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Induction of Pyrophosphate:Fructose 6-Phosphate 1-Phosphotransferase by Anoxia in Rice Seedlings[1]

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ABSTRACT

Rice (*Oryza sativa*) seeds were imbibed for 3 days and the seedlings were further incubated for 8 days in the presence of either air or nitrogen. In aerobiosis, the specific activity of pyrophosphate:fructose 6-phosphate 1-phosphotransferase and that of the ATP-dependent phosphofructokinase increased about fourfold. In anaerobiosis, the specific activity of ATP-dependent phosphofructokinase remained stable, whereas that of pyrophosphate:fructose 6-phosphate 1-phosphotransferase increased as much as in the presence of oxygen and there was also a fourfold increase in the concentration of fructose 2,6-bisphosphate, a potent stimulator of that enzyme. These data suggest a preferential involvement of pyrophosphate:fructose 6-phosphate 1-phosphotransferase rather than of ATP-dependent phosphofructokinase in glycolysis during anaerobiosis.

There is a general agreement that a major regulatory point of glycolysis is the reaction catalysed by PFK 1[2] (reviewed in Hers and Hue [7]). In most animal cells, the activity of PFK 1 is greatly stimulated by Fru-2,6-P_2, which appears as the main regulator of glycolysis under aerobic conditions. By contrast, in higher plants, PFK 1 is completely insensitive to stimulation by Fru-2,6-P_2, but the cells contain a pyrophosphate:fructose-6-phosphate 1-phosphotransferase, also called "PPi-dependent phosphofructokinase" (PPi-PFK), which catalyses the reversible formation of Fru-1,6-P_2 and Pi from Fru-6-P and PPi (4). Remarkably, plant PPi-PFK is strongly stimulated by Fru-2,6-P_2 (16). PFK 1 and PPi-PFK coexist in the cytoplasm of plants, and their maximal activities are of the same order (5).

Because it catalyses a freely reversible reaction, PPi-PFK could operate *in vivo* in the gluconeogenic as well as in the glycolytic direction. The first hypothesis has been favored by several groups (2, 8, 24), who considered that the role of the reaction is to provide PPi for the pyrophosphorolysis of UDPG when glycolysis occurs at the expense of sucrose; this sugar is indeed assumed to react with UDP, under the action of sucrose synthase, to form fructose and UDPG. On the other hand, the glycolytic role of PPi-PFK is supported by a series of experimental data: (a) The fact that, in several plant tissues, PPi-PFK coexists with fructose-1,6-bisphosphatase (which is strongly inhibited by Fru-2,6-P_2) in the cytosol (5), whereas these two enzymes are inversely affected by the presence of Fru-2,6-P_2 can only make sense if they catalyse opposite reactions (19); (b) The concentration of Fru-2,6-P_2, which is the cofactor of PPi-PFK, is greatly increased in conditions under which glycolysis is expected to be intense, such as wounding (21), anaerobiosis (13), and in the presence of an uncoupler (6); (c) A series of protozoa which, although belonging to different groups, have in common the absence of oxidative phosphorylation, contain no detectable PFK 1 but a high amount of a fully active PPi-PFK, indicating that the latter enzyme is appropriate to its glycolytic role (14).

The purpose of the present work was to further investigate this problem, using rice seedlings exposed to anaerobiosis as an experimental model. Indeed, rice seeds are able to germinate and grow under long periods of anaerobiosis, during which they display a strong alcoholic fermentation (18). This adaptation of rice plantlets to anaerobiosis is reflected by their ability to synthetize a limited number of proteins during this treatment (15). These proteins include two glycolytic enzymes, alcohol dehydrogenase, and pyruvate decarboxylase, the activities of which greatly increase under anoxia (1, 9). Our aim was, therefore, to determine if prolonged anoxia could induce either PFK 1 or PPi-PFK, and to obtain in this way an indication concerning their respective glycolytic function.

MATERIALS AND METHODS

Materials

DTT was from Janssen Chimica (Beerse, Belgium). Enzymes and biochemicals were from Boehringer (Mannheim, FRG); others chemicals were from Merck (Darmstadt, FRG) and were of analytical grade. Fru-2,6-P_2 (20) and PPi-PFK (22) were prepared as described.

