Fructose 2,6-bisphosphate and carbohydrate metabolism during the life cycle of the aquatic fungus *Blastocladiella emersonii*

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Removal of the growth medium and resuspension of *Blastocladiella emersonii* vegetative cells in a sporulation medium resulted in an abrupt fall of fructose 2,6-bisphosphate concentration to about 2% of its initial value within 10 min. The concentrations of hexose 6-phosphate and of fructose 1,6-bisphosphate also decreased by, respectively, three and tenfold over the same period. All these values remained at their low level throughout the sporulation phase and during the subsequent germination of zoospores when performed in the absence of glucose. In contrast, the concentration of cyclic AMP was low during the sporulation period and exhibited a transient increase a few minutes after the initiation of germination. Other biochemical events occurring during sporulation were a 70% reduction in glycogen content and the complete disappearance of trehalose. The remaining glycogen was degraded upon subsequent germination of the zoospores. *B. emersonii* phosphofructo 2-kinase (PFK-2) and fructose-2,6-bisphosphatase (FBPase-2) could not be separated from each other by various chromatographic procedures, suggesting that they were part of a single bifunctional protein. On anion-exchange chromatography, two peaks of PFK-2 and FBPase-2 were resolved. Upon incubation of fractions from the two peaks or of a crude extract in the presence of [2-³²P]fructose 2,6-bisphosphate, two radiolabelled subunits with molecular masses close to 90 and 54 kDa were obtained. The labelling of the subunit of higher molecular mass was greater than that of the lower one in extracts prepared in the presence of protease inhibitors and in the first peak of the Mono Q column. PFK-2 and FBPase-2 displayed kinetic properties comparable to those of mammalian enzymes, but no indication of a cyclic AMP-dependent regulation could be obtained. Phosphofructo 1-kinase and fructose-1,6-bisphosphatase from *B. emersonii* were, respectively, stimulated and inhibited by micromolar concentrations of fructose 2,6-bisphosphate. The physiological significance of these properties is discussed. A simple method for the determination of trehalose is also reported.

Introduction

Blastocladiella emersonii is a unique experimental model for developmental studies. The life cycle of this aquatic fungus alternates between two functionally and morphologically distinct types of cells: a motile, non-growing

zoospore, which is formed upon starvation from a sessile, multinucleate vegetative cell (Lovett, 1975). The life cycle starts with the germination of the zoospore into the vegetative cell, which is capable of syncytial growth if incubated in the presence of glucose. A key event in this differentiation of the zoospores is their rapid (within 20 min) conversion into round cells or cysts. This conversion, which occurs even in the absence of glucose, is accompanied by a *de novo* formation of a cell wall, whose major component is chitin. Because this morphological change occurs in the absence of either RNA or protein synthesis (Soll & Sonneborn, 1971a; Lovett, 1975), it has been suggested that post-translational control mechanisms are involved. The observation of a transient accumulation of cyclic AMP (cAMP) (Vale *et al.*, 1976) and the activation of a cAMP-dependent

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Abbreviations: Fru-6-P, fructose 6-phosphate; Fru-2,6-P₂, fructose 2,6-bisphosphate; Fru-1,6-P₂, fructose 1,6-bisphosphate; Glc-6-P, glucose 6-phosphate; PEP, phosphoenolpyruvate; FBPase-1, fructose-1,6-bisphosphatase, EC 3.1.3.11; FBPase-2, fructose-2,6-bisphosphatase, EC 3.1.3.46; PFK-1, ATP:D-fructose-6-phosphate phosphotransferase, EC 2.7.1.11; PFK-2, ATP:D-fructose-6-phosphate phosphotransferase, EC 2.7.1.105; PP_i-PFK, pyrophosphate:fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.1.

protein kinase (Behrens & Maia, 1986) during encystment, as well as the effectiveness of cAMP and of inhibitors of cAMP phosphodiesterase in eliciting the conversion of zoospores into cysts (Gomes *et al.*, 1980), suggest a role for cAMP in this morphogenetic transition. However, substrates for cAMP-dependent protein kinase still remain to be found.

