On a possible role of fructose 2,6-bisphosphate in regulating photosynthetic metabolism in leaves

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1. INTRODUCTION

In photosynthesis two distinct FBPases are involved: a stromal enzyme participating in the Calvin cycle and a cytosolic enzyme involved in sucrose synthesis. Regulation of these enzymes is needed to permit a balanced withdrawal of carbon from the chloroplast stroma, provided by the Calvin cycle or by the degradation of assimilatory starch, for synthesis of sucrose and other carbon utilizing reactions in the cytosol. The stromal FBPase is known to be activated in the light by an interconversion catalysed by thioredoxin [1], and also to be stimulated by alkalization of the stroma [1] but it is unclear to what extent a fine control of this enzyme occurs in the light. The cytosolic FBPase, as will be shown in the following, does not appear to be modified by illumination. It is weakly inhibited by AMP and P_i [2,3] but only at concentrations above those occurring in situ. In the present article it is demonstrated that F2,6BP, which plays an important role in regulating glycolysis and gluconeogenesis in liver [4–6], is not only found in plant cells but also strongly inhibits cytosolic FBPase from wheat leaves, and to lesser extent also the chloroplastic enzyme. These data indicate that F2,6BP may be involved in regulating photosynthetic metabolism.

Abbreviations: P, phosphate; FBP, fructose 1,6-bisphosphate; F2,6BP, fructose 2,6-bisphosphate; PFK, phosphofructokinase; TX100, Triton X-100; Chl, chlorophyll; FBPase, fructose 1,6-bisphosphatase

2. MATERIALS AND METHODS

Wheat leaf protoplast preparation, incubation media, light sources, protoplast fractionation by membrane filtration and assay of marker enzymes were as in [7], wheat chloroplast isolation as in [8], liver PFK was purified as in [9] and F2,6BP synthesized as in [6].

Assays (total volume 600 μl, containing 50–100 μl protoplast suspension or filtrate) were carried out with a Hitachi double wavelength photometer with automatic cuvette changes. For FBPase, all assays included 1.7 mM EDTA, 0.1% TX100, 0.4 mM NADP, 3 U/ml glucose6P dehydrogenase and 14 U/ml phosphoglucoisomerase. Further additions were 50 mM imidazole–HCl (pH 7), 4 mM $MgCl_2$ and 50 μM FBP for the cytosolic enzyme; or 50 mM HEPES (pH 8.1), 20 mM $MgCl_2$ and 1 mM FBP for the stromal enzyme. PP_i dependent PFK was assayed in 50 mM imidazole (pH 7.1), 4 mM $MgCl_2$, 1.7 mM EDTA, 0.1% TX100, 0.3 mM NADH, 0.8 mM PP_i, 0.8 mM fructose 6-P, 2.4 mM glucose6-P, 5 U/ml aldolase, 13 U/ml TrioseP isomerase, 4 U/ml glycerol 3-P dehydrogenase. The stimulation of activity in the presence of F2,6BP required the presence of both PP_i and fructose 6-P.

F2,6BP was extracted from spinach grown in hydroponic culture in 9 h light/15 h dark cycle. Leaf material (0.5 g) was frozen in liquid N_2, powdered, heated 5 min in 5 ml methanol plus 0.6 ml 20 mM HEPES (pH 8.5) in an 80°C waterbath, cooled, 1 ml $CHCl_3$ added, shaken, 3 ml H_2O added, shaken, centrifuged (5 min, 1500 × *g*), the supernatant dried down in 5–10 min in a rotary

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evaporator, redissolved in 400 μl H_2O and treated with activated charcoal (6 mg). For the bioassay of F2,6BP [10] 10–25 μl aliquots were measured in parallel with a control (10 min at pH 2 before reneutralisation) and an internal standard (5–15 pmol F2,6BP added to an acid-treated sample after reneutralisation). In some experiments, extracts were pooled, applied to a Dowex AG 1–X8 (200–400 mesh, Cl form) anion exchange column and eluted with a 100–400 mM NaCl gradient [11] to confirm that the PFK-activating factor eluted just behind authentic FBP. The extraction method was checked by adding small amounts of F2,6BP (0.2 nmol/mg Chl) to the hot methanol with the plant material and showing that 80–90% could be recovered. The measurements were carried out with spinach leaves because of technical difficulties in recovering F2,6BP in the presence of wheat leaves.

