Diurnal Changes of Fructose-6-phosphate,2-kinase and Fructose-2,6-bis-phosphatase Activities in Spinach Leaves

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Extracts have been rapidly prepared from spinach leaves, and the activity of fructose-6-phosphate,2-kinase (Fru6P,2kinase) and fructose-2,6-bisphosphatase (Fru2,6P_2ase) measured. The enzyme activities do not change during light-dark transitions, but there is an increase of Fru6P,2-kinase and decrease of Fru2,6P_2ase activity over several hours in the light. This increase of the Fru6P,2kinase:Fru2,6P_2ase ratio shows that a previously unrecognized mechanism, which may include protein modification, controls Fru2,6P_2 levels in leaves. It operates as sucrose accumulates in the leaf, and will be involved in regulating the partitioning of photosynthate.

Introduction

Alterations of Fru2,6P_2 concentrations contribute to controlling glycolysis and gluconeogenesis in many eukaryotic tissues. Studies with liver have revealed how this regulator metabolite is synthesized and degraded by specific enzymes called Fru6P,2kinase and Fru2,6P phosphatase [1]. These two activities reside on the same protein and are regulated in a reciprocal way by selected metabolites and by protein phosphorylation.

Fru2,6P_2 also plays an important role in controlling photosynthetic sucrose synthesis in leaves. It acts by inhibiting the cytosolic fructose-1,6-bisphosphatase [2, 3], which catalyses the first irreversible reaction leading to sucrose. A decrease of Fru2,6P_2 at the start of the day stimulates sucrose synthesis [4, 5], while later in the day when sucrose has accumulated in the leaf there is an increase of Fru2,6P_2 so that more of the photosynthate is diverted towards starch [4, 6]. It has been shown how metabolites can control Fru2,6P_2 levels in leaves [5–9]. However, we do not yet know whether additional mechanisms, such as alteration of the amount of activity of Fru6P,2kinase and Fru2,6P_2ase, also contribute to the changes of Fru2,6P_2 found during the day.

Abbreviations: Fru2,6P_2, fructose-2,6-bisphosphate; Fru2,6P_2ase, fructose-2,6-bisphosphatase; Fru6P,2kinase, fructose-6-phosphate,2-kinase; Fru6P, fructose-6-phosphate; Glc6P, glucose-6-phosphate; PFP, pyrophosphate-fructose-6-phosphate-phosphotransferase; FPLC, fast protein liquid chromatography.

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Materials and Methods

Spinach (*Spinacea oleracea* var. Mazurka) was grown in water culture [4] with a 9 h light/15 h dark cycle (light intensity 340 $mE \cdot m^{-2} \cdot s^{-1}$, temperature 22 °C in light, 16 °C in dark).

Leaf discs (36 mm diameter) from fully expanded leaves of 5–7 week-old plants were squashed against the base of a 5 ml plastic syringe containing a 200 µl nylon net and a modified capillary outlet [10]. Sap (30–50 µl) was collected in 150 µl extraction buffer (100 mM HEPES-KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol), 60 µl aliquots snap-frozen in plastic centrifuge tubes in aluminium blocks at −185 °C, and stored in liquid N_2 until assay. Rapid freezing did not inhibit enzyme activity. For reference, chlorophyll was measured [10]. The extract contained 3–5 µg Chl per 20 µl.

Samples were desalted within 5 min with less than 10% alteration of their volume by centrifuging through G-25 Sephadex (coarse). Sephadex was preswollen in extraction buffer (without Triton X-100), pipetted (1.0 ml bed volume) into a 2 ml plastic syringe with a cushion of quartz wool, and centrifuged to remove buffer from the void volume, but not from inside the beads (1500 rpm, swingout rotor), before pipetting 60–120 µl thawed extract onto the Sephadex and recentrifuging (3 min, 1500 rpm).

