig. 2B) as compared to incubation with EDTA. In the presence of 4 M NaCl membrane-bound activity decreased by approx. 90%, yet only an estimated 20% of this activity was recovered in the soluble form.

To determine if high salt concentrations alone or high salt concentrations plus chelator affected the activity of Triton-solublized 6-phosphofructokinase, this enzyme was incubated for 1 h on ice, in the presence of 2 M NaCl and in the presence of 2 M NaCl/0.6 mM EDTA. Activity of the enzyme was not altered under either condition. Similarly, 6-phosphofructokinase solubilized by high salt conc./EDTA was incubated with 1% Triton. After 1 h no change in activity was observed.

Membrane pellets were also incubated for 60 min at 30°C in 67 mM potassium phosphate buffer with increasing concentrations of EDTA or EGTA to determine if 6-phosphofructokinase is solubilized by a chelator in the absence



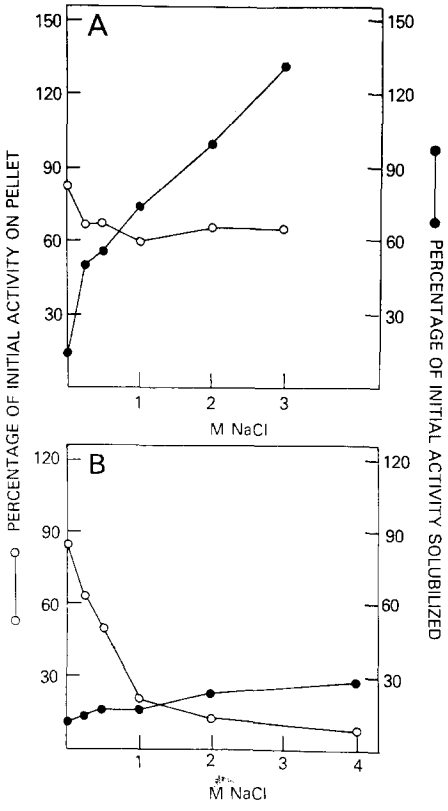


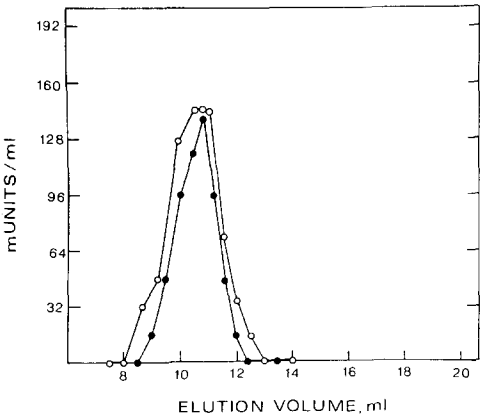
Fig. 1. Solubilization of membrane-bound 6-phosphofructokinase using Triton X-100. Membrane pellets were incubated with Triton X-100 for 30 min on ice with gentle stirring, and centrifuged for 1 h at 100 000 × *g*. 6-Phosphofructokinase activity was measured in the supernatant fractions and in the resuspended pellets.

Fig. 2. Solubilization of membrane-bound 6-phosphofructokinase using NaCl. Membrane pellets were incubated in 0.2 M potassium phosphate buffer (pH 7.2) containing increasing concentrations of NaCl, (A) 0.6 mM EDTA and (B) no EDTA. After mixing gently for 30 min on ice and centrifuging for 1 h at 100 000 × *g*, 6-phosphofructokinase activity was measured in the supernatant fractions and in the resuspended pellets.

of high salt concentrations. Under such conditions, no solublized activity could be measured. Also, washing membrane pellets collected at pH 7.2 with buffers from pH 7.2—8.2 failed to solubilize 6-phosphofructokinase activity (data not shown).

