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Hypothesis

Pyrophosphate-dependent phosphofructokinase, an anaerobic glycolytic enzyme?

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Recent evidence indicates that in as diverse organisms as unicellular eukaryotes, higher plants and prokaryotes, anaerobic glycolysis relies on a pyrophosphate-dependent phosphofructokinase instead of the classical ATP-dependent enzyme. This difference in phosphoryl donor specificity does not necessarily reflect a primitive metabolism, as thought earlier, but could rather be the result of convergent evolution, fostered by the energetic advantage conferred to the cell when glycolysis is the sole source of ATP.

Pyrophosphate; Phosphofructokinase; Glycolysis; Anaerobic metabolism

1. INTRODUCTION

Glycolysis is a central pathway of carbohydrate metabolism, present in almost all cells. Its importance is reflected in its remarkable conservation throughout evolution. In eukaryotes, one of the most significant deviations from the usual glycolysis is the replacement of the widespread phosphofructokinase that uses ATP as phosphoryl donor (ATP-PFK):

ATP:D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11)

$$\text{ATP} + \text{fructose-6-P} \rightarrow \text{fructose-1,6-P}_2 + \text{ADP} \qquad (\Delta G^{\circ\prime} = -4.41 \text{ kcal/mol})$$

by a peculiar one that uses inorganic pyrophosphate (PP_i-PFK):

Inorganic pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90)

$$PP_i + \text{fructose-6-P} \leftrightarrow \text{fructose-1,6-P}_2 + P_i \qquad (\Delta G^{\circ\prime} = -2.08 \text{ kcal/mol})$$

ATP-PFK catalyses the first irreversible reaction of glycolysis (first committed step) and is the main control point of this pathway. In contrast, the reaction catalyzed by PP_i-PFK is easily reversible and could be close to equilibrium in vivo.

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As discussed in this article, the use of PP_i as a phosphate donor raises important questions on the metabolism of PP_i, the regulation of glycolysis, and more generally the *raison-d'être* of PP_i-PFK.

2. RESULTS AND DISCUSSION

2.1. *Distribution of PP_i-PFK among the living organisms*

PP_i-PFK was first discovered in 1974 by Reeves et al. [1] in the parasitic amoeba *Entamoeba histolytica*. It was subsequently found in the bacterium *Propionibacterium shermanii*, in the photosynthetic protist *Euglena gracilis* [3], and in much more evolved higher plants [4]. More recently, PP_i-PFK was detected in *Isotricha prostoma* (a ciliate living in the rumen [5]), in the parasitic flagellates *Giardia lamblia* [6], *Tritrichomonas foetus* and *Trichomonas vaginalis* [5], and in some prokaryotes from various phyla (see [7,8]).

Two different situations are found in organisms possessing PP_i-PFK :

(1) higher plants and *E. gracilis* contain comparable cytoplasmic activities of PP_i-PFK, ATP-PFK, and often fructose-1,6-bisphosphatase (FBPase). In addition, their PP_i-PFK is allosterically regulated (see below).

(2) all other PP_i-PFK-containing organisms have little if any ATP-PFK and FBPase, and their PP_i-PFK is apparently not controlled by allosteric effectors.

2.2. *Stimulation by fructose-2,6-bisphosphate*

A striking characteristic of the PP_i-PFK from the

first group of organisms (plants [9] and *E. gracilis* [3]) is its ability to be stimulated by fructose-2,6-bisphosphate (Fru-2,6-P_2). Fru-2,6-P_2 is a regulatory phosphate ester present in eukaryotes but not in prokaryotes, which enhances glycolysis and inhibits gluconeogenesis (reviewed in [10]). Plant PP_i-PFK is nearly inactive in the absence of Fru-2,6-P_2 and is extremely sensitive to it, a half-maximal effect being observed at nanomolar concentrations. This high sensitivity is exploited in the most commonly used Fru-2,6-P_2 assay [10]. In the presence of physiological concentrations of organic or inorganic anions, the sensitivity is shifted to the micromolar range, which is of the order of the Fru-2,6-P_2 concentrations found in vivo.