Discovered in 1980, fructose 2,6-bisphosphate (Fru-2,6-P₂) appears to play an important role in the regulation of carbohydrate metabolism in a wide variety of eukaryotic cells (for a review, see Van Schaftingen, 1987). It is a potent stimulator of phosphofructo 1-kinase (PFK-1) of animal or fungal origin and of pyruvate kinase from *Trypanosoma brucei* as well as an inhibitor of fructose-1,6-bisphosphatase (FBPase-1) from various sources. Fru-2,6-P₂ is formed from fructose 6-phosphate (Fru-6-P) and ATP by phosphofructo 2-kinase (PFK-2) and hydrolysed back to Fru-6-P and P_i by fructose-2,6-bisphosphatase (FBPase-2). In liver (El-Maghrabi *et al.*, 1982, Van Schaftingen *et al.*, 1986b), muscle (Van Schaftingen *et al.*, 1986b) and plants (Larondelle *et al.*, 1986), but not in yeast (Kretschmer *et al.*, 1987; François *et al.*, 1988) or in *T. brucei* (Van Schaftingen *et al.*, 1986a), PFK-2 and FBPase-2 are associated in a bifunctional protein. The activity of these enzymes is controlled by the concentration of several metabolites and, in liver and yeast, by covalent modification (for reviews, see Van Schaftingen, 1987; Hue & Rider, 1987).

The observation of a several-fold increase in the concentration of Fru-2,6-P₂ upon germination of *Phycomyces blakesleeanus* spores (Van Laere *et al.*, 1983) or of oat seeds (Larondelle *et al.*, 1987) and after slicing of Jerusalem artichoke tubers (Van Schaftingen & Hers, 1983) gave rise to the idea that Fru-2,6-P₂ may play a role in developmental biology (Hers *et al.*, 1985). The purpose of the present work was, therefore, to investigate the potential role of Fru-2,6-P₂ during the life cycle of *B. emersonii* and, more specifically, to determine whether the two critical periods of the cycle, i.e. sporulation and germination, were accompanied by changes in the concentration of Fru-2,6-P₂ and whether these changes could be regulated by a cAMP-dependent process. We also analysed the variations in the concentrations of important glycolytic metabolites and carbohydrate stores during these two developmental phases and made a kinetic study of key glycolytic and gluconeogenic enzymes and of their sensitivity to Fru-2,6-P₂.

Methods

Materials. The Mono Q column (HR 5/5) and the FPLC system were from Pharmacia LKB Biotechnology, blue Trisacryl was from IBF biotechnics and SDS-PAGE molecular mass standards were from Bio-Rad. Dithiothreitol and phenylmethylsulphonyl fluoride (PMSF) were purchased from Janssen Chimica; antipain and bovine heart type

II cAMP-dependent protein kinase were from Sigma. Enzymes and other biochemicals were from Boehringer. Other chemicals were from Merck or Fluka and were of analytical grade. Potato tuber pyrophosphate:fructose-6-phosphate 1-phosphotransferase (PP_i-PFK) (Van Schaftingen *et al.*, 1982b), Fru-2,6-P₂ (Van Schaftingen & Hers, 1981a) and [2-³²P]Fru-2,6-P₂ (Van Schaftingen *et al.*, 1982a) were prepared as described previously.

Cell growth conditions and sampling procedures. *B. emersonii* Cantino & Hyatt (CBS 561.70) was kept at 24 °C on standard Cantino peptone/yeast/glucose (PYG) (1.25 g Bacto-peptone, 1.25 g yeast extract, 3 g glucose and 2 g agar l⁻¹) agar plates and subcultured daily on a 16 h, 24 °C life cycle schedule. Freshly released zoospores were harvested by flooding the surface of agar cultures with sterile distilled water. For sporulation and germination experiments (Figs 1-3), 2.3 × 10⁸ zoospores were inoculated in 1 litre of DM₄ liquid medium (Maia & Camargo, 1974) and cultivated overnight at 19 °C in a rotary shaker at 170 r.p.m. The cell suspension was then filtered through a Nitex cloth (20 × 30 cm; pore size, 10 µm), which retained the vegetative cells as a gelatinous mass. The cells were washed with 200 ml of the sporulation medium (1 mM-Tris/maleate buffer, pH 6.8, containing 1 mM-CaCl₂), resuspended in the same solution to a final volume of 400 ml and incubated at 27 °C. At various times, samples (50 ml) of the cell suspension were filtered under vacuum on a cellulose nitrate filter (47 mm diameter, 0.45 µm pore size, Gelman Science). The cakes were scraped off with a spatula and immediately frozen in liquid N₂. As determined by microscopic examination, sporulation was usually complete after 4 h. The remaining suspension (100 ml), which contained the released zoospores, was then filtered on the Nitex cloth, which retained the vegetative cell ghosts. The zoospores were collected by brief centrifugation and resuspended in about 1 litre of germination medium (same as the sporulation medium but supplemented with 1 mM-MgCl₂ and 50 mM-KCl) at a cell density of 1.5 × 10⁶ ml⁻¹. At various times, a sample (8 ml) was collected by centrifugation (10 s at 10000 g in an Eppendorf centrifuge); the supernatant was discarded and the pellet was frozen in liquid N₂.

