Localization of Phosphofructokinase on the Mitochondria of Tetrahymena pyriformis*

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SUMMARY

At least three-fourths of the phosphofructokinase activity in homogenates of Tetrahymena pyriformis is localized on the mitochondria. The mitochondrial phosphofructokinase activity is stabilized by ATP and by fructose 6-phosphate and is inhibited by ATP and by citrate.

Although several authors have claimed that the entire glycolytic sequence can be observed in some mitochondria (1, 2; also see references in 1), it is almost universally accepted that glycolysis occurs primarily, if not exclusively, in the cytosol. In the ciliated protozoon Tetrahymena pyriformis, however, virtually all of the lactate dehydrogenase (3) and hexokinase (4) is mitochondrial and about half of the glyceraldehyde 3-phosphate dehydrogenase is particulate (5), presumably localized on the mitochondria. Since phosphofructokinase is frequently a rate-limiting step in glycolysis, and has been reported to be the glycolytic enzyme with lowest activity in Tetrahymena (6), it was of interest to initiate studies on factors that might regulate this enzyme in Tetrahymena. In this communication we report that at least three-fourths of the phosphofructokinase activity in this cell is on the mitochondria and present some preliminary information on the kinetic properties of the particulate enzyme.

MATERIALS AND METHODS

T. pyriformis, strain HSM, was grown in a medium consisting of 1% proteose-peptone and 0.05% liver extract in 0.02 M KH₂PO₄ adjusted to pH 6.5 with NaOH. Cells were grown at 26° in Erlenmeyer flasks with shaking, unless otherwise specified. Cells were counted with a Coulter Counter (Coulter Co., Hialeah, Fla.).

Preparation of Particulate Fractions— Cultures were mixed with an equal volume of ice-cold Buffer A (20 mM glycylglycine, pH 8.0, 0.5 mM MgCl₂, and 0.25 mM sucrose) and were centrifuged at 200 x g for 3 min. This and all subsequent steps were done at 0–4°. The supernatant was decanted, the cells were resuspended in about 5 ml of Buffer A containing 0.5 mM ATP and 0.5 mM dithiothreitol. A cell count was taken and the remaining cells were then broken by mechanical homogenization or by sonication. Mechanical homogenization was accomplished in a Potter-Elvehjem grinder with a Teflon pestle rotating at about 100 rpm; over 90% of the cells were broken after 100 strokes. Sonication treatment consisted of two 30 s exposures in a model LS 75 Sonifier (Branson Ultrasonic Corp.) at a setting of 7. The disrupted cell preparations were centrifuged at 12,000 x g for 20 min and the pellet was resuspended in a small volume of the Buffer A with ATP and dithiothreitol. The volumes of the supernatant and of the resuspended pellet were measured.

Zonal centrifugation was carried out in a TiXIV rotor equipped with a 29 liner essentially as described by Porter et al. (7). Four hundred milliliters of a continuous 10 to 50% (w/w) sucrose gradient in Buffer A plus 0.5 mM ATP and 0.5 mM dithiothreitol were loaded in from the edge, and rested on a cushion of 55% sucrose in Buffer A. The sample volume was 15 ml and was overlaid with about 90 ml of a 1:1 dilution of Buffer A with water. Centrifugation was at 5000 rpm for 16 min. Fractions of about 11 ml were collected from the outer edge.

Enzyme Assays—Glutamate dehydrogenase, lactate dehydrogenase, isocitrate lyase, and catalase were assayed as described by Porter et al. (7). Phosphofructokinase was assayed essentially as described by Ho and Anderson (8). The assay mixture contained, in 1.0 ml: 0.5 μmole of ATP, 0.25 μmole of NADH, 0.5 μmole of MgSO₄, 1 μmole of fructose 6-phosphate, 20 μmoles of glycylglycine (pH 8.0), 0.2 unit of aldolase, 12.3 units of triosephosphate isomerase, and 1.4 units of α-glycerophosphate dehydrogenase. The reaction was started by adding the solution to be assayed for phosphofructokinase. For each assay a control with fructose 6-phosphate omitted was performed. The coupling enzymes were dialyzed overnight against 20 mM glycylglycine at pH 8.0 prior to use. It was ascertained at the end of each day that an excess of each coupling enzyme was present. The reaction rate was computed from the change in absorbance at 340 nm, using cells with a 1-cm light path in a thermostatted cell compartment maintained at 26°. One unit of phosphofructokinase activity is equal to 1 μmole of fructose 1,6-diphosphate produced per hour. Activity was expressed as units per 10⁸ cells or units per ml of fraction.