Fru6P,2kinase was assayed (for details see [11]) by adding 20 µl extract to assay mix (final volume 100 µl) containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 2 mM Fru6P, 10 mM Glc6P, 1 mM ATP, 5 mM KH_2PO_4. Two 20 µl aliquots were immediately alkalized with 30 µl 0.25 M KOH, one also receiving 2 pmol $Fru2,6P_2$ (internal standard). Further 20 µl aliquots were alkalized later, typically after 3 and 6 min. $Fru2,6P_2$ in the alkalized aliquots was measured using the pyrophosphate-fructose-6-phosphate-phosphotransferase (PFP) bioassay [11, 12]. $Fru2,6P_2$ synthesis was estimated by comparing the activation of PFP by the internal standard (2 pmol, added to a zero-time sample) and by samples which were alkalized after incubation of extracts with Fru6P and ATP. There was a linear dependence of activity on the amount of extract used (5–40 µl, equivalent to 1–8 µg Chl), and on the time (1–15 min).

$Fru2,6P_2$ase was assayed by adding 30 µl extract to a final volume of 120 µl containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, removing and alkalizing one 20 µl aliquot (control for the PFP bioassay), and then starting the assay by adding 20 µl 0.6 µM $Fru2,6P_2$ to give a concentration of 0.1 µM $Fru2,6P_2$ in the assay. Duplicate 20 µl aliquots were alkalized immediately, and further 20 µl aliquots at later times, typically 6, 12 and 18 min. $Fru2,6P_2$ in each alkalized aliquot was assayed using the PFP bioassay [11, 12]. $Fru2,6P_2$ hydrolysis was estimated by comparing the activation of PFP by the samples alkalized immediately after addition of $Fru2,6P_2$ (equivalent to 2 pmol of $Fru2,6P_2$) and by samples alkalized after incubation for increasing lengths of time. Provided desalted extracts were used (see results) and under 30% of the added $Fru2,6P_2$ was being hydrolyzed, there was a linear dependence of activity on amount of extract (20–60 µl, equivalent to 4–12 µg Chl), and on the time (6–48 min). When the $Fru2,6P_2$ concentration in the assay was above 0.1 µM, the alkalized samples were diluted with 0.05 M NaOH so that a 20 µl aliquot contained 2 pmol $Fru2,6P_2$ when added to the PFP bioassay (see [8]). However, normally 0.1 µM $Fru2,6P_2$ was used to avoid this additional step.

For application to FPLC, 0.8 g spinach leaves were homogenized for 1 min with 3 ml extraction medium (as above, but 50 mM HEPES-KOH) for 30 sec, centrifuged (18,000 × *g*, 2 min), passed through G-25 Sephadex, filtered (0.45 µM, cellulose acetate) and applied to a Pharmacia Mono-Q anion exchange column. The column was washed with 10 ml extraction buffer (without Triton X-100, 50 mM HEPES-KOH) and eluted with a NaCl gradient (0 to 0.5 M in 10 min, 0.5–1 M in 5 min, flow rate 1 ml·min^{-1}, 0.5 ml fractions). The partially purified protein was stored in 50% glycerol at −20 °C.

Results

Activities of Fru6P,2kinase and $Fru2,6P_2$ase in raw extracts

We first investigated whether the activity of Fru6P,2kinase or $Fru2,6P_2$ase changed in conditions when the $Fru2,6P_2$ content was altering in leaves. Measurements were made at the end of the night, after 20 min light, and after 9 h light, corresponding to the times at which the largest changes of $Fru2,6P_2$ are found [4]. Extracts were prepared rapidly by squashing leaves in a syringe [10] to obtain a drop of sap which was collected in buffer and assayed or sap frozen within 15 secs of disrupting the leaves. No large changes of enzyme activities were found im-

Table I. Activities of synthesis and hydrolysis of $Fru2,6P_2$, and $Fru2,6P_2$ levels in extracts from spinach leaves obtained at different times of the day and night.