Inoculation of zoospores (7 × 10⁷ l⁻¹) into PYG phosphate medium (Suberkropp & Cantino, 1973) followed by growth for 12 h at 24 °C gave rise to vegetative cells, which were collected under vacuum as described above and used for the purification of PFK-2 and FBPase-2.

Measurement of metabolites, cAMP and glycogen. Fru-2,6-P₂ (Van Schaftingen & Hers, 1983) and glycogen (François *et al.*, 1987) were assayed in alkaline extracts prepared by mixing frozen cells (~5 mg protein) in 1 ml hot 0.25 M-Na₂CO₃ and further heating in a water bath at 90 °C with occasional shaking. Extraction of cells for the determination of glycolytic intermediates, cAMP and trehalose was as follows. A frozen sample (equivalent to 5-25 mg protein) was transferred to 1 ml cold 6% (w/v) trichloroacetic acid and homogenized by sonication or, in the case of zoospores, by pipetting up and down four times with a Pasteur pipette. After centrifugation, the pellet was discarded and the trichloroacetic acid was eliminated from the supernatant by five successive extractions with 5 vols diethyl ether. The solution was then evaporated to dryness by incubation at 60 °C under a stream of nitrogen. The residue was dissolved in 1 ml 0.1 M-Tris/HCl buffer, pH 7.4, containing 1 mM-EDTA. Glucose 6-phosphate (Glc-6-P) and Fru-6-P (Lang & Michal, 1974), ATP (Lamprecht & Trautschold, 1974), fructose 1,6-bisphosphate (Fru-1,6-P₂) (Michal & Beutler, 1974) and phosphoenolpyruvate (PEP) (Czok, 1974) were measured by published procedures. cAMP was measured with the Amersham assay kit as described by Tovey *et al.* (1974) and trehalose by an original procedure described in the next paragraph.

Determination of trehalose. Our method is based on the report by Courtois & Demelier (1966) of the high activity (5 U g⁻¹) of trehalase in rabbit kidney. The two kidneys of one rabbit were homogenized in a

Potter-Elvehjem apparatus with 10 vols 0.25 M-mannitol. The pH was brought to 5 with 1 M-HCl and the resulting suspension was centrifuged for 10 min at 1000 g. The pellet, which contained the agglutinated subcellular organelles, was washed twice with 5 vols 10 mM-sodium acetate, pH 5.0, containing 0.25 M-mannitol, resuspended in 5 vols of the same buffer and centrifuged for 10 min at 1000 g. The supernatant was discarded and the pellet was frozen and thawed three times (to liberate the lysosomal enzymes), resuspended in 5 vols cold water and centrifuged for 10 min at 1000 g. The resulting pellet was resuspended in 5 vols 0.16% (w/v) sodium deoxycholate and the suspension was centrifuged for 10 min at 1000 g. The supernatant was used as a source of trehalase and could be kept for at least 1 year at -20°C .

Trehalose was assayed in a total volume of 0.2 ml containing 25 mM-sodium acetate buffer, pH 5.5, and 50–100 μl of an extract prepared as described above. The reaction was started by adding 50 μl (0.1 U) of the rabbit kidney trehalase preparation. After 30 min incubation at 30°C , the reaction was stopped by adding 50 μl 0.15 M-Ba(OH)₂, 50 μl 0.15 M-ZnSO₄ and 0.2 ml water. The resulting mixture was vigorously shaken and then centrifuged for 10 min at 1500 g. The liberated glucose was assayed in 0.2 ml of the supernatant by the glucose oxidase method (Huggett & Nixon, 1957). Glycogen, lactose, raffinose, sucrose and Glc-6-P were not hydrolysed by the rabbit kidney preparation; only trehalose and, to a lesser extent, maltose were hydrolysed. If necessary, maltose could be destroyed by heating the solution at 90°C for 5 min in the presence of 0.1 M-NaOH, but this precaution was not required in our study, since maltose was not present in the samples.

Enzyme studies. Except for the study of PFK-1, zoospores (equivalent to 5–10 mg protein) were suspended in 1 ml of a solution containing 100 mM-KCl, 2 mM-EDTA, 1 mM-dithiothreitol, 1 mM-PMSF, 50 μg ml⁻¹ antipain and 20 mM-potassium phosphate, pH 7.5 (buffer A). The cells were homogenized by pipetting the suspension up and down several times with a Pasteur pipette. The extracts were then centrifuged at 4°C for 15 min at 10000 g and the supernatants were used for the enzyme assays.