Hexokinase was assayed according to the method of Risse and Blum (4) except that glycylglycine buffer was used instead of Tris. The reaction mixture was contained, in 0.5 ml at pH 8.0, 0.5 mM MgCl₂, 0.25 mM ATP, 0.25 mM dithiothreitol, and 0.5 mM hexokinase. The reaction was started by adding the solution to be assayed for hexokinase. After the supernatant was decanted, the cells were resuspended in a small volume of ice-cold Buffer A containing 0.5 mM ATP and 0.5 mM dithiothreitol. A cell count was taken and the remaining cells were then broken by mechanical homogenization or by sonication. Mechanical homogenization was accomplished in a Potter-Elvehjem grinder with a Teflon pestle rotating at about 100 rpm; over 90% of the cells were broken after 100 strokes. Sonication treatment consisted of two 30 s exposures in a model LS 75 Sonifier (Branson Ultrasonic Corp.) at a setting of 7. The disrupted cell preparations were centrifuged at 12,000 x g for 20 min and the pellet was resuspended in a small volume of the Buffer A with ATP and dithiothreitol. The volumes of the supernatant and of the resuspended pellet were measured.

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of Tris-HCl. Glucose 6-phosphate isomerase activity was measured by coupling the hexokinase assay with the phosphofructokinase assay.

Reagents—ATP, dithiothreitol, aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, and fructose 6-phosphate were purchased from Sigma. All other reagents were of highest purity available.

RESULTS

Table I shows the results of an experiment in which half of the cells from a culture were disrupted by sonication and half by the gentler procedure of homogenization in a Teflon glass homogenizer. Most of the phosphofructokinase activity was "soluble" (i.e. stayed in solution after centrifugation for 20 min at 12,000 × g) in cells disrupted by ultrasound, but three-fourths of the phosphofructokinase activity was localized in the crude mitochondrial fraction. Washing the crude mitochondrial fraction by centrifugation and resuspension in fresh buffer with or without 0.04% Triton X-100 did not solubilize an appreciable amount of the activity. Centrifugation of the supernatant of the sonicated aliquot at 100,000 × g for 1 hour in a Beckman model L ultracentrifuge did not lead to the sedimentation of any activity; by this criterion, sonication leads to a true solubilization of the enzyme without any loss of activity.

The particulate phosphofructokinase activity decayed rapidly after homogenization. Since it is well known (9) that this enzyme from other species is stabilized by its substrates, ATP and fructose 6-phosphate were added to the homogenate. As shown in Fig. 1, 1 mM fructose 6-phosphate reduced the rate of loss of activity while 0.5 mM ATP almost prevented loss of activity of particles kept for over 5 hours at pH 8.0. In all subsequent experiments ATP was added to the washed cell suspension just prior to homogenization. It should be noted, however, that even in the presence of 0.5 mM ATP some activity is frequently lost. It was also observed that the greater the dilution of the crude mitochondrial preparation, the greater the tendency for loss of activity.

Localization of Phosphofructokinase Activity—To determine the nature of the particles containing the phosphofructokinase activity, particles obtained from a homogenate were resolved by zonal gradient centrifugation as described in Fig. 2. Isocitrate lyase and catalase served as peroxisomal markers; lactate and glutamate dehydrogenases served as mitochondrial markers (7). It can be seen that an almost complete separation between mitochondria and peroxisomes was achieved, and that the phosphofructokinase activity was localized on the mitochondria. The

Table I

Localization of phosphofructokinase after disruption of Tetrahymena by sonication or by mechanical homogenization

| Method of disruption | PFK units in supernatant | PFK units in pellet | Total units |
|----------------------|--------------------------|---------------------|-------------|
| Sonication           | 27.5                     | 3.9                 | 31.4        |
| Homogenization       | 6.8                      | 24.9                | 31.7        |

Fig. 1. Decay of phosphofructokinase (PFK) activity with time. Cells were homogenized in a Potter-Elvehjem grinder as described under "Materials and Methods." Aliquots of the particles were resuspended in Buffer A (□—□), in Buffer A plus 1.0 mM fructose 6-phosphate (△—△), or Buffer A plus 0.5 mM ATP (○—○), and phosphofructokinase activity was assayed at the times shown on the abscissa. All solutions used were at pH 8.0.