	Activity [pmol·mg Chl^{-1}·min^{-1}]		$Fru2,6P_2$ [pmol·mg Chl^{-1}]
	Fru6P,2kinase	$Fru2,6P_2$ase	
14 h dark	341 ± 20	54 ± 5	161 ± 17
20 min light	391 ± 21	45 ± 4	81 ± 14
8 h light	512 ± 16	33 ± 2	176 ± 19

Activities are mean ± SE of 15 samples, each from a different leaf. $Fru2,6P_2$ was assayed in parallel samples as in [4].

mediately after illumination (Table I) but at the end of the day the Fru6P,2kinase activity had increased by 50%, while Fru2,6P_2ase decreased by 30%. Parallel measurements confirmed that Fru2,6P_2 decreased after illumination and increased again during the day (Table I) as in previous studies [4]. The question arose whether these measurements with raw extracts represent real changes of the amount of enzyme activity.

Assay specifity

Assay of these enzymes involves measuring the synthesis of Fru2,6P_2 from ATP and Fru6P (Fru6P,2-kinase) or the degradation of Fru2,6P_2 (Fru2,6P_2ase) in a two-step procedure. The extract and substrates are first incubated to allow Fru2,6P_2 to be synthesized and degraded, and samples are removed and alkalized at various times to stop the reaction. The Fru2,6P_2 in the individual samples is then estimated by determining how far each activates potato tuber PFP. We routinely checked that enzyme activity was linearly dependent on time and the amount of extract used (see Methods). The accuracy of the results also depended on the raw extract not interfering with the determination of Fru2,6P_2 in the PFP bioassay. This was minimized (see [11]) by desalting extracts (see below) before starting the assay procedure, and by inactivating enzymes by alkalization before adding samples to the PFP bioassay. In addition, internal standards were always included in the PFP bioassay (see Methods).

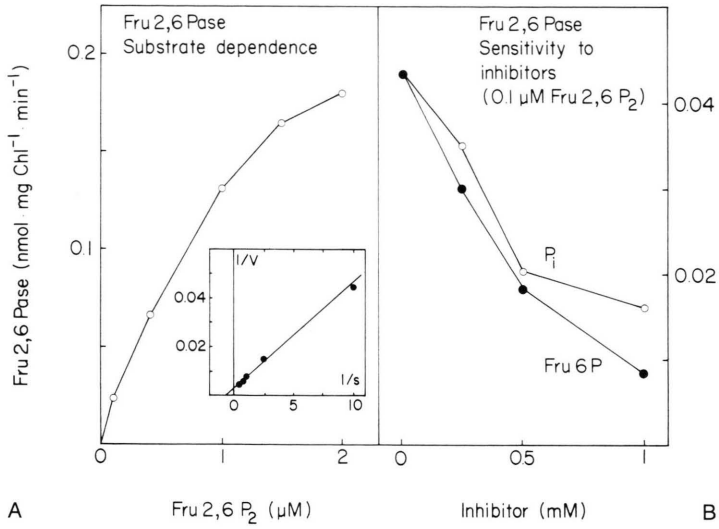
The specifity of the Fru6P,2kinase assay was checked using criteria which are described in detail elsewhere [11]. There was no stimulation of PFP if Fru6P or ATP were omitted from the assay mix for Fru6P,2kinase, nor if samples from the Fru6P,2-kinase assay were treated with alkaline phosphatase or acid before adding them to the PFP bioassay. The activation of PFP can therefore be ascribed to formation of Fru2,6P_2. Moreover, synthesis of Fru2,6P_2 was stimulated by P_i and was inhibited by glycerate-3-P and dihydroxyacetone-P (not shown) in the same way as the partially purified spinach leaf Fru6P,2-kinase [7, 9]. Rehydrolysis of Fru2,6P_2 during the assay of Fru6P,2kinase is prevented by the high concentrations of Fru6P and P_i which are present (see below).