3. RESULTS

3.1. *Specific assay of cytosolic and stromal FBPase*

Since isolated stromal FBPase has a higher K_m, pH optimum and Mg^{2+} requirement than the cytosolic enzyme [2] we investigated whether the cytosolic and stromal enzymes could be differentially assayed in raw extracts by variation of the assay conditions. The experiments were carried out with chloroplasts or protoplasts from wheat leaves. A protoplast is an isolated, intact plant cell from which the cell wall has been removed. At pH 8.1, 20 mM Mg^{2+} and 1 mM FBP (assay of stromal enzyme), illuminated chloroplasts and protoplasts had a high FBPase activity, but in an assay at pH 7.0, 2.4 mM free Mg^{2+} and 50 μM FBP (assay of cytosolic enzyme), there was negligible activity in chloroplasts, but high activity in protoplasts, which was not altered by removing 85% of the chloroplasts by membrane filtration (table 1). The stromal enzyme is sufficiently inactive under the cytosolic assay conditions, conversely the cytosolic enzyme is almost 85% inhibited under the stromal assay conditions.

Table 1

Assay of stromal and cytosolic fructose 1,6-bisphosphatases

	Pretreatment	FBPase (μmol/mg Chl . h)	
		Cytosolic assay	Stromal assay
Chloroplasts	Light	< 3	100
Protoplasts		47	99
Chloroplasts	Dark	< 1	22
Protoplasts		45	22
Enriched cytosol		44	8 (2)

Isolated chloroplasts or protoplasts were incubated 8 min in the light or dark and pipetted directly into assays containing 2.4 mM Mg^{2+} and 50 μM FBP at pH 7.0 (cytosolic) or 20 mM Mg^{2+} and 1 mM FBP at pH 8.1 (stromal). Enriched cytosol was obtained by membrane filtration, and contained only 15% of the GAPDH present in the protoplasts. The figure in parentheses shows the activity in the cytosol extract ascribable to the stromal enzyme (for calculation see [6])

When protoplasts (or chloroplasts) are illuminated, a marked increase in the stromal FBPase activity occurs, which is due to a light-dependent enzyme interconversion catalysed by thioredoxin [1]. In contrast, there is no change in the cytosolic FBPase activity. Apparently, this enzyme is not directly controlled by light-dependent interconversion.

3.2. *Effect of F2,6BP*

Chloroplast FBPase was assayed by pipetting pre-illuminated chloroplasts directly into assay mix containing TX100. Inhibition was found by F2,6BP, provided low FBP levels were used (table 2) as the inhibition was competitive (K_m 40 μM, K_i 2 μM, data not shown). The less active form of FBPase which is present in chloroplasts in the dark has a higher K_m for FBP than the active form [12], and F2,6BP was a more effective inhibitor of the enzyme from darkened chloroplasts (table 2). As previously reported [2], AMP is a very weak inhibitor of the stromal FBPase, even 10 mM AMP producing only a weak inhibition with 0.1 mM FBP, and no interaction was found between F2,6BP and AMP (table 2, compare fig.2). In other experiments (not shown) it was checked that the inhibition by AMP remained weak even at limiting (1.2 mM) Mg^{2+} and that in these conditions there was no synergism between weakly inhibiting levels of AMP and F2,6BP.