Molecular weight determination

Fig. 3 shows the elution profile of the Triton-solubilized enzyme passed through the Bio-Gel column equilibrated with 0.5% Triton, and that of the Triton-solubilized enzyme, from which the Triton was removed by the use of Bio-Beads, passed through the same column equilibrated without detergent. It can be seen that the elution volume of the two enzymes is the same. Hence, in the absence of Triton the enzyme neither aggregates nor inactivates. The peak of activity of the NaCl-solubilized enzyme is the same as that of Triton-released



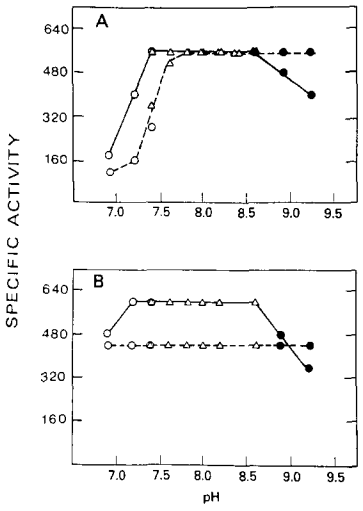


Fig. 3. Elution profiles of Triton-solubilized 6-phosphofructokinase. The Triton-solubilized enzyme was passed through a Bio-Gel A 0.5 M column equilibrated with buffer containing 0.5% Triton (○———○). The Triton was removed from a solubilized enzyme preparation by use of Bio-Beads, and the sample applied to the same column equilibrated with buffer containing no Triton (●———●).

Fig. 4. Profiles of activity of membrane-bound 6-phosphofructokinase (A) and Triton-solubilized 6-phosphofructokinase (B) as a function of pH (solid lines). Dotted lines indicate activity of the enzyme incubated at the designated pH for 5 min at 30°C and assayed in 0.1 M Tris-HCl (pH 7.8). The symbols indicate the buffer used: ○, 0.1 M imidazole-HCl; △, 0.1 M Tris-HCl and ●, 0.1 M glycine-NaOH.

6-phosphofructokinase. The approximate molecular weight of the solublized enzyme was estimated from a calibration curve, using proteins of known molecular weights, to be 300 000.

pH optimum

Both membrane-bound and Triton-solubilized 6-phosphofructokinase exhibit maximal activity over a wide pH range (Fig. 4). The decrease in activity, seen with both forms of the enzyme, at high pH is not due to an irreversible inactivation of the enzyme since incubation of the enzyme at high pH and subsequent assay at pH 7.8 does not result in decreased activity. Membrane-bound 6-phosphofructokinase responds more drastically to assay at lower pH than does the solubilized enzyme. It can also be seen that the membrane-bound enzyme is irreversibly inactivated at low pH, whereas the solubilized enzyme is not.

In vitro inactivation of 6-phosphofructokinase

The activity of particulate 6-phosphofructokinase does not change upon incubation of a resuspended membrane pellet for 1 h at 30°C (Fig. 5A). Addition of 10 mM $MgCl_2$, 10 mM ATP and 20 mM KF to the resuspended membrane also did not alter the activity of the enzyme (Fig. 5A). Activity of membrane-bound 6-phosphofructokinase is also not affected during incubation for 1 h at 30°C in the presence of the crude supernatant, $MgCl_2$, ATP, and the crude supernatant, or KF and the crude supernatant (Fig. 5B). The particulate