Treatment of the Seeds

Rice seeds (*Oryza sativa,* var Martelli), kindly provided by Dr. M. Boutry from the Faculty of Agronomy of this university, were stirred in 60% ethanol for 2 min, washed in sterile

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[2] Abbreviations: PFK 1, 6-phosphofructo 1-kinase (EC 2.7.1.11); PFK 2, 6-phosphofructo 2-kinase (EC 2.7.1.105); PPi-PFK, pyrophosphate:fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90); Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; PPi, inorganic pyrophosphate; Glc-6-P, glucose 6-phosphate.

water, stirred in 0.1% $HgCl_2$ for 2 min, and finally washed twice in sterile water. The seeds were then put on a wet filter paper and set in the dark at 20 °C, in a vacuum flask through which a gas (air or nitrogen) was circulated after bubbling in sterile water. The seeds were allowed to germinate aerobically for 3 d and then placed in a nitrogen atmosphere during 8 d, or left in air. At the indicated times, samples were removed, dropped in liquid nitrogen and stored at −80 °C until further processing.

Extraction of the Seeds and Analytical Procedures

To measure Fru-2,6-P_2, a batch of five seedlings was weighed and homogenized with an Ultra-Turrax in 2 mL of ice-cold, 50 mM NaOH; the homogenate was centrifuged for 5 min at 10,000*g*, and the resulting supernatant was heated for 5 min at 80 °C, cooled, and centrifuged again for 2 min at 10,000 *g*. Fru-2,6-P_2 was determined in the last supernatant by the stimulation of potato PPi-PFK, according to Van Schaftingen and Hers (21). It has been verified that the recovery of various amounts of Fru-2,6-P_2 added to an homogenate of seedlings after 3 or 11 d of germination exceeded 90%.

For the assay of enzymes, a batch of 5 seedlings was weighed and homogenized at low speed with an Ultra-Turrax, in 2 mL of an ice-cold solution containing 5 mM DTT, 5 μg/mL antipain, 0,5% PVP, 5 mM magnesium acetate, and 50 mM Hepes (pH 7.1). The homogenate was centrifuged for 10 min at 10,000 *g*, and the resulting supernatant was used the same day for the measurement of enzymic activities.

PPi-PFK was assayed spectrophotometrically by monitoring the oxidation of NADH (0.15 mM) in the presence of 4 mM magnesium acetate, 2 mM Fru-6-P, 7 mM Glc-6-P, 2 μM Fru-2,6-P_2, 1 mM PPi, 50 μg/mL aldolase, 1 μg/mL triosephosphate isomerase, 10 μg/mL glycerol 3-P dehydrogenase, 2 mM DTT, 0.2% bovine serum albumin, and 50 mM Tris/Cl (pH 7.8). The same mixture was used for the determination of PFK 1, except that PPi was replaced by 5 mM ATP, that magnesium acetate was 10 mM and that Fru-2,6-P_2 was omitted. PFK 2 was assayed by the formation of Fru-2,6-P_2, as described by Larondelle *et al.* (11). Pyruvate kinase was assayed by pyruvate-dependent oxidation of NADH (0.15 mM), in the presence of 5 mM magnesium acetate, 2 mM DTT, 2 mM ADP, 1 mM P-enolpyruvate, 5 μg/mL lactate dehydrogenase, and 50 mM Hepes (pH 7.1). Glc-6-P dehydrogenase was measured spectrophotometrically by the reduction of NADP (0.15 mM) in the presence of 2 mM DTT, 5 mM magnesium acetate, 5 mM Glc-6-P, and 50 mM Hepes (pH 7.1). Phosphoglucose isomerase was assayed by the same procedure, except that Fru-6-P was used instead of Glc-6-P and that 5 μg/mL Glc-6-P dehydrogenase was also present. All enzyme measurements were made at 30 °C. It was checked that gentle extraction, made in a Potter-Elvejhem device, of seeds which were dehulled by hands, gave similar PPi-PFK and PFK 1 activities as that measured after extraction with an Ultra-Turrax. One unit is the amount of enzyme that catalyses the conversion of 1 μmol of substrate/min under the standard conditions of assay. Protein was determined according to Bradford (3) with bovine γ-globulin as a standard.