For the partial purification of PFK-2 and FBPase-2, vegetative cells were disrupted for two 30 s periods in an MSK cell homogenizer (Braun) with 10 g glass beads (0.5 mm diameter) in 5 ml buffer A. The beads were removed and washed once with 5 ml of the same solution. The washing and the extract were pooled and centrifuged at 25000 g for 15 min in the cold. The supernatant was applied to a Sephadex G-25 column (1.4 $\times$ 28 cm) equilibrated in buffer A, except that the KCl concentration was 20 mM, and potassium phosphate, pH 7.5, was replaced by Tris/HCl, pH 7.4. The filtrate was applied to a blue Trisacryl column (1.4 $\times$ 10 cm) which had been equilibrated with this buffer and developed (Fig. 4) with a linear gradient of NaCl (0.2 M in 100 ml of buffer) at a rate of 100 ml h⁻¹ or on a Mono Q column equilibrated with the same buffer and developed with a gradient of NaCl programmed in an FPLC system (Fig. 5).

All enzyme assays were performed, unless otherwise indicated, at 30°C by the following procedures. PFK-1 was assayed spectrophotometrically by coupling with aldolase, triose phosphate isomerase and glycerol 3-phosphate dehydrogenase according to Van Schaftingen *et al.* (1981), except that Tris/HCl, pH 7.4, was used instead of HEPES, pH 7.1, and that NH₄Cl was omitted from the incubation mixture. FBPase-1 was assayed spectrophotometrically according to Gancedo & Gancedo (1971). The reaction was initiated by the addition of Fru-1,6-P₂. PFK-2 was measured by the formation of Fru-2,6-P₂ according to François *et al.* (1984). FBPase-2 was measured by the release of ³²P_i from [2-³²P]Fru-2,6-P₂ according to Van Schaftingen *et al.* (1982a), except that the assay mixture contained 2 mg bovine serum albumin ml⁻¹, 5 mM-MgCl₂, 20 mM-glycerol 2-phosphate, 2 μM -[2-³²P]Fru-2,6-P₂ and 50 mM-HEPES, pH 7.1, in a total volume of 0.1 ml. The formation of a [³²P]phosphoenzyme and SDS-PAGE analysis were performed as described by François *et al.* (1988). Pyruvate kinase was

assayed as in Van Schaftingen *et al.* (1986a). One unit of enzyme is the amount that catalyses the conversion of 1 μmol substrate min⁻¹ under the assay conditions or describes an activity of 1 μmol substrate converted min⁻¹ under variable experimental conditions.

Other methods. The concentration of protein was measured by the method of Bradford (1976) with bovine immunoglobulin G as a standard. The intracellular concentration of metabolites was calculated according to Selitrennikoff *et al.* (1976, 1980). Results shown are representative of at least three different experiments which yielded similar results.

Results

Concentrations of Fru-2,6-P₂, glycolytic intermediates, cAMP and reserve carbohydrates during sporulation and germination

As shown in Fig. 1(a), the concentration of Fru-2,6-P₂ in vegetative cells after 12 h of growth was close to 0.8 nmol (mg protein)⁻¹, a value which corresponds to an intracellular concentration of 60 μM according to the formulation of Selitrennikoff *et al.* (1976, 1980). Removal of the growth medium and resuspension of vegetative cells in a sporulation medium resulted in an abrupt fall in the concentration of Fru-2,6-P₂ to less than 0.02 nmol (mg protein)⁻¹ within 10 min. At the same time, there was also a fourfold decrease in the concentration of hexose 6-phosphates and an almost complete disappearance of Fru-1,6-P₂ (Fig. 1b). These metabolites remained at a low level throughout sporulation and during the subsequent germination of zoospores in the absence of glucose. In contrast, the content of cAMP, which was close to 20 pmol (mg protein)⁻¹ in vegetative cells, exhibited no change during sporulation (not shown), and showed a transient threefold increase during the first minute after induction of the germination of zoospores (Fig. 2). When 17 mM-glucose was present in the germination medium, the rise in cAMP concentration was similar, but the concentrations of Fru-2,6-P₂ and hexose phosphate progressively increased, at rates of 0.2 and 4 nmol h⁻¹ (mg protein)⁻¹, respectively (not shown). Fig. 1(c) also shows that ATP remained at a steady-state concentration of 25 nmol (mg protein)⁻¹ (i.e. an intracellular concentration close to 2 mM) during the major part of sporulation, and decreased by fourfold during the last hour of this process. It then remained at this low level during the subsequent germination. Other metabolites such as AMP, PEP and glycerate 3-phosphate were also measured in the same experiment. Their concentrations were close to 1.25 nmol (mg protein)⁻¹ in vegetative cells and were not significantly changed during sporulation and germination (not shown).