To study the fine regulation of cytosolic FBPase without interference from the stromal enzyme, protoplasts were pre-incubated in the dark to inac-

Table 2

Effect of F2,6BP on the activity of stromal fructose 1,6-bisphosphatase extracted from isolated chloroplasts as assayed in the presence of various concentrations of AMP and FBP

Incubation of chloroplasts	Assay conditions	FBPase activity (μmol/mg Chl . h) F2,6BP in assay (μM)		
		0	1	10
Light	2 mM FBP	102	98	88
Light	0.1 mM FBP	70	55	30
Light	25 μM FBP	45	32	5
Dark	2 mM FBP	25	18	9
Light	0.1 mM FBP 10 mM AMP	61	48	20

Assays performed as in Materials and Methods after 8 min illumination (O_2 uptake 110 μmol/mg Chl . h) or darkness by directly pipetting wheat chloroplasts into assay mix containing TX100.

tivate the stromal FBPase before fractionating them by membrane filtration. The filtrate obtained, which had only a low chloroplast contamination, was used in the selective assay of cytosolic FBPase. Cytosolic FBPase has a low K_m for FBP (table 3, see also [2]) and was competitively inhibited by F2,6BP (fig.1). It should be noted that this does not necessarily imply a common binding site for FBP and F2,6BP on the cytosolic enzyme (or on the chloroplast enzyme, see above). At low FBP levels there were indications of sigmoidal substrate dependence with F2,6BP, as has been reported for liver FBPase [6] but further experiments on the purified enzyme are needed to clarify this point. As with liver FBPase [5,6], F2,6BP strongly enhances the inhibition by AMP (fig.2), F2,6BP

Table 3

Regulatory properties of the cytosolic fructose 1,6-bisphosphatase

Inhibitor	Further additions	K_m (FBP) (μM)	K_i (Inhibitor) (μM)
F2,6BP	–	3.6	0.1 μM
P_i	–	3.7	8.4 mM
AMP	0.1 μM F2,6BP	8.3*	0.1 mM
P_i	1 μM F2,6BP 0.25 mM AMP	94*	9.1 mM

For assay conditions see Materials and Methods, further additions in table, apparent K_m* measured in presence of further addition

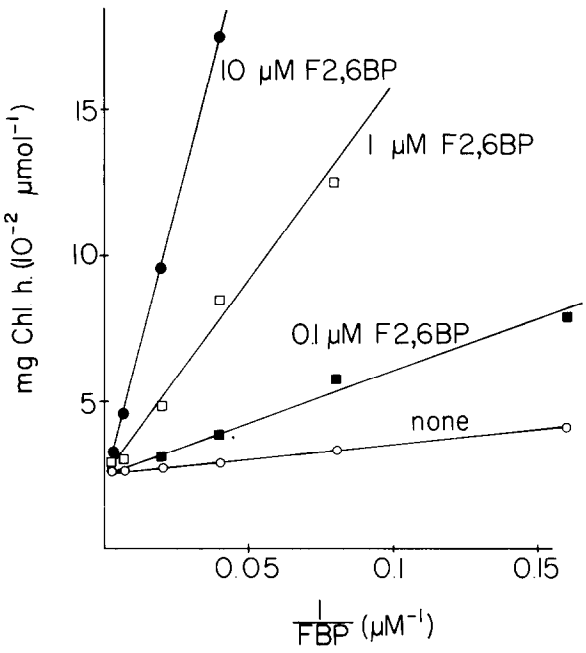

Fig.1. Lineweaver–Burk plot of the inhibition of cytosolic FBPase by F2,6BP. The V_{max} was 40 μmol/mg Chl . h, K_m for FBP 4 μM and K_i for F2,6BP was 0.1 μM.