Fru-2,6-P_2 is synthesized from ATP and Fru-6-P by a specific phosphofructokinase, and hydrolysed back to fructose-6-P and P_i by a specific fructose-2,6-bisphosphatase [10,11]. Fig. 1 provides a summary of these relationships for plants.

2.3. *Role of Fru-2,6-P_2-insensitive PP_i-PFK*

Since it catalyzes an easily reversible reaction, PP_i-PFK is theoretically able to replace both ATP-PFK and FBPase (see Fig. 1). Organisms of the second group, which have an unregulated PP_i-PFK, have also in common an obligate fermentative metabolism and rely on glycolysis for the supplying of ATP [12]. The metabolic function of PP_i-PFK in these organisms is, therefore, clearly glycolytic [5,6].

It is important to note that glycolytic and gluconeogenic fluxes are almost ubiquitously controlled at the level of the fructose-6-phosphate/fructose-1,6-bisphosphate interconversion, catalyzed by the tightly controlled and physiologically irreversible enzymes ATP-PFK and FBPase. Obviously, such fluxes must be regulated in basically different ways in organisms that rely on PP_i-PFK rather than on these rate-limiting enzymes; such mechanisms as yet remain virtually unexplored.

2.4. *Role of Fru-2,6-P_2-sensitive PP_i-PFK*

Elucidation of the role of PP_i-PFK in higher plants and in *E. gracilis* is much more complex, primarily because, as mentioned above, this enzyme coexists with ATP-PFK and FBPase. Comparison of the mass action ratio with the equilibrium constant should reveal whether PP_i-PFK catalyses the forward or the reverse reaction. Such an analysis is however made more complicated not only by the presence of ATP-PFK and FBPase, but also by cellular compartmentalisation, which prevents the exact quantification of metabolites in the cytosol. We are therefore left with indirect arguments.

On the one hand, PP_i-PFK is thought to perform the net synthesis of PP_i, as part of an alternate pathway of sucrose degradation. Instead of being hydrolyzed by invertase, the disaccharide would be converted together with UDP by sucrose synthase to fructose and UDPG.

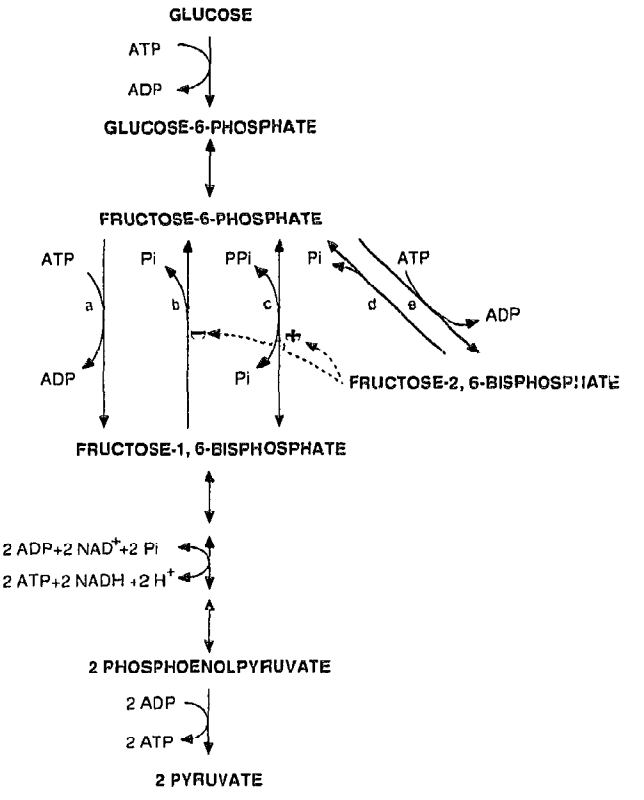

Fig. 1. Involvement of pyrophosphate-dependent phosphofructokinase (PP_i-PFK) and fructose-2,6-bisphosphate in the carbohydrate metabolism in higher plants. (a) ATP: D-fructose 6-phosphate 1-phosphotransferase (ATP-PFK1); (b) D-fructose 1,6-bisphosphate 1-phosphohydrolase (FBPase 1); (c) PP_i: D-fructose 6-phosphate 1-phosphotransferase (PP_i-PFK); (d) D-fructose 2,6-bisphosphate 2-phosphohydrolase; (e) ATP: D-fructose 6-phosphate 2-phosphotransferase. Broken lines show the regulatory effects of fructose-2,6-bisphosphate. Reactions between fructose-1,6-bisphosphate and phosphoenolpyruvate have been omitted for the sake of clarity.