The content of glycogen and trehalose in vegetative cells after 12 h of growth was close to 400 μg (mg protein)⁻¹ (15% of dry weight) and 130 μg (mg pro-

Fig. 1. (a) Activities of PFK-2 ($\square$) and of FBPase-2 ($\triangle$) and concentration of Fru-2,6-P₂ ($\circ$); (b) concentration of hexose 6-phosphates ($\bullet$) and of Fru-1,6-P₂ ($\blacktriangle$); (c) concentration of ATP ($\circ$) during sporulation and germination of *B. emersonii*. After 12 h of growth at 19 °C ($t = 0$), the vegetative cells were harvested and resuspended in the sporulation medium. After 4 h (arrow), the liberated zoospores were transferred to the germination medium.

Fig. 2. Concentration of cAMP during the encystment of *B. emersonii* zoospores. General procedure as in Fig. 1.

Fig. 3. Glycogen ($\triangle$) and trehalose ($\circ$) contents of *B. emersonii* during sporulation and germination. General procedure as in Fig. 1.

tein)⁻¹, respectively (Fig. 3). After transfer of vegetative cells to sporulation medium, the two storage carbohydrates were rapidly mobilized, but the patterns of their degradation were different. Trehalose disappeared almost completely during sporulation, at a rate of approximately 0.2 $\mu\text{mol glucose unit h}^{-1}$ (mg protein)⁻¹. In contrast, the degradation of glycogen followed two distinct phases. The first phase, which lasted for about 2 h after the induction of sporulation, was characterized by the degradation of 70% of the glycogen at a rate of 1.0 $\mu\text{mol glucose unit h}^{-1}$ (mg protein)⁻¹. After this period and until the end of sporulation, the degradation of the polysaccharide occurred at a slower rate. Upon germination, the remaining glycogen was broken down at a rate similar to that observed during the first 2 h of sporulation.

Characterization of PFK-2 and FBPase-2

A crude extract of vegetative cells contained about 35 μU of PFK-2 and 25 μU of FBPase-2 (mg protein)⁻¹ and the two enzymic activities increased about twofold during sporulation but remained stable during germination (Fig. 1a). As has been shown for other low- K_m FBPase-2 (Larondelle *et al.*, 1986; François *et al.*, 1988), the *B. emersonii* enzyme could be identified in the crude extract by its ability to form a phosphoprotein when incubated in the presence of [2-³²P]Fru-2,6-P₂. Autoradiography after SDS-PAGE revealed one major 90 kDa subunit, a tiny band of 54 kDa and a faint band of 70 kDa. In

Fig. 4. Elution profile of PFK-2 and FBPase-2 from the blue Trisacryl column. An extract of vegetative cells containing 160 mg protein (4.8 mU PFK-2 and 3.9 mU FBPase-2) was applied to the gel. Fractions of 3 ml were collected. Δ , Protein; $\circ$, PFK-2; $\bullet$, FBPase-2; —, NaCl gradient.

Fig. 5. Separation of two peaks of PFK-2 and FBPase-2 by chromatography on the Mono Q column and characterization of FBPase-2 by SDS-PAGE analysis. Four millilitres of gel-filtered crude extract from vegetative cells containing 0.7 mU PFK-2 and 0.56 mU FBPase-2 was applied to the Mono Q column, and 1 ml fractions were collected. In some of these fractions, FBPase-2 was labelled by incubation with $1 \mu\text{M}$ -[2- ^{32}P]Fru-2,6- P_2 and submitted to SDS-PAGE, which was developed by autoradiography (lower panel). Δ , Protein; $\circ$, PFK-2; $\bullet$, FBPase-2; —, NaCl gradient.

contrast, if the extract had been prepared in the absence of protease inhibitors and then incubated at 22 °C for 20 min, the major labelled band corresponded to the 54 kDa subunit (results not shown).

PFK-2 and FBPase-2 were partially purified from vegetative cells and characterized. Like the plant

(Larondelle *et al.*, 1986) and yeast (François *et al.*, 1988) enzymes, *B. emersonii* PFK-2 and FBPase-2 were strongly retained on blue Trisacryl and they could be eluted together at a high salt concentration with an activity ratio close to 1 (Fig. 4). This procedure resulted in a purification of about tenfold with a recovery of 60%.

In addition, PFK-2 and FBPase-2 could not be separated by gel filtration of an extract through a Superose 6B column (not shown). When an extract was applied to a Mono Q column, two peaks with different PFK-2 and FBPase-2 activity ratios were obtained (Fig. 5). The first peak of activity was eluted at 150 mM-NaCl. It contained 55% of the total amount of PFK-2 and FBPase-2 applied to the column, with an activity ratio close to 1.5. A second peak was eluted at 280 mM-NaCl with an activity ratio close to 3. SDS-PAGE analysis of the [32 P]phosphoprotein formed upon incubation of the fractions with [2- 32 P]Fru-2,6-P₂ revealed two major subunits with molecular masses close to 90 and 54 kDa (Fig. 5). In the first peak the labelling of the 90 kDa subunit was more intense than that of the 54 kDa fragment; in the second peak, the opposite was true.