The specifity of the Fru2,6P_2ase assay is investigated in Fig. 1. These experiments were carried out to assess how much of the Fru2,6P_2 hydrolysis might be due to non-specific phosphatases, rather than to Fru2,6P_2ase. The reaction saturated with μmolar concentrations of Fru2,6P_2 (Fig. 1A), and the apparent K_m (1.1 μM) resembled that reported for the partially purified Fru2,6P_2ase [8, 9]. Hydrolysis was inhibited by Fru6P and P_i (Fig. 1B) at concentrations similar to those needed to inhibit the partially purified enzyme in similar conditions [8]. Finally, when raw extract was directly applied to an anion exchange column, 94% of the Fru6P,2kinase which had been applied was recovered with 17-fold enrichment in one band at about 0.29 M NaCl (Fig. 1C). The majority of the Fru2,6P_2-hydrolysing activity (78% of that applied) copurified with Fru6P,2kinase as expected if it represents a specific Fru2,6P_2ase located on the same protein as Fru6P,2kinase (see Introduction, and [8]). These results show that the majority of the Fru2,6P_2 hydrolysis in the standard assay conditions is due tu Fru2,6P_2ase.

The relative activity of these two enzymes in raw extract resembles that previously described in a partially purified preparation where the Fru6P,2kinase: Fru2,6P_2ase ratio in V_{max} conditions was 4 for the partially purified enzyme [9]. In raw extracts in our standard assay conditions, which allow 50% and 14% of the V_{max} activities of Fru6P,2kinase (not shown) and Fru2,6P_2ase (Fig. 1A) respectively, the ratio of Fru6P,2kinase:Fru2,6P_2ase activities is between 7–20, predicting a minimum Fru6P,2kinase: Fru2,6P_2ase ratio of 2–5 in V_{max} conditions.

Removal of low molecular weight components

The changes of Fru6P,2kinase and Fru2,6P_2ase shown in Table I could have been caused by low molecular weight compounds occurring at different levels in the extracts prepared at different times of the day. We therefore investigated whether these changes are still seen after desalting extracts. For this, extracts were centrifuged through G-25 Sephadex (see Methods). As shown in Table II, desalting did not affect the activity of Fru6P,2kinase, but increased the activity of Fru2,6P_2ase. The difference between the activities of Fru2,6P_2ase determined in the light and dark samples was even larger after desalting than in the untreated extracts. Obviously, the changes of Fru6P,2kinase and Fru2,6P_2ase observed in Table I are not due to the direct effect of low molecular weight compounds in the extract. The



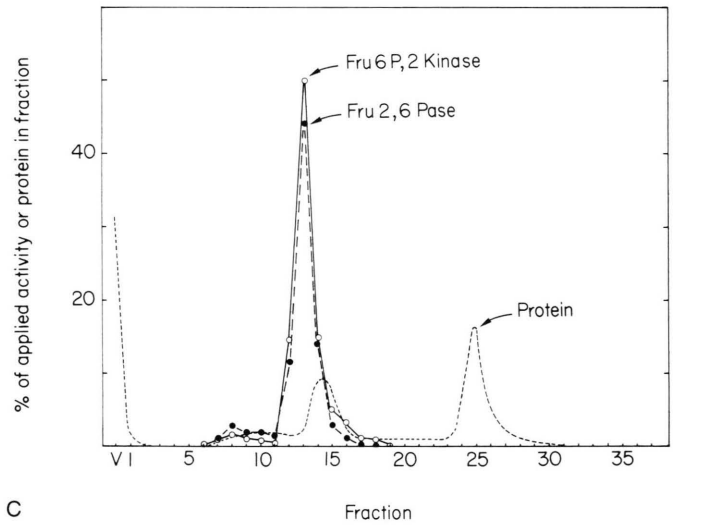


Fig. 1. Properties of Fru2,6P_2ase. (A) Saturation with Fru2,6P_2. Inset shows a double reciprocal plot. (B) Inhibition by Fru6P and P_i in presence of 0.1 μM Fru2,6P_2. (C) Cochromatography of Fru6P,2kinase and Fru2,6P_2ase after FPLC chromatography. Activities were measured in the washout (V) and in fraction 1–36 as the NaCl concentration rose, and the enzymes eluted at 0.29 M NaCl. Recovery of activity in summed fractions (as % of applied) was 97% for Fru6P,2kinase, 87% for Fru2,6P_2ase.