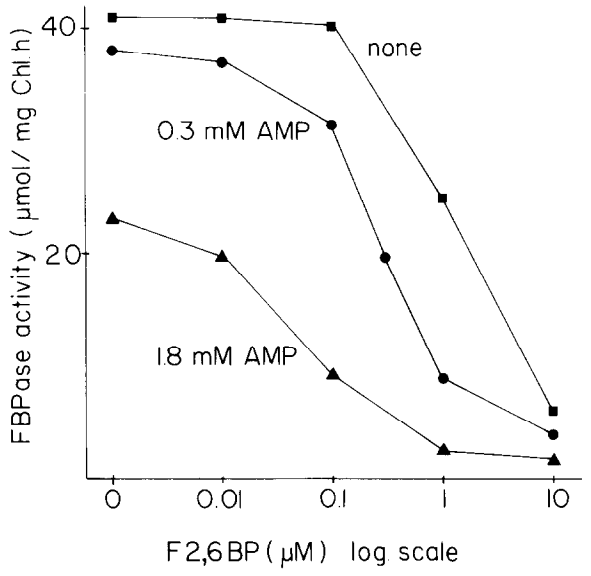

Fig.2. Effect of increasing F2,6BP concentration on the inhibition of cytosolic FBPase by AMP.

and AMP acting synergistically, and the inhibition by AMP is competitive to FBP (table 3). Like most FBPases, cytosolic FBPase is inhibited by P_i [3]. This inhibition is weak, but competitive to FBP (table 3) and can be potentiated (fig.3) by mildly

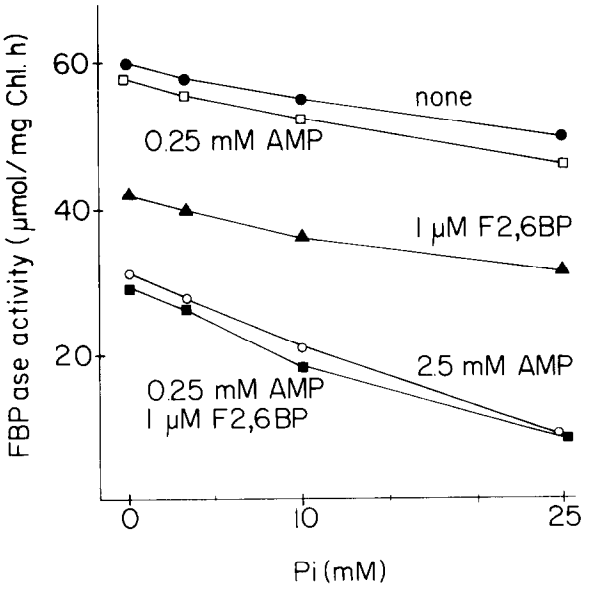

Fig.3. Inhibition of cytosolic FBPase in the presence of AMP or F2,6BP. Conditions as in Materials and Methods with 50 µM FBP, additions or alterations see figure.

inhibitory levels of AMP, or a combination of low AMP and F2,6BP. In the presence of F2,6BP alone, the inhibition by P_i is only weakly enhanced (fig.3). The inhibition by P_i in the presence of F2,6BP and AMP is markedly competitive to FBP (table 3) and under these conditions almost mM levels of FBP are required to saturate the enzyme (fig.4). In other experiments (not shown) pre-illuminating protoplasts for 10 min (O_2 evolution 160 µmol/mg Chl . h) did not alter the concentrations of F2,6BP, AMP or P_i needed to inhibit the cytosolic FBPase by 40% to 60%, indicating that the regulatory properties of the cytosolic FBPase are not altered by a light-dependent enzyme modification.

Recently, the PP_i-dependent PFK from mung bean seedlings [11] and the plastid isoenzyme of the ATP dependent PFK from the endosperm of *Ricinus communis* [13] were found to be activated by F2,6BP. We investigated the activity and subcellular distribution of the PP_i-dependent PFK in wheat protoplasts. An activity of 10.6 µmol/mg