Fractions 16 (peak 1) and 21 (peak 2) eluted from the Mono Q column were used for a study of the kinetic properties of PFK-2. The enzyme of peak 2 displayed a threefold higher affinity for the substrates, ATP (K_m 0.17 mM) and Fru-6-P (K_m 0.12 mM), and was tenfold more sensitive to inhibition by glycerol 3-phosphate (K_i 0.6 mM) and dihydroxyacetone phosphate (K_i 0.5 mM) than the enzyme of the first peak. However, both peaks of activity were inhibited to the same extent by PEP and citrate. The PFK-2 eluted from the blue Trisacryl column had kinetic properties remarkably similar to the enzyme present in peak 1.

Some kinetic properties of FBPase-2 were studied with the enzyme from fractions 24–28 of the blue Trisacryl chromatography or with fraction 16 of the Mono Q column. The partially purified enzyme displayed typical Michaelis–Menten kinetics, with a K_m of about 0.4 μ M, and was non-competitively inhibited by Fru-6-P, with a K_i of 12.5 μ M (results not illustrated). This inhibition was partially (20%) relieved by 20 mM-glycerol 3-phosphate, 20 mM-glycerol 2-phosphate or 5 mM-P_i. Nucleoside triphosphates (GTP, ATP), which are activators of the liver FBPase-2 (Van Schaftingen *et al.*, 1982a), were without effect on the activity of the *B. emersonii* enzyme in our assay conditions. Characterization of FBPase-2 from peak 2 was not possible because of the low activity of the enzyme.

Finally, the activities of PFK-2 and FBPase-2 partially purified on Mono Q or on blue Trisacryl were not modified upon incubation with beef heart cAMP-dependent protein kinase, cAMP and ATP-Mg.

Stimulation of PFK-1 and inhibition of FBPase-1 by Fru-2,6-P₂

The activity of PFK-1 from *B. emersonii* could be satisfactorily assayed when the extraction of the zoo-

Fig. 6. Effects of Fru-2,6-P₂ on the saturation curve of *B. emersonii* PFK-1 for (a) Fru-6-P and (b) ATP. In (a), the concentration of ATP-Mg was 1 mM and Glc-6-P was present at a concentration four times higher than that of Fru-6-P; in (b), Fru-6-P and Glc-6-P were present at concentrations of 0.5 and 2 mM, respectively. The concentrations of Fru-2,6-P₂ (μ M) are shown next to the curves. Similar results were obtained with AMP instead of Fru-2,6-P₂, with a K_a of 0.2 mM.

spores was performed in buffer A containing 20 mM-HEPES, pH 7.1, and 1 mM-Fru-6-P. The use of other extraction media such as potassium phosphate, pH 7.5, or Tris/HCl, pH 7.4, resulted in low recovery and in instability of PFK-1, and in a reduced sensitivity of this enzyme to AMP and Fru-2,6-P₂ (results not shown). As observed with PFK-1 from other sources, the *B. emersonii* enzyme displayed a sigmoidal saturation curve for Fru-6-P, with an $S_{0.5}$ of 0.8 mM (Fig. 6a) and was inhibited by excess ATP (Fig. 6b). The effects of Fru-2,6-P₂ and of AMP were to decrease the K_m of the enzyme for Fru-6-P, to increase the maximum velocity, V , two- to three-fold and to relieve the inhibition by ATP. At 0.2 mM-Fru-6-P and 1 mM-ATP, half-maximal stimulation was obtained with 2 μ M-Fru-2,6-P₂ (Fig. 6a) and 0.2 mM-AMP (not shown). These two effectors, when present at suboptimal concentrations, had additive but not synergistic effects (not shown). The activity of

Fig. 7. Effect of Fru-2,6-P₂ on the saturation curve of *B. emersonii* FBPase-1 for Fru-1,6-P₂ in the absence (open symbols) or in the presence (filled symbols) of 50 μM-AMP. The concentrations of Fru-2,6-P₂ (μM) are shown next to the curves. The inset shows the synergistic effect of Fru-2,6-P₂ and AMP on FBPase-1 activity measured at 25 μM-Fru-1,6-P₂ (○, 0 μM-AMP; ●, 50 μM-AMP).

PFK-1 was not affected by 5 mM-P_i, 5 mM-citrate or 20 mM-NH₄Cl.