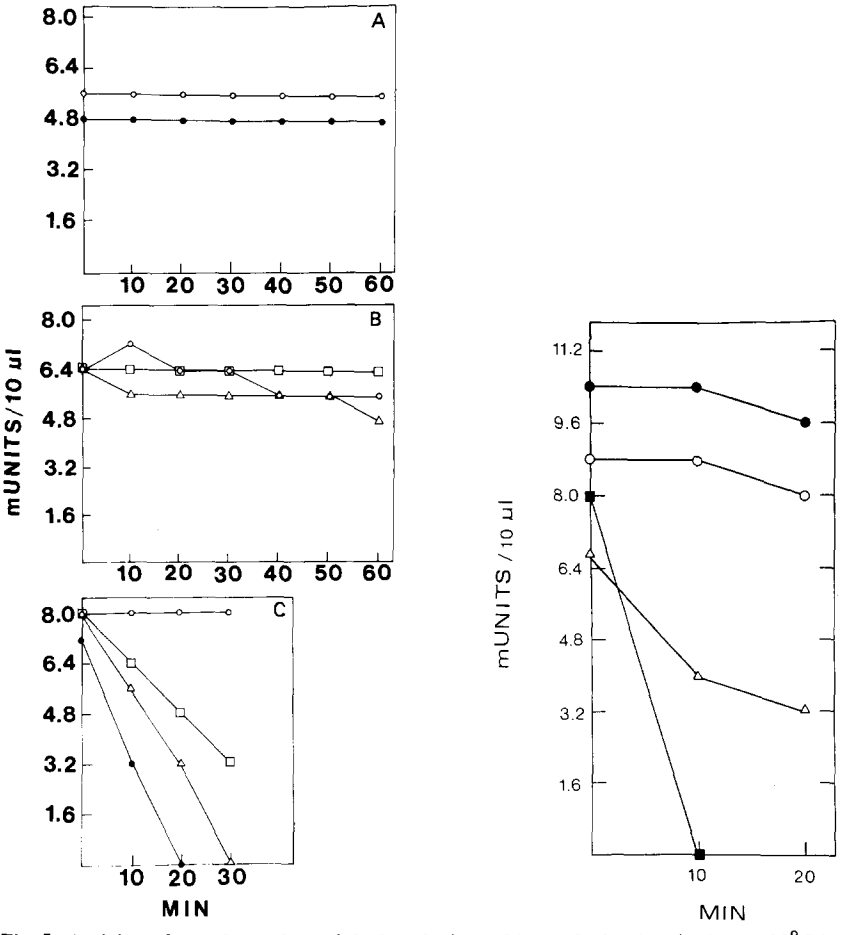


Fig. 5. Activity of membrane-bound 6-phosphofructokinase during incubation at 30° C in the presence of (A); ○, 100 mM Tris-HCl buffer (pH 7.8); ●, buffer, as above, 10 mM $MgCl_2$/10 mM ATP/20 mM KF: (B); ○, 100 µl crude supernatant; △, 100 µl crude supernatant plus 10 mM $MgCl_2$/10 mM ATP; □, 100 µl crude supernatant plus 20 mM KF: (C), ○, buffer, as above, 10 mM $MgCl_2$/10 mM ATP/20 mM KF; □, 5 µl crude supernatant/10 mM $MgCl_2$/10 mM ATP/20 mM KF; △, 10 µl crude supernatant/10 mM $MgCl_2$/10 mM ATP/20 mM KF and ●, 25 µl crude supernatant/10 mM $MgCl_2$/10 mM ATP/20 mM KF. Cells were washed and broken in 100 mM Tris-HCl buffer (pH 7.8).

Fig. 6. The effect of ATP analogues on the inactivation of membrane-bound 6-phosphofructokinase. Particulate 6-phosphofructokinase was incubated with crude supernatant, 20 mM NaF, 10 mM $MgCl_2$ and (■) 10 mM ATP, (○) 10 mM Ado*P*[CH_2]*PP*, and (△) 10 mM Ado*PP*[NH]*P*. Membrane-bound 6-phosphofructokinase was also incubated with only crude supernatant (●).

enzyme is inactivated when incubated with the crude supernatant, $MgCl_2$, ATP, and KF (Fig. 5C). NaF can replace KF in the inactivation mixture. GTP and ADP can each replace ATP in the inactivation mixture, but as with ATP, the ADP- and GTP-dependent loss of activity required the soluble inactivating factor, Mg^{2+} and NaF or KF. AMP, adenosine, pyrophosphate, cyclic AMP, inorganic phosphate, UTP or CTP could not replace ATP in the inactivation mixture.

As seen in Fig. 6 no loss of activity occurred upon incubation with the ATP

analogue Ado*P*[CH_2]*PP*, suggesting that hydrolysis of the α,β-anhydride bond may be involved in the ATP-dependent loss of 6-phosphofructokinase activity. Incubation of the membrane pellet with the analogue, Ado*PP*[NH]*P*, $MgCl_2$, NaF and the soluble factor did result in a slight loss of 6-phosphofructokinase activity; therefore, it is possible that cleavage of the α,β-bond is necessary for the ATP effect. As with ATP, the loss in activity with Ado*PP*[NH]*P* requires Mg^{2+}.

The in vitro, ATP-dependent inactivation of particulate 6-phosphofructokinase is observed in cell extracts prepared in 67 mM potassium phosphate buffer (pH 7.2) as well as in 100 mM Tris-HCl buffer (pH 7.8). Occasionally, membrane preparations in the latter buffer system had endogenous inactivating activity, in that loss of some 6-phosphofructokinase activity occurred during incubation of pellet fractions, fluoride and $MgCl_2$, and ATP (data not shown).

Incubation of Triton-solubilized 6-phosphofructokinase or NaCl-solubilized 6-phosphofructokinase with NaF, $MgCl_2$, ATP and the crude supernatant did not affect enzyme activity. Inclusion of membrane pellets in the above incubation mixtures failed to promote inactivation of the solubilized enzyme (data not shown).