PP_i-PFK would provide the PP_i needed for the subsequent pyrophosphorolysis of UDPG to glucose 1-phosphate and UTP[13]. This function of PP_i-PFK rests, however, mainly on the teleological argument that a source of PP_i is needed, and conclusive experimental evidence is not yet available.

On the other hand, the following observations suggest a glycolytic function of PP_i-PFK:

(1) PP_i-PFK is also present in plant tissues in which gluconeogenic flux is unlikely, such as non-starchy, non-photosynthetic tissues (*Helianthus tuberosus* tubers, onion bulbs, ...).

(2) Co-existence in the same compartment of FBPase and PP_i-PFK, which are inversely regulated by Fru-2,6-P_2, is more understandable if the two enzymes catalyze opposite reactions.

(3) Since PP_i-PFKs from higher plants and *E. gracilis* are strongly stimulated by Fru-2,6-P_2, the changes in the concentration of this regulator that occur in vivo are of critical importance. In various plant tissues, the concentration of Fru-2,6-P_2 increases in response to different treatments known to stimulate glycolysis, as a

result of a drop in the ATP concentration. These include: anaerobiosis [14], uncoupling of mitochondrial respiration [15] and wounding [14]. Remarkably, a similar anoxia-induced increase in the concentration of Fru-2,6-P_2 was also observed in *E. gracilis* [16]. Furthermore, in rice seedlings, which are well adapted to prolonged anaerobiosis, prolonged oxygen deprivation not only increases the concentration of Fru-2,6-P_2, but also induces the synthesis of PP_i-PFK although not of ATP-PFK [17].

These observations strongly indicate that, in plants and *E. gracilis*, PP_i-PFK plays a role in anaerobiosis or in other situations where the ATP/ADP ratio is lowered.

One attractive possibility is that PP_i-PFK is used instead of ATP-PFK under these conditions because it provides a significant energetic advantage. As was pointed out some time ago [2,18], PP_i-PFK uses as a high-energy phosphate donor a by-product of biosynthetic reactions (PP_i), which would otherwise be simply hydrolyzed to inorganic phosphate. On this basis, replacement of ATP-PFK by PP_i-PFK can theoretically improve the ATP yield of glucose degradation. This energetic advantage would be important (50%, three ATP vs two per glucose) when glycolysis is the sole source of ATP, as in fermentative metabolism. In contrast, the improvement of the energetic yield would be negligible (39 ATP vs 38 per glucose) when oxidative phosphorylation is the main source of ATP[5]. This hypothesis is supported by the observation that the diverse organisms sharing a full dependence on PP_i-PFK, have also in common a low ATP yield from glucose because of their obligate fermentative metabolism [5,6].

A sort of *a contrario* example of the energy conserving role of PP_i-PFK is to be found in the spectacular acceleration of carbohydrate degradation that occurs during thermogenesis in *Arum maculatum* clubs. This process is not accompanied by any change in the Fru-2,6-P_2 or PP_i-PFK content, but by a dramatic increase in that of ATP-PFK. Furthermore, the PP_i-PFK activity cannot account for the very high glycolytic flux in thermogenic clubs [19]. Since thermogenesis is by definition an energy-wasteful process leading to the production of heat and not of ATP, it would be paradoxical that an energy-saving enzyme such as PP_i-PFK were involved in it [17].

Nevertheless, other as yet unknown functions of PP_i-PFK in plants should be examined; particularly intriguing is the large excess of PP_i-PFK over ATP-PFK found in leaves of some plants with a crassulacean acid metabolism [4].