An enzyme catalysing the Mg-dependent hydrolysis of Fru-1,6-P₂ was detected in both vegetative cells and zoospores. This FBPase-1 displayed a hyperbolic saturation curve for Fru-1,6-P₂, with a K_m close to 5 μM (Fig. 7), and was inhibited by high concentrations of substrate (40% at 500 μM; not shown). It was non-competitively inhibited by AMP (K_i close to 0.75 mM; not shown). As shown in Fig. 7, it was also inhibited by Fru-2,6-P₂; this inhibition strikingly resembled that described for the liver enzyme (Van Schaftingen & Hers, 1981*b*), i.e. it was more pronounced at low concentrations of substrate, changed the saturation curve from hyperbolic to sigmoidal and was synergistic with the inhibition by AMP. When the enzyme was assayed at 25 μM-Fru-1,6-P₂, the K_i for Fru-2,6-P₂ was reduced from 0.25 to 0.05 μM in the presence of a non-inhibitory concentration of 50 μM-AMP (inset in Fig. 7). It is worth mentioning that the activity of pyruvate kinase was not affected by Fru-2,6-P₂.

Discussion

Relationship between glucose, Fru-2,6-P₂, and cAMP

The general rule that Fru-2,6-P₂ is a signal formed in response to glucose appears also to apply to *B. emersonii*. Indeed, the presence of glucose was required for the formation of Fru-2,6-P₂ during germination of zoospores, whereas the removal of the nutritive medium

was accompanied by a rapid decrease in the concentration of Fru-2,6-P₂. It is known, however, that the mechanism by which glucose increases Fru-2,6-P₂ concentration is very different from one type of cell to the other, as is also the effect of cAMP on this system. In the liver, the glucose effect is believed to be mediated by an increase in the concentration of Fru-6-P, a substrate of PFK-2 and a potent inhibitor of FBPase-2, whereas the cAMP-dependent phosphorylation of these two enzymes causes the rapid disappearance of Fru-2,6-P₂. In *Saccharomyces cerevisiae*, in contrast, the effect of glucose is to cause a rise in cAMP concentration and the secondary activation of PFK-2 by a cAMP-dependent phosphorylation. Our work indicates that, in *B. emersonii*, as in *Dictyostelium discoideum* (Aragon *et al.*, 1986) and *Neurospora crassa* (Dumbrava & Pall, 1987), the changes in the levels of Fru-2,6-P₂ during the cycle are independent of those of cAMP (see Figs. 1 and 2), but, as also in the liver, tightly related to those of hexose 6-phosphate.

Biosynthesis and biodegradation of Fru-2,6-P₂

As in other cells, the biosynthesis and the biodegradation of Fru-2,6-P₂ in *B. emersonii* are catalysed by, respectively, a 6-phosphofructo 2-kinase (PFK-2) and one or possibly (see below) several fructose-2,6-bisphosphatase(s) (FBPase-2). Several experimental data suggest that in *B. emersonii*, as in liver, muscle and in plants (see Introduction), PFK-2 and FBPase-2 are associated in a single bifunctional protein. Upon brief incubation in the

presence of $[2\text{-}^{32}\text{P}]\text{Fru-2,6-P}_2$, the low- K_m FBPase-2 formed a labelled phosphoprotein characterized by two subunits of respectively 90 and 54 kDa. The smaller fragment probably results from proteolytic degradation occurring during the preparation of the extracts, since it was a minor component of homogenates prepared in the presence of protease inhibitors. Furthermore, two peaks of PFK-2/FBPase-2 with different activity ratios were separated by chromatography on a Mono Q column. Since the 90 kDa subunit was predominant in the first peak, it is suggested that this peak contained the native form of the protein. The relatively low and variable FBPase-2 activity of peak 2 indicates that, contrary to what is known for the liver (El-Maghrabi *et al.*, 1984) and for the plant (Larondelle *et al.*, 1986) bifunctional protein, *B. emersonii* FBPase-2 is more sensitive to proteolysis than PFK-2. It is also surprising that the PFK-2 present in peak 2 had more affinity for its two substrates, ATP and Fru-6-P, and was more sensitive to inhibitors than the enzyme present in peak 1.

The kinetic properties of *B. emersonii* PFK-2 and FBPase-2 were in many respects similar to those of the animal, plant and yeast enzymes. The fact that the K_m of PFK-2 for Fru-6-P was in the physiological range of concentration and that FBPase-2 was strongly inhibited by the same phosphate ester is in agreement with the hypothesis that the effect of glucose to increase the concentration of Fru-2,6-P₂ is mediated by a change in the concentration of Fru-6-P. The fact that there was no effect of beef heart cAMP-dependent protein kinase on the activity of either PFK-2 or FBPase-2 reinforces the idea that no interrelationship exists between these two regulatory molecules in *B. emersonii*.