Characterization of the soluble factor

Table II summarizes the results of preliminary studies of the properties of the soluble factor. Dialyzed and undialyzed supernatant were equally effective in causing inactivation of membrane-bound 6-phosphofructokinase in the presence of $MgCl_2$, ATP and NaF. The filtrate obtained from pressure filtration of crude supernatant, through a membrane of 10 000 porosity, could not replace the requirement for the crude supernatant. Boiling the supernatant for 7 min in a water bath, however, destroyed the inactivating factor. Pretreatment of the crude supernatant with trypsin followed by inhibition of protease activity with TLCK destroyed the inactivating capacity. Partial purification of the soluble inactivating factor (data not shown) has shown that it is precipitated by

TABLE II

EFFECTS OF PRETREATED CRUDE SUPERNATANT ON INACTIVATING ACTIVITY

The incubation mixture in these experiments consisted of a membrane pellet washed with 100 mM Tris-HCl buffer (pH 7.8)/10 mM $MgCl_2$/10 mM ATP/20 mM NaF.

Addition to incubation mixture	% Loss of 6-phosphofructokinase activity
Expt. 1	
1. Buffer	25
2. Crude supernatant	100
3. Boiled supernatant	40
4. Dialyzed supernatant	100
5. Ultrafiltration dialysate	30
Expt. 2	
1. Buffer	0
2. Crude supernatant	87
3. Crude supernatant + TLCK	93
4. Crude supernatant + trypsin + TLCK	13

$(NH_4)_2SO_4$. Taken together, these results indicate that the inactivating factor is a non-dialyzable, heat labile protein of greater than 10 000 daltons.

Role of the supernatant protein

To examine the possibility that ATP is required to activate an enzyme in the supernatant fraction, which in turn catalyzes the inactivation of 6-phosphofructokinase, the crude supernatant was incubated with $MgCl_2$, ATP and NaF. After 20 min, the mixture was dialyzed then incubated with a membrane pellet containing active 6-phosphofructokinase. No change in 6-phosphofructokinase activity was noted for 1 h under these conditions (data not shown).

To determine if a small molecule is produced in the inactivation mixture which inhibits 6-phosphofructokinase activity, following inactivation of 6-phosphofructokinase in the presence of the crude supernatant, NaF, $MgCl_2$ and ATP, the inactivation mixture was centrifuged and the supernatant subjected to pressure filtration. The filtrate was then incubated with a membrane fraction containing active 6-phosphofructokinase. No loss of 6-phosphofructokinase activity was observed for 1 h (data not shown).

The crude supernatant and a partially purified inactivating factor were assayed for fructose diphosphatase activity. No activity was found in either sample.

Role of fluoride in the inactivation of 6-phosphofructokinase

The inactivation of particulate 6-phosphofructokinase was not complete when low levels of ATP were used in the inactivation mixture. Such incomplete inactivation could be due to exhaustion of the nucleotide by endogenous ATPase activity. If so, fluoride might be required to inhibit the ATPase activity and thereby maintain a high level of ATP. Preliminary experiments (data not shown) did show that fluoride does inhibit the ATPase activity in *T. pyriformis*. Therefore, to determine if fluoride was needed to maintain a high ATP level during incubation, an ATP-generating system consisting of 10 mM ADP and 1.25 units creatine kinase (EC 2.7.3.2) was incubated with a pellet, partially purified soluble factor, $MgCl_2$ and ATP. No loss of 6-phosphofructokinase activity occurred during 20 min, whereas in the presence of fluoride, $MgCl_2$, ATP and partially purified soluble fractor, particulate 6-phosphofructokinase was completely inactivated within 10 min. Hence, the continued presence of ATP did not replace the requirement for fluoride. These results suggest that fluoride is not serving to inhibit ATPase activity.

Attempts to reactivate inactive 6-phosphofructokinase

The inactivation of particulate 6-phosphofructokinase may be due to a covalent modification of the enzyme. If the ATP-dependent inactivation of 6-phosphofructokinase from *T. pyriformis* involves phosphorylation of the enzyme, then the effect of phosphorylation is just opposite to that obtained with rat liver 6-phosphofructokinase [14]. The requirement for fluoride in the inactivation mixture is consistent with a phosphorylation mechanism, since the fluoride could be required to inhibit a phosphatase activity and thereby shift the equilibrium toward the phosphorylated, inactive form of the enzyme. Attempts were made, therefore, to reactivate the enzyme using acid phos-

phatase and alkaline phosphatase. Both enzymes failed to reactivate 6-phosphofructokinase.