Table II. Effect of desalting on activities of Fru6P,2kinase and Fru2,6P_2ase in extracts from spinach leaves.

	Conditions	Activity [pmol · mg Chl^{-1} · min^{-1}] Untreated	Desalted
Fru6P,2kinase	14 h dark	217 ± 24	214 ± 17
	9 h light	432 ± 31	430 ± 21
Fru2,6P_2ase	14 h dark	25 ± 2	33 ± 6
	9 h light	21 ± 1	26 ± 3

Extracts were prepared from leaves after 9 h light or 14 h dark and analysed directly, or after desalting by centrifugation through sephadex (see Methods). Results are mean ± SE of three separate treatments.

effect of desalting on Fru2,6P_2ase can be explained by removal of P_i, known to inhibit this enzyme (see Fig. 1B), from the extract. Spinach leaves contain enough P_i (5 μmol · mg Chl^{-1}) to give a concentration of 0.2 mM in the assay when extracts are used without desalting. For this reason, extracts were routinely desalted in subsequent experiments.

Stability of activity in the extract

Despite the rapid extraction procedure it was possible that the results of Table I could reflect a differing stability of these enzymes in extracts prepared at different times of the day. We therefore prepared extracts and measured enzyme activities immediately, or after 3 h at 4 °C (Table III). During this time there was no significant change of activity in an extract prepared at the start of the day. However, the activity of Fru6P,2kinase decreased and that of Fru2,6P_2ase increased when an extract from leaves at the end of the day was left on ice. These results make it unlikely that the results of Table I could be an artefact due to differential deactivation of enzymes. Instead, the changes which occur as an extract stands decrease the difference between the extracts. After 3 h, an extract prepared at the end of the day resembles one from the start of the day (see below for discussion).

Diurnal changes of activity

These results suggested that the alterations of Fru6P,2kinase and Fru2,6P_2ase activity are real. We therefore studied how these activities varied throughout a 9 h light/15 h dark cycle (Fig. 2, solid line, open symbols show points taken in the light).

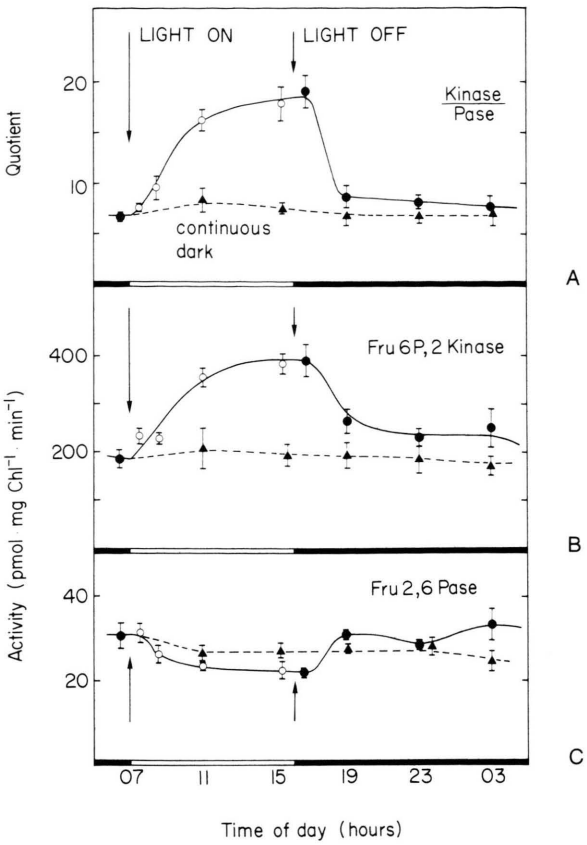


Fig. 2. Alterations of Fru6P,2kinase and Fru2,6P_2ase during the day and night in leaves of spinach plants. (A) Quotient of Fru6P,2kinase : Fru2,6P_2ase. (B) Fru6P,2-kinase activity. (C) Fru2,6P_2ase activity. Results are mean ± SE of three samples, each sample from 2 different plants. The plant were in a normal 9 h light/15 h dark rhythm (solid line, open symbol for samples taken during light) or were transferred into continuous darkness at the start of the experiment (dotted line).