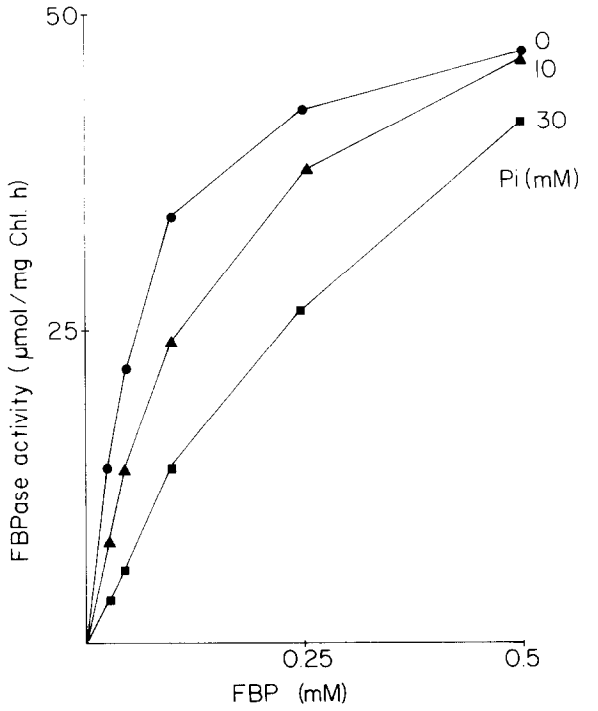

Fig.4. FBP dependence of cytosolic FBPase in the presence of 1 µM F2,6BP, 0.25 mM AMP and varying P_i concentrations. For other conditions see Materials and Methods.

Chl . h was found in protoplasts in the presence of 1 μM F2,6BP. Activity was negligible without F2,6BP. After passage through an 8 μm membrane filter, 91% of the activity was recovered in the filtrate, compared to 92% of the phosphoenolpyruvate carboxylase and 12% of the NADP-glyceraldehyde 3-P dehydrogenase (data not shown). When the chloroplasts were eluted from the 8 μm membrane with assay medium, negligible PP_i dependent PFK activity was present. Further experiments with chloroplast free filtrates showed a high affinity for PP_i (K_m = 14 μM) and a low K_a for F2,6BP (K_a = 0.25 μM) under the assay conditions used (data not shown). The PP_i-dependent PFK appears to play a role in sucrose metabolism which is reciprocal to that of the cytosolic FBPase. This is indicated by its location in the cytosol, its reciprocal modulation by F2,6BP as well as by the consideration that it is presumably dependent upon sucrose phosphate synthetase as a source of PP_i in the cytosol.

3.3. *F2,6BP levels in leaves*

To measure the F2,6BP levels in leaves, an extraction method was developed to minimise alterations in the levels during the quench and extraction process (see Materials and Methods). Leaves were frozen in liquid N_2, the F2,6BP extracted in slightly alkaline methanol, and chloroform added to precipitate protein. F2,6BP was found to be present in leaf material in the light and the dark in levels between 0.05–0.2 nmol/mg Chl. Bearing in mind that the chloroplast volume is about 25 μl/mg Chl [14] and that the cytosol volume may be 2–3 times smaller (Douce, personal commun.) these would be equivalent to 1–5 μM concentration, or even more if the F2,6BP were restricted to one compartment only.

Further experiments are needed to establish the subcellular distribution of F2,6BP and the metabolic conditions under which the concentration varies, but the observation that leaves contain F2,6BP in both the light and the dark makes it unlikely that F2,6BP operates simply as an on-off switch of metabolism. In this context, it is of interest that F2,6BP, AMP and P_i interact in their inhibition of the cytosolic FBPase, and are competitive to FBP, so that enzyme activity could be altered by variation in any one of these metabolites. The published cytosolic levels of about 0.3–0.5 mM AMP [15] lie in the range which could effectively modulate the cytosolic FBPase when micromolar levels of F2,6BP are present (see above). As will be shown elsewhere, the total esterified P in the cytosol varies greatly, indicating reciprocal alterations of the cytosolic P_i of the order of 10–20 mM (Stitt, unpublished). Thus variation of P_i, or AMP might modulate the enzyme in the presence of F2,6BP, and any changes in the F2,6BP level itself could amplify or override these effects as well as directly affecting the enzyme.

NOTE ADDED

During the preparation of this manuscript an abstract has been published where, without any experimental details being given, it is announced that F2,6BP has been found in spinach leaf cells, but not in chloroplasts, and that this substance inhibited the cytosolic FBPase, but not the stromal one [16].

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