Biological actions of Fru-2,6-P₂

As found in many other eukaryotic cells, the effects of Fru-2,6-P₂ in *B. emersonii* were to stimulate PFK-1 and to inhibit FBPase-1. The action of Fru-2,6-P₂ on the *B. emersonii* PFK-1 closely resembled that reported for the yeast enzyme (Bartrons *et al.*, 1982), in that it affected both $V_{\max}$ and K_m . Interestingly, this enzyme displayed a tenfold higher sensitivity to AMP than PFK-1 from other sources. However, the potent action of AMP on *B. emersonii* PFK-1 probably has no physiological role during sporulation, since the concentration of AMP did not change during this period while those of Fru-2,6-P₂ and Fru-1,6-P₂ decreased in parallel. This result is, therefore, in favour of a major role of Fru-2,6-P₂ in the control of *B. emersonii* PFK-1 activity. Particularly striking was also the finding that the inhibition of *B. emersonii* FBPase-1 by Fru-2,6-P₂ resembles in all respects that described for the liver enzyme (Van

Schaftingen & Hers, 1981*b*), except that Fru-2,6-P₂ was 25 times more effective. This strong similarity may allow the conclusion that the inhibition of *B. emersonii* FBPase-1 is of the allosteric competitive type (François *et al.*, 1983).

Metabolic changes during sporulation

As expected, *B. emersonii* responded to starvation by completely redirecting its carbohydrate metabolism. Upon removal of the growth medium, the glycolytic capacity of the vegetative cells was dramatically reduced, as indicated by a parallel drop in the concentration of Fru-2,6-P₂ and of Fru-1,6-P₂, whereas glycogen, which is known to accumulate during syncytial growth (Plessmann Camargo *et al.*, 1969), and trehalose were degraded. As explained above, the dramatic drop in Fru-2,6-P₂ concentration can probably be explained by the simultaneous decrease in the concentration of Fru-6-P. It must be noted, however, that the rate of the *in vivo* disappearance of Fru-2,6-P₂ under these conditions was at least fourfold higher than the activity of the low- K_m FBPase-2 measured in a gel-filtered crude extract. This suggests that in *B. emersonii*, as in plants (MacDonald *et al.*, 1987; Larondelle *et al.*, 1989) and in yeast (François *et al.*, 1988), a high- K_m FBPase-2 (not yet identified in *B. emersonii*) may be responsible for the degradation of Fru-2,6-P₂.

While glycolysis was inhibited, the ATP concentration remained unchanged during the major part of the sporulation period, suggesting that mitochondrial activity was sustained by the utilization of amino acids derived from the degradation of proteins which took place during this period (Lodi & Sonneborn, 1974; Lovett, 1975). Conversely, the decrease in the concentration of ATP during the last hour of sporulation was probably the consequence of the energetic expenditure, for their motility in water, of the zoospores which are escaping from the sporangia.

The degradation of reserve carbohydrates during sporulation is a unique feature of *B. emersonii*, since in most other fungi sporulation is accompanied by the synthesis of either glycogen or trehalose or both (Thevelein, 1984). The question remains as to what are the factors triggering the glycogenolysis and the mobilization of trehalose since cAMP, which is involved in these processes in other fungi (Thevelein, 1984; Pall, 1984), remained unchanged during sporulation.

Metabolic changes during germination

In contrast to what has been found during germination of *Phycomyces blakesleeanus* spores (Van Laere *et al.*, 1983)

and of oat seeds (Larondelle *et al.*, 1987), the concentration of Fru-2,6-P₂ did not change during this process in *B. emersonii*. This major difference probably reflects the fact that, although quiescent with respect to growth-related functions (Soll & Sonneborn, 1971*b*; Lovett, 1975), zoospores of *B. emersonii* are not dormant as are fungal spores or oat seeds, but are ephemeral cells precariously poised between lysis and germination. They differ from dormant forms in that they maintain some catabolic activity (Suberkropp & Cantino, 1973). In contrast, the encystment of the zoospores was accompanied by a transient increase in the concentration of cAMP, corroborating the recent observation that cAMP-dependent protein kinase was activated during this morphogenetic process (Behrens & Maia, 1986). Furthermore, one might suggest that the sudden increase in cAMP concentration could be responsible for the resumption of the degradation of glycogen. The glucose moieties derived from the polysaccharide degradation are probably used for the biosynthesis of chitin, since the amount of polysaccharide degraded during germination (about 3% of dry weight) closely correlated with the amount of chitin synthesized during this process (2.5 g%, according to Selitrennikoff *et al.*, 1976, 1980).

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