Table III. Alteration of enzyme activity in desalted extracts.

Plant material	Time between extraction and assay	Activity [pmol · mg Chl^{-1} · min^{-1}] Fru6P,2kinase	Fru2,6P_2ase	Quotient $\frac{\text{Kinase}}{\text{Pase}}$
14 h dark	5 min	196 ± 23	33 ± 3	6.2 ± 0.8
	3 h	214 ± 31	32 ± 3	6.9 ± 0.8
9 h light	5 min	341 ± 18	26 ± 3	13.6 ± 2.1
	3 h	264 ± 17	37 ± 3	7.2 ± 0.5

Extracts were prepared from spinach leaves after 9 h light or 14 h dark and assayed (after desalting) immediately or after 3 h at 4 °C. Results are mean ± SE of experiments with 4 separate extracts.

For comparison, some plants were left in dark on the day of the experiment (dotted line). There was no large response after illumination, but there was a gradual increase of the Fru6P,2kinase : $Fru2,6P_2$ase ratio during the day (Fig. 2A). This involved both an increase of Fru6P,2kinase activity (Fig. 2B) and a decrease of $Fru2,6P_2$ase activity (Fig. 2C). After darkening, the enzyme activities did not change immediately. Between 0.5–3 h after darkening, the activities reverted towards those found at the start of the day. None of these changes occurred in plants which were not illuminated. These results suggested that the reciprocal alterations of Fru6P,2kinase and $Fru2,6P_2$ase are not due to an endogenous rhythm, nor an immediate response to illumination, but occur as a consequence of a period of photosynthesis.

On the regulation of $Fru2,6P_2$ in leaves

The significance of these changes is revealed by comparing them with earlier studies of $Fru2,6P_2$ metabolism and carbohydrate synthesis in spinach leaves during the day [4]. Immediately after illumination $Fru2,6P_2$ decreases 2-fold ([10], see also Table I) but the Fru6P,2kinase : $Fru2,6P_2$ase ratio does not alter or even starts to increase. This rapid decrease of $Fru2,6P_2$ can be attributed to a regulation of these enzymes by metabolites. As previously discussed [5, 9], rising levels of compounds like dihydroxyacetone-P and glycerate-3-P inhibit Fru6P,2kinase, and stimulate their own use for synthesis of sucrose. This provides a rapid way of coordinating the rates of sucrose synthesis and photosynthesis [3, 5].

The gradual increase of the Fru6P,2kinase : $Fru2,6P_2$ase ratio resembles the gradual rise of $Fru2,6P_2$ during the day [4] which occurs as sucrose accumulates in the leaf [4, 6]. This increase of $Fru2,6P_2$ is involved in altering the partitioning of photosynthate, so that more starch and less sucrose is made [4, 6]. In earlier studies we suggested that an inhibition of sucrose P synthase [13] led to an increase in the concentration of its substrates [6] which, in turn, led to the increase of $Fru2,6P_2$ because Fru6P activated Fru6P,2kinase and inhibits $Fru2,6P_2$ase [7–9]. The results of Fig. 2 show there is also a more direct control of the $Fru2,6P_2$ level, operating via an unknown mechanism which alters the relative activities of Fru6P,2kinase and $Fru2,6P_2$-ase in desalted extracts.

While protein turnover cannot be excluded, the reciprocal changes of Fru6P,2kinase and $Fru2,6P_2$ase activities, and the preliminary evidence that the changes revert in a crude extract (Table III) suggest that these results may be due to protein modification. The decrease of the Fru6P,2kinase : $Fru2,6P_2$-ase ratio in the absence of ATP cannot be easily explained if this ratio is decreased by phosphorylation as in liver [1]. We are currently investigating whether the control in leaves is analogous to that in yeast [14, 15] where phosphorylation leads to an increased Fru6P,2kinase : $Fru2,6P_2$ase ratio in conditions when more carbohydrate is available.

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