2.5. Metabolism of PP_i

The energetic advantage of PP_i-PFK discussed here rests on the assumption that the PP_i used is a by-product of the biosynthesis of macromolecules (proteins, nucleic acids, polysaccharides). The biochemical dogma states that, to allow such reactions to occur, the concentration of PP_i must be kept at a low level and that this is achieved through hydrolysis by inorganic pyrophosphatase, which is supposed to be ubiquitously present. However, PP_i can be removed by PP_i-PFK just as well as by pyrophosphatase. Interestingly, in contrast to most organisms, the cytoplasm of higher plants, *E. gracilis*, *E. histolytica*, *G. lamblia* and *T. vaginalis* contains no significant pyrophosphatase activity, whereas the presence of this enzyme has been shown to be essential for an ATP-PFK-bearing organism (*E. coli*) [20].

Until recently, the metabolism of PP_i had drawn little attention. Experimental evidence that anabolic reactions are the major source of PP_i has yet to be obtained. One should bear in mind that fermentative organisms have a high rate of glycolysis, and therefore require an intense PP_i supply. Theoretical calculation of the rate of PP_i-formation in the course of macromolecular synthesis [21] applied to *T. vaginalis* [22] gives a rate of approximately 50 nmol $PP_i \cdot min^{-1} \cdot (mg\ of\ protein)^{-1}$, which is comparable to the glycolytic flux of 50–90 $nmol \cdot min^{-1} \cdot (mg\ of\ protein)^{-1}$. Limited information available for *E. histolytica* and plants indicate a cytosolic PP_i concentration in the 0.1–1 mM range, i.e. saturating for PP_i-PFK.

Alternative sources of PP_i have been proposed, none of them being truly energy-conserving:

(1) Metabolite cycling between glycogen and glucose-1-P through the action of UDPG pyrophosphorylase, glycogen synthase and glycogen phosphorylase could result in the net conversion of nucleotide triphosphate and phosphate into nucleotide diphosphate and PP_i [18]. Interestingly, in contrast to the general situation, regulation of glycogen phosphorylase and synthase activities by covalent modification in PP_i-PFK-dependent organisms has not yet been demonstrated.

(2) *P. shermanii* and *E. histolytica* contain, in addition to PP_i-PFK, other unusual PP_i-dependent transphosphorylases. P-enolpyruvate carboxytransphosphorylase, which catalyzes the following reaction:

P-enolpyruvate + P_i + CO_2 ↔ PP_i + oxaloacetate

is thought to perform the net synthesis of oxaloacetate in the amoeba, therefore being a possible source of PP_i [18]. This enzyme has, however, not yet been identified in other PP_i-PFK containing protists. Furthermore, in *E. histolytica* [18] and *I. prostoma* (Mertens, E., unpublished results), pyruvate kinase is absent and replaced by pyruvate orthophosphate dikinase, which catalyses the following reaction:

P-enolpyruvate + AMP + PP_i ↔ pyruvate + ATP + P_i

Such a reaction is expected to work towards pyruvate synthesis, therefore requiring additional PP_i.