Fig. 2. Zonal centrifugation of particles prepared from a homogenate of Tetrahymena. Shaken cells in the log phase of growth (448,000 cells per ml) were collected and homogenized in a Potter-Elvehjem grinder in Buffer A plus 0.5 mM ATP and 0.5 mM dithiothreitol. Particles from 115 × 10⁶ cells were put into the zonal rotor and zonal centrifugation performed as described under "Materials and Methods." Fractions of about 11 ml were collected and assayed for the following activities: lactate dehydrogenase (LDH); glutamate dehydrogenase (GDH); phosphofructokinase (PFK); isocitrate lyase (IL); catalase. The sucrose density at selected points on the gradient is shown across the top of the graph. Per cent activities recovered, obtained by summing the values for each fraction (interpolating when necessary) and comparing to the activities measured for a sample of the whole particle fraction were: phosphofructokinase, 33%; glutamate dehydrogenase, 72%; lactate dehydrogenase, 30%; catalase, 82%; isocitrate lyase, 140%.
FIG. 3. Variation of mitochondrial phosphofructokinase (PFK) activity with pH and time after mechanical homogenization. A crude mitochondrial fraction was prepared as described under "Materials and Methods" and kept at 0°. For these measurements 20 mM KH₂PO₄ was added to the assay mixture and the final pH was adjusted to the values shown on the abscissa. The numbers on each curve indicate the time, in minutes, after preparation of the resuspended mitochondrial pellet. Assays were made at pH values chosen at random. A and B show the results of two different experiments. ATP (10 mM) was included in the assay mixture for the assays performed at 60 min and 275 min in A and at 130 min in B.

Small amount of phosphofructokinase activity appearing in the peroxisomal peak is almost certainly due to contamination of the peroxisomes by mitochondria, as indicated by the presence of small amounts of lactate dehydrogenase and glutamate dehydrogenase activities. It should also be noted that only half of the total phosphofructokinase activity put onto the gradient was recovered, presumably due to inactivation resulting from dilution as mentioned above.

Some Properties of Mitochondrial Phosphofructokinase—In a freshly prepared crude mitochondrial pellet phosphofructokinase activity is low at pH about 6 and increases to a fairly flat maximum as the pH is increased to pH 8 (Fig. 3). Although the presence of 0.5 mM ATP in the homogenization buffer appears to protect the activity measured at pH 8, this may not be true of the activity measured below pH 7. Two different kinds of results were obtained with time in ice after homogenization. In Fig. 3A the shape of the activity versus pH curve appears to change so that with time after homogenization the activity decreases more at low pH than at high pH. In Fig. 3B, however, the activity decreased with time more or less uniformly in the pH range studied. Increasing the ATP concentration in the assay medium to 10 mM caused a marked inhibition of activity, but the highest activity still occurred at about pH 8.

Mitochondrial phosphofructokinase was inhibited by citrate (Fig. 4). The enzyme was much more sensitive to inhibition by citrate at pH 6 than at pH 8. In experiments not shown, it was established that both orthophosphate and ammonium sulfate enhance the activity of this enzyme. There was no effect of cyclic 3':5'-AMP on the activity of the mitochondrial phosphofructokinase whether it was added to the homogenization medium or to the assay medium.

Several experiments were done in which the sensitivity of the supernatant phosphofructokinase to pH, citrate, and ATP was compared to the sensitivity of the mitochondrially localized enzyme. These very preliminary experiments did not indicate any major differences between the two forms of phosphofructokinase, and the subject was not pursued further.

