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STUDY OF SOME KINETIC PROPERTIES OF SUCROSE PHOSPHATE SYNTHASE FROM RICE LEAVES

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Abstract

Rice (*Orya sativa* L. cv. IR-30) was grown hydroponically in growth chambers at 28/23°C day/night temperatures with 14-h photoperiod and quantum irradiance of 450 μmol m-2 S-1. Sucrose phosphate synthase (SPS) activity in desalted extracts from 21 days old leaves was measured in saturating and "limiting" (P_i included) substrate conditions. Evidence is presented that SPS activity via UDP estimation underestimates the enzyme activity in rice. Substrate UDP-Glc and Fru-6-P saturation curves in presence of Glc-6-P were hyperbolic with apparent Km(UDP-Glc) of 2.7 mM and S0.5 (Fru-6-P) of 1.2 mM. Glc-6-P activated SPS and apparent K_{act} was 3.2 mM. Diel SPS profiles indicated that rice SPS was activated in light and this activation was more marked under "limiting" assay conditions. P_i inhibition of SPS was dependent upon substrate conditions; being more pronounced in "limiting" conditions. Mannose and P_i feeding experiments indicated that rice SPS was activated by mannose in darkness and the activated enzyme was not sensitive to P_i. In contrast, SPS from leaves fed with P_i was substantially inhibited when P_i was included in the assay medium. The data suggests that rice SPS may exist in different forms. It may also be possible that P insensitive form is more active in light while P_i sensitive in the dark.

Introduction

Sucrose plays a pivotal role in plant growth and development and there is increasing evidence that sucrose (or some metabolite derived from it) may play a non-nutritive role as a regulator of cellular metabolism, possibly by acting at the level of gene expression (Sheen, 1994; Huber & Huber, 1996). The pathway of sucrose synthesis involves a cytoplasmic enzyme, sucrose phosphate synthase (SPS, EC.2.4.1.14)

Fru-6-P + UDP-glucose $\longleftrightarrow$ Sucrose-6-P + UDP (1)
Sucrose-6-P $\longleftrightarrow$ Sucrose + P_i (2)

where SPS catalyzes reaction (1) above and sucrose phosphate phosphatase catalyzes reaction (2). The latter enzyme has little or no regulatory function. In contrast SPS is one of the key enzymes that serves as a major control point in the regulation of sucrose synthesis. Consequently, the activity of SPS allows the plant to balance the photosynthetic carbon partitioning between starch and sucrose (Huber *et at.*, 1984; Stitt *et al.*, 1987).

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There are reports where SPS has been isolated and partially purified from wheat germ (Salerno & Pontis, 1978), spinach (Amir & Preiss, 1982; Doehlert & Huber, 1983a; Stitt *et al.*, 1988; Salvucci *et al.*, 1990), maize (Kalt-Torres *et al.*, 1987; Huber *et al.*, 1989a) and soybean (Kerr & Huber, 1987). SPS activity in barley (Sicher & Kremer, 1984), *Lolium temulentum* (Pollock & Housley, 1985) and maize (Lunn & Hatch, 1997) changes rapidly with light/dark transitions suggesting light modulation. In other species like soybean there is a pronounced endogenous rhythm in SPS activity which is independent of light/dark changes (Rufty *et al.*, 1983). Such changes are considered an important mechanism that adjusts the capacity of sucrose biosynthetic pathway to substrate availability (Huber & Huber, 1991).

In spinach Glc-6-P activates SPS by increasing V_{max} and decreasing the K_m(Fru-6-P). Such an activation was also observed in maize but with a decrease in affinity for UDP-Glc rather than Fru-6-P (Doehlert & Huber, 1983a and 1983b). There are conflicting reports concerning SPS substrate saturating profiles and differences among species also exist with regard to P_i inhibition of the enzyme (Stitt *et al.*, 1987; Crafts-Brander & Saluucci, 1989). It is hypothesized that changes in maximum extractable activity level of SPS are due, in part, to changes in its kinetic properties. Alterations in the kinetic properties may not be observed when assays are conducted (as is typically done) with saturating substrate concentrations. Therefore, characterization of SPS under "limiting conditions" of substrate UDP-Glc and Fru-6-P and in the presence of activator Glc-6-P and inhibitor P_i (to simulate *in vivo* conditions) might better reflect how changes in substrate availability *in situ* might affect regulatory property of SPS. The objectives of the present study were (i) to establish the best assay method for rice SPS, (ii) to evaluate substrate saturation profiles especially under limiting-substrate conditions, which could possibly reflect *in vivo* regulation, and, (iii) to assess mannose activation and the P_i inhibition patterns to help understand the biochemical basis for possible light/dark changes in rice SPS activity.