2.6. PP_i-PFK: an evolutionary enigma

The unfolding story of PP_i-PFK is also intriguing

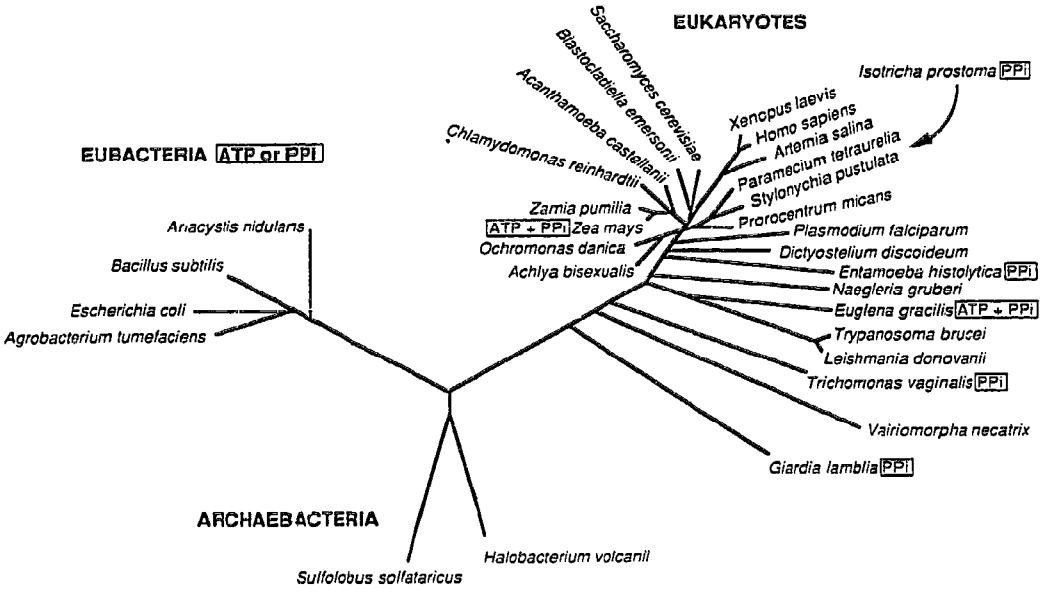

Fig. 2. Distribution of PP_i-dependent phosphofructokinases among prokaryotes and eukaryotes. PP_i-PFK is present alone: [PP_i], or with ATP-PFK: [ATP + PP_i]. The phylogenetic position of organisms is shown on a multi-kingdom tree derived from 16S-like rRNA sequences (modified from reference [24], with permission). Position of the ciliate *Isotricha prostoma* is inferred from 23S-like rRNA sequences (A. Baroin and R. Perasso, personal communication).

because of its evolutionary aspects. Soon after its discovery in *E. histolytica* and in *P. shermanii*, this enzyme was regarded as a remnant of ancestral metabolism restricted to a 'primitive' eukaryotic parasite and prokaryotes [1,2,18]. Recent identification of PP_i-PFK in *Giardia lamblia* [6] and trichomonads [5], which represent early branches of the evolutionary tree, agree with this suggestion.

The recent discovery of PP_i-PFKs in more evolved organisms, such as higher plants and ciliates, does however not support this view. The distribution of PP_i-PFK, in such remotely related groups as eubacteria, unicellular eukaryotes and higher plants, does not follow a direct lineage on the generally accepted phylogenetic tree (Fig. 2) based on rRNA sequence homologies [23,24]. Noteworthy in this figure is the large evolutionary distance between the various PP_i-PFK-containing unicellular eukaryotes, as well as between plants and *E. gracilis*.

The distribution of PP_i-PFK in the living world appears to correlate better with metabolic characteristics (anaerobiosis) than with phylogenetic relationships. An interesting possibility is, therefore, that PP_i-PFK derived from ATP-PFK, probably not once but on several independent occasions. Particularly suggestive is the case of *I. prostoma*, a ciliate without mitochondria, which lives in the anaerobic rumen. Recent rRNA sequence data show the close relationship of *I. prostoma* to aerobic ciliates (Baroin, A., Perasso, R., personal communication) such as *Tetrahymena pyriformis* and *Paramecium caudatum*, which contain mitochondria and ATP-PFK but no PP_i-PFK (Mertens, E. and Müller, M., unpublished results).

The possibility that PP_i-PFK has derived on several occasions from ATP-PFK can be tested by sequence comparisons. Sequences of ATP-PFKs from a number of different organisms show a high degree of homology between eubacterial and eukaryotic ATP-PFKs [25]. Full sequence of potato PP_i-PFK [26] and partial sequences of PP_i-PFKs from *I. prostoma* and *T. vaginalis* (Mertens, E., Ladror, U., Kemp, R. and Müller, M., unpublished results) reveal significant homology between these PP_i-PFKs and ATP-PFKs from various organisms, suggesting that both phosphofructokinases have a common ancestry.

As emphasized elsewhere [27], the glycolytic pathway is particularly well suited for studies of enzyme evolution. The vast amount of information available indicates that glycolysis is not only almost ubiquitous, but also that its enzymes evolved very slowly, at a rate comparable to the highly conserved protein cytochrome *c* [27]. Therefore, the evolutionary history of PP_i- PFK could be a unique example of how such remote organisms as plants, protists and bacteria had a similar adaptive response to anaerobiosis.

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