RESULTS

During the 8 d of the experimental period, the aerobically growing seedlings showed a rapid expansion of both radicle and coleoptile, whereas the lack of oxygen prevented radicle and leaf elongation, allowing only a modest coleoptile elongation. As expected, the weight and the protein content of the seedlings increased much less in anoxia than under aerobic conditions (Fig. 1)

The maximal activities of PFK 1 and PPi-PFK were measured on whole seedlings exposed to both gas phases. It is shown in Figure 2 that, in the presence of oxygen, the specific activity of both enzymes increased about 4-fold, corresponding to an increase in enzyme content close to 8-fold. The

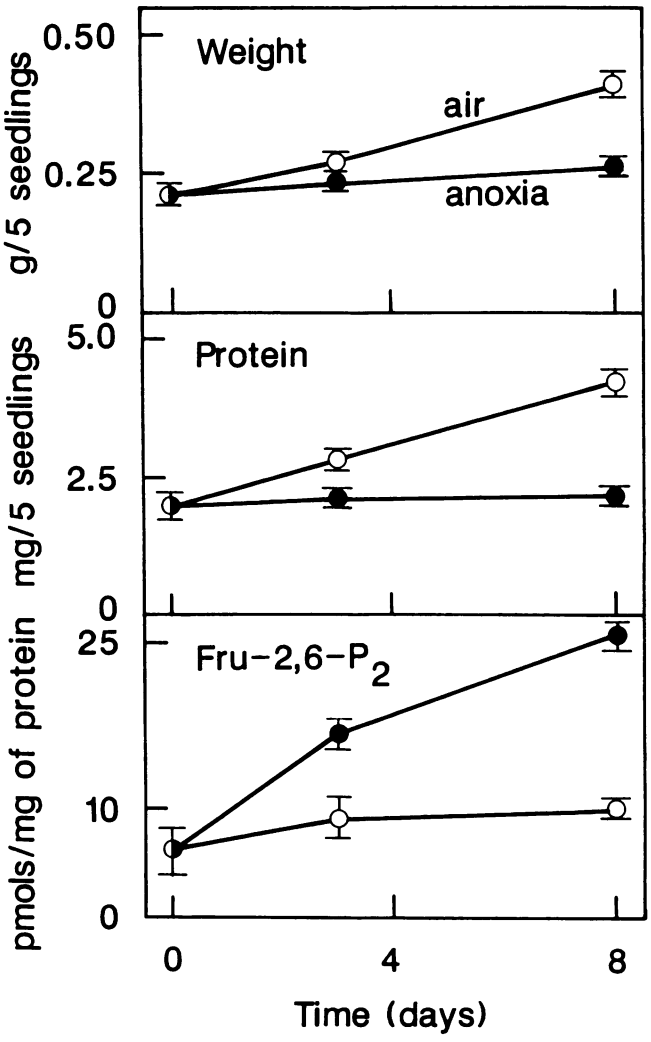

Figure 1. Effect of anoxia on weight, protein content, and Fru-2,6-P_2 concentration of rice seedlings. Anaerobiosis was applied to rice seeds imbibed aerobically for 3 d. Values are mean ± SEM of three samples of five seedlings.

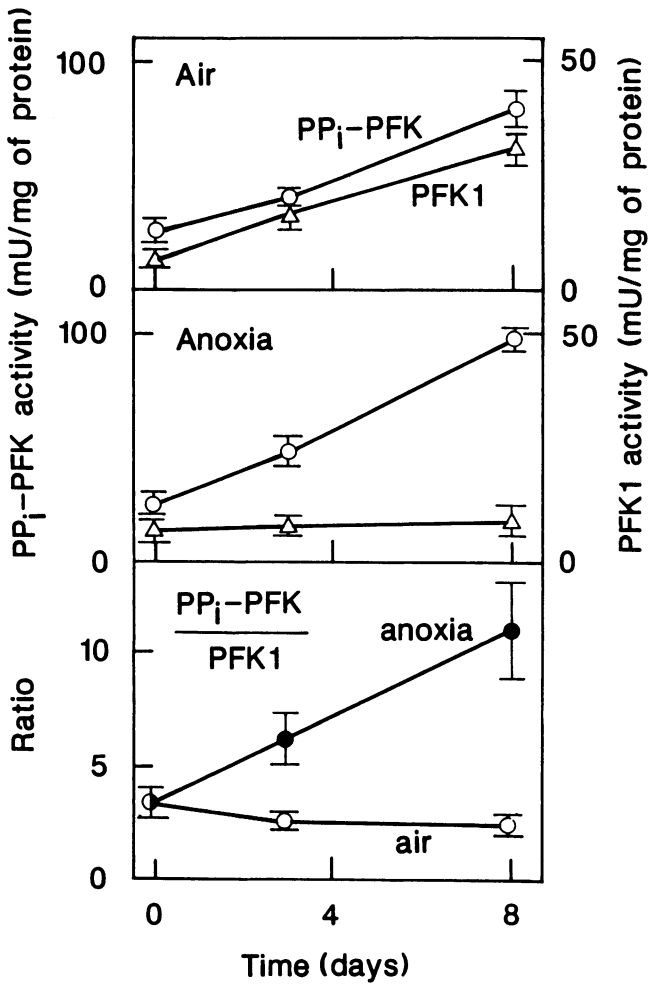

Figure 2. Effect of anoxia on maximal activities of PFK 1 and PPi-PFK from rice seedlings. Same procedure as described in Figure 1.