It was found that in homogenates of Tetrahymena prepared in a Potter-Elvehjem grinder at pH 7.9 most of the hexokinase activity is localized on the mitochondria, whereas in homogenates prepared at pH 6.0 most of the activity was soluble (4). The effect of pH of the homogenization buffer on localization of phosphofructokinase was therefore studied. No change in the distribution of phosphofructokinase was observed when a homogenate made at pH 7.0 or 7.5 was compared with one made at pH 8.0. The possibility that phosphohexose isomerase was present on the mitochondria was tested for by adding glucose and some more ATP to the assay mixture for phosphofructokinase, but leaving out the fructose 6-phosphate. It was ascertained that the hexokinase was active under these conditions and that if fructose 6-phosphate was added, the phosphofructokinase was active. In the absence of added fructose 6-phosphate, however,
no oxidation of NADH was observed, indicating that phospho-
hexose isomerase was not present.

Several experiments were done in which the cells were grown
in medium supplemented with 6 mM acetate or 12 mM glucose,
or were grown under conditions of partial anaerobiosis, or were
grown to stationary phase. In no case did we find a large and
reproducible change in total amount of phosphofructokinase
activity nor was there any reproducible change in per cent of
distribution between the mitochondrial pellet and the super-
natant. The amount of activity varied from 10 to 25 nmoles
per min per mg of cell protein, comparable to the values obtained
by Warnock and Van Eys (6).

DISCUSSION

It is generally found that phosphofructokinase in many species
is a soluble enzyme (9), although Mansour et al. (10) noted that
this enzyme was localized on an insoluble fraction of sheep heart
homogenates. In so far as we have been able to ascertain, how-
ever, *Tetrahymena* is the first cell for which a mitochondrial
localization of this glycolytic enzyme has been established.
Hexokinase is relatively loosely bound to the mitochondria of
*Tetrahymena*; changing the pH from 7.9 to 6.9 leads to an almost
complete solubilization of this enzyme. Lactate dehydrogenase,
also localized on the mitochondria of this cell, is at the opposite
extreme, and remains particulate even after sonication. Phos-
phofructokinase appears to be intermediate. It is entirely solu-
bilized by sonication but remains on mitochondria prepared by
mechanical homogenization even after washing with 0.04% Triton X-100. It is therefore very unlikely that the phospho-
fructokinase was localized in the cytosol and was adsorbed onto
the mitochondria because of changes brought about by the
mechanical homogenization procedure. We have also noticed
that the distribution is very sensitive to the particular set of
Potter-Elvehjem homogenizers used. The maximum yield we
have obtained for the mitochondrial form is about 80% of the
total, but, in view of the sensitivity to mechanical disruption,
it is possible that almost all of the phosphofructokinase of *Tetra-
hymena* is localized on the mitochondria in vivo. This possibility
is further strengthened by our failure to note any differences in
kinetic behavior between the enzyme in the supernatant of
homogenates as compared to the pellet. Regardless of the na-
ture of the non-mitochondrially bound enzyme, the fact that at
least 75% of this enzyme, most of the hexokinase and lactate
dehydrogenase (4, 7) and probably over half of the glyceralde-
hyde 3-phosphate dehydrogenase (5) activities are on the mito-
chondria raise important questions as to the structure of gluco-
neogenesis and glycolysis in this cell. It is possible that with
gentler methods of disruption, other glycolytic enzymes will also
be found to be mitochondrial in this cell and, indeed, available
evidence suggests that *Tetrahymena* is not unique in this re-
spect (1).

The properties of the mitochondrial phosphofructokinase of
*Tetrahymena* in general conform to those of this enzyme from
other sources (9): (a) it is inhibited at pH 8.0 and more strongly
at pH 6.0 by citrate and by ATP; (b) it is relatively unstable
but can be stabilized by the presence of fructose 6-phosphate
and, to a greater extent, by ATP; (c) activity is enhanced by
(NH₄)₂SO₄ and by orthophosphate; (d) its stability decreases
with increasing dilution. Unlike the enzyme from liver flukes,
however (9), no effect of cyclic 3':5'-AMP on activity was ob-
served. The similarity of the effect of pH, citrate, ATP,
(NH₄)₂SO₄, and orthophosphate on the activity of the mito-
chondrial phosphofructokinase of *Tetrahymena* to the effect of
these modifiers on this enzyme from other sources suggests that
the enzyme is comparable to the other enzymes in most of its
kinetic properties and differs primarily in its localization.
Further kinetic studies on this enzyme as well as localization studies
on other cells are indicated.

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