As noted above, the results of studies with ATP analogues suggest that hydrolysis of the α, β-bond of ATP could be involved in the inactivation of particulate 6-phosphofructokinase. However, the possibility that 6-phosphofructokinase is adenylylated, as occurs with glutamine synthetase [33], is contraindicated by the observation that treatment of the inactive enzyme with snake venom phosphodiesterase failed to restore activity.

Extensive washing of the membrane pellet following 6-phosphofructokinase inactivation failed to restore 6-phosphofructokinase activity. Dialysis of the inactivation mixture following inactivation also failed to reactivate the enzyme as did gel filtration of Triton-solubilized membrane proteins following inactivation. (Maurizi reports that a 20-fold dilution of the inactivation mixture results in the restoration of 6-phosphofructokinase activity. Personal communication.)

Discussion

Sedimentation of 6-phosphofructokinase at $100\,000 \times g$ for 1 h following sonication indicates that 6-phosphofructokinase is particulate in *T. pyriformis*. Lack of 6-phosphofructokinase activity in the microsomal supernatant of homogenized cells further implies that the enzyme is not soluble. The presence of other glycolytic enzymes exclusively in the soluble portion of the cell extract indicates that nonspecific entrapment of 6-phosphofructokinase by membrane vesicles does not occur, but rather that the enzyme is specifically bound to a membrane.

The 2-fold increase in 6-phosphofructokinase activity following solubilization of activity by Triton X-100 may reflect an activation of activity, due to conformation changes of the enzyme induced by the detergent as occurs with brain acetylcholinesterase [34]. However, the finding that Triton failed to alter the activity of the NaCl-solubilized enzyme casts doubt on this possibility. The increased activity may be due instead to activity of latent enzyme molecules as occurs with membrane-bound hexokinase [35] and cyclic 3′,5′-nucleotide phosphodiesterase [36] in rat brain.

Enzymes bound to cell membranes by hydrophobic interactions generally aggregate and are inactive in aqueous solution [37]. The finding that 6-phosphofructokinase is the same size in the presence and absence of Triton indicates that the enzyme does not aggregate in aqueous solution. Retention of activity following removal of Triton further suggests that 6-phosphofructokinase is not bound to the membrane by hydrophobic interactions.

Since chelators in the absence of high salt concentrations do not release 6-phosphofructokinase activity from the membrane, a divalent cation is most likely not responsible for the direct attachment of the enzyme to the membrane. Solubilization of 6-phosphofructokinase by high ionic strength in the presence of a chelator does suggest the involvement of ionic interactions in the binding of 6-phosphofructokinase to the membrane. The increased activity seen with increasing ionic strength in the presence of EDTA reflects an activation of enzyme activity under these conditions, perhaps by removal of a cation inhibitory to the solubilized enzyme. The increased activity may instead be due to

the release of latent enzyme. It is curious that incubation of membranes in high ionic strength, in the absence of a chelator, results in a greater loss of membrane-bound activity than is found in membranes incubated with the chelator. This may reflect dissociation of the membrane-bound enzyme at high ionic strength, in the absence of chelator, as occurs with soluble sheep heart 6-phosphofructokinase [38]. Since high salt concentration failed to reduce the activity of the Triton-solubilized enzyme, the effect exerted by the high ionic strength is instead perhaps on the membrane. If this is the case, protection from membrane disruption may be conferred by the chelator. The lesser recovery of solubilized activity in the absence of chelator may also be due to dissociation of the solubilized enzyme or the presence of a cation that inhibits solubilized enzyme activity. The results of Fig. 2 also suggest that in the presence of EDTA, 6-phosphofructokinase is bound more tightly to the membrane. This may be the result of chelation of a cation in the membrane environment of 6-phosphofructokinase that results in a tighter 6-phosphofructokinase-membrane association.

Dissociation of proteins from membranes by high salt concentration is characteristic of peripheral proteins [37] and hence 6-phosphofructokinase from *T. pyriformis* can be classified in this category of membrane-bound enzymes. It is possible, though, that the high salt concentration solubilizes membrane lipid and releases lipoprotein fragments as is the case with acetylcholinesterase of erythrocyte membranes [39]. The finding that the enzyme released by high NaCl concentration is the same size as the Triton-solubilized enzyme, following removal of detergent, argues against this possibility. One would expect an enzyme attached to lipoprotein fragments to be larger than the enzyme in aqueous solution.