same figure shows that anoxia deeply reduced the formation of PFK 1, whereas it had little effect on the rise in the specific activity of PPi-PFK. Therefore, the PPi-PFK/PFK 1 ratio increased more than 3-fold in anoxia, whereas it declined slightly in air, and the maximal activity of PPi-PFK was more than 10-fold higher than that of PFK 1 after 8 d of oxygen deprivation (Fig. 2). Similarly to what we have described for PFK 1, the specific activity of a series of enzymes involved in carbohydrate metabolism, including pyruvate kinase, glucose 6-phosphate dehydrogenase, phosphoglucomutase, and PFK 2, remained stable during the 8 d of anoxia and increased more than twice during the same time in the presence of oxygen (not shown).

The kinetic properties of the anaerobically induced PPi-PFK did not differ from that of its aerobic counterpart; indeed, the K_m values, measured in the presence of a saturating (2 μM) concentration of Fru-2,6-P_2, were similarly in the range of 0.3 mM for Fru-6-P and 10 μM for PPi for both seedling extracts. Remarkably, however, the concentration of Fru-2,6-P_2, which is nearly a cofactor for PPi-PFK in plants, increased fourfold in the seedlings in anoxia, whereas it remained much more stable in the controls (Fig. 1).

We have also observed that a up to 48 h anaerobic treatment of Jerusalem artickoke tubers at 4 °C, which is known to cause a more than 10-fold increase in the concentration of Fru-2,6-P_2 (13), was not accompanied by a change in the maximal activities of PFK 1 and PPi-PFK (not shown).

DISCUSSION

Increased Activity of PPi-PFK in Prolonged Anoxia

The main observations made in this work are that not only is the maximal activity of PPi-PFK increased in rice seedlings during prolonged anoxia, but that the activity of this enzyme in the cell can be further increased because of a higher concentration of Fru-2,6-P_2. The rise in maximal activity is not accompanied by a change in the kinetic properties of the enzyme and, therefore, presumably reflects the formation of an increased amount of enzyme. It is similar to that previously shown for two glycolytic enzymes, alcohol dehydrogenase, and pyruvate decarboxylase (9). The specific synthesis of these enzymes, occurring when that of most other proteins, including PFK 1, is severely restricted, indicates that they have a role to play in the response to anoxia.

It is worth mentioning that the increased concentration of Fru-2,6-P_2 reported in this paper is not associated with an induction of PFK 2; this is not surprising if we consider that PFK 2 and the low K_m FBPase 2 from higher plants are associated in a single bifunctional protein (11, 12). Therefore, similarly to what has been observed on castor bean endosperm (10), Jerusalem artichoke tubers, and soja seedlings (13), this increase in Fru-2,6-P_2 concentration in anoxia might be the consequence of a lower concentration of glycerate 3-P, a powerful inhibitor of its synthesis (11).

It is also interesting to recall the observation made by Smyth *et al.* (17) that the maximal activity of PPi-PFK was about 50% higher in maize roots submerged in water (a condition expected to reduce oxygen tension), by comparison to roots growing in moist vermiculite.

Role of PPi-PFK in Higher Plants

As discussed elsewhere (14, 23), the potential advantage of the use of PPi-PFK rather than of PFK 1 in anoxia could be to allow an increase of up to 50% in the ATP yield of glycolysis, thanks to the use of PPi, a byproduct of several biosynthetic reactions, as a phosphate donor.

This energetic advantage is, however, minimal in aerobic conditions, as illustrated by the observations made during thermogenesis of *Arum maculatum* clubs. Indeed, the tremendous acceleration of carbohydrate oxidation which occurs under this condition is accompanied by a large increase in the maximal activity of PFK 1, whereas that of PPi-PFK and the concentration of Fru-2,6-P_2 remain stable. Furthermore, the maximal activity of PPi-PFK cannot account for this largely increased glycolytic flux (2). These enzymatic changes, although they are the opposite of what we found in rice seedlings during anoxia, are not inconsistent with our hypothesis. Indeed, the acceleration of the glycolytic flux during

thermogenesis is an energy-wasting process leading to the production of heat, but not of ATP.

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