Materials and Methods

Plant Material: Rice (*Oryza sativa* L. cv. IR-30 and cv. Gulfmont) seeds were soaked in aerated water overnight and then transferred to a moist filter paper in a Petri dish for germination at 28°C in the dark for 24-h. Subsequently, sprouting seeds were planted on nylon nets mounted on large jars containing water which was replaced by nutrient solution after two days (Yoshida *et al.*, 1972). The nutrient solution was renewed on alternate days and the pH adjusted daily to 5.0. The plants were maintained in a growth chamber at ambient [CO_2], with 28/23°C day/night air temperatures, and quantum irradiance of 450 μmol m-2 s-1 with incandescent and fluorescent bulbs with a 14-h photoperiod.

At 21 days after planting, and in the middle of the light period, 2 fully expanded leaves from 50 plants were rapidly excised at the base and immersed in liquid N_2 for SPS kinetic studies. Leaf samples (2 fully expanded leaves from 10 plants) were also frozen in liquid N_2 every 3 hours, over a 24-h period, to determine diel SPS activity. For each sampling procedure all the leaves were pooled. Unless otherwise indicated, the cultivar Gulfmont was used in some experiments for a comparative study of UDP

and resorcinol methods of SPS activity with the IR-30 cultivar. Such a study was essential because the assay conditions optimized for SPS activity in different cultivars differ. Kinetic studies were not conducted on Gulfmont. The results are presented as the means ± SE of three to four replications, with each replication representing one grind of material from the pooled sample. Error bars were omitted in the Figures when they were smaller than the symbols.

SPS Extraction: Extracts were prepared by grinding the leaf tissue in a precooled mortar using a 1:7 tissue-to-buffer ratio in a medium containing 50 mM Hepes (N-[hydroxyethyl] piperazine-N'- [2-ethanesulfonic acid]), 4 mM $MgCl_2$, 1% PVP-40 (polyvinyl pyrrolidone, 1 mM EDTA (ethylenediamine tetraacetic acid sodium salt), 0.01% Trition X-100, 1 mM PMSF (phenylmethly sulfony1 fluoride), 1 mM Na_2MoO_4, 10 μM leupeptin and 5 mM DTT (dithiothreitol) at pH 7.5. The extract was centrifuged at 12,000g for 90 s and the supernatant was immediately desalted on a Sephadex G-25 column equilibrated with extraction buffer at 4°C. This crude extract was then used for subsequent kinetic analyses in order to reflect as closely as possible the *in vivo* state of the enzyme.

SPS Assay: UPD method. SPS activity was estimated by the Fru-6-P dependent release of UPD as described by Stitt *et al.*, (1988).

Resorcinol method. Modified method of Huber *et al.*, (1985) was used to determine SPS activity. SPS was assayed with (a) saturating concentrations of substrates and activators, and (b) limiting substrates together with Glc-6-P as activator and P_i as inhibitor (to simulate physiological components and concentrations). For the saturating assay, 100 μL of desalted enzyme extract was incubated with 12 mM UDP-Glc, 4 mM Fru-6-P, 20 mM Glc-6-P, 50 mM Hepes-NaOH, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_2MoO_4, 5 mM DTT at pH 7.5 in a final volume of 200 μL. Under limiting conditions, enzyme extracts were assayed in the presence of 2 mM UDP-Glc, 2 mM Fru-6-P, 10 mM Glc-6-P and 10 mM P_i, otherwise the assay was identical to that used for the saturating condition. For kinetic studies, varied concentrations of substrate UDP-Glc and Frc-6-P and activator Glc-6-P were used. In both limiting and saturating assays the enzyme was incubated at 30°C and the reaction was terminated after 15 min by the addition of 150 μL of 1.2 N NaOH. Unreacted Fru-6-P was degraded by placing the tubes in boiling water for 10 min. In order to measure the sucrose and sucrose-P produced, after cooling, 0.25 mL of 0.1% resorcinol in 95% ethanol and 0.75 mL of HCL (9N) were added, and the tubes incubated at 80°C for 8 min (Roe, 1934). Blanks for saturating and limiting conditions as well