The broad pH optimum of activity of 6-phosphofructokinase from *T. pyriformis* is similar to that of 6-phosphofructokinase found in rabbit muscle [40], pig spleen [41] and rat thyroid cells [42]. The more pronounced response to low pH exhibited by the membrane-bound enzyme, as compared to that of the solubilized enzyme, coincides with the irreversible inactivation of the particulate enzyme upon incubation at low pH and with the absence of such inactivation upon similar incubation of the solubilized enzyme. The difference noted may be due to the effect of low pH directly on the enzyme molecules. If this is the case, differences in the conformation of the two enzymes are realized. It is also possible that the different response to low pH is due to local, environmental changes on the membrane affecting activity of the particulate enzyme. Since 6-phosphofructokinase is bound to the membrane by electrostatic forces, ionization in the vicinity of the enzyme could well influence enzyme activity.

Membrane-bound 6-phosphofructokinase from *T. pyriformis* is inactivated in a system containing $MgCl_2$, ATP, fluoride and a soluble factor which appears to be a protein, for it is (1) heat labile, (2) nondialysable, (3) precipitated by $(NH_4)_2SO_4$ and (4) inactivated by a protease. The role of the supernatant protein is not known at this time. The protein may serve to covalently modify the membrane-bound enzyme. It is conceivable that in this system active 6-phosphofructokinase is being phosphorylated and inactivated. However, the results of experiments using analogues of ATP suggest that the β, γ-bond of ATP is not hydrolyzed during the inactivation, but rather that the α, β-bond is being

hydrolyzed. If the findings with the analogues do reflect specific bond cleavage, an argument against phosphorylation of 6-phosphofructokinase can be made. Hydrolysis of the α, β-bond would occur during adenylylation of 6-phosphofructokinase.

If 6-phosphofructokinase is not being covalently modified by the soluble protein or inhibited by ATP, it is possible that a product of ATP hydrolysis inhibits the enzyme. Hydrolysis of the α, β-bond may be due to adenyl cyclase activity with the production of cyclic AMP and pyrophosphate. Either product may subsequently inhibit 6-phosphofructokinase. Since NaF activates membrane-bound adenyl cyclase in other systems [43] this possibility seemed reasonable. Absence of an effect of cyclic AMP or pyrophosphate on particulate 6-phosphofructokinase activity, however, casts doubt on the involvement of adenyl cyclase activity in the inactivation of 6-phosphofructokinase. The finding, that the other products of ATP hydrolysis cannot replace the requirement for ATP in the inactivating system, strongly suggests that inhibition of the enzyme by such a product is not occurring. As well, failure of the filtrate of an inactivation mixture to inactivate particulate 6-phosphofructokinase, further suggests that the enzyme is not being allosterically inhibited by a small molecule produced during the incubation. The inactivation of 6-phosphofructokinase seen with ADP may be due to the production of ATP by endogenous adenyl kinase activity. It is also possible that the soluble protein binds to 6-phosphofructokinase, produces a conformational change in the enzyme molecule and consequently inactivates it.

The finding that solubilized 6-phosphofructokinase cannot be inactivated when incubated with NaF, $MgCl_2$, ATP and crude supernatant indicates a direct role of the membrane in the inactivation of the membrane-bound form of the enzyme. Failure of the addition of a membrane pellet to the above incubation mixture to inactivate solubilized 6-phosphofructokinase, further suggests that proximity to membrane components is needed for inactivation. The membrane may serve to maintain a conformation of 6-phosphofructokinase needed to interact with the soluble protein, if a protein-protein interaction is taking place. On the other hand, modification of the membrane components in the vicinity of bound 6-phosphofructokinase may be occurring, which subsequently changes the conformation of 6-phosphofructokinase to an inactive form.

While the precise mechanism of the inactivation of phosphofructokinase in the system described here is not clear, it is apparent that the supporting membrane of the enzyme contributes to the regulation of enzyme activity. Further investigations will hopefully delineate the mechanism of inactivation and the role the membrane plays.

Acknowledgements

Sincere appreciation is extended to Earl R. Stadtman for his advice throughout this work and to Maria J. Mazón for many helfpul discussions. This investigation was supported by National Institutes of Health National Research Service Award 5 F32 AI 05053 from the National Institute of Allergy and Infectious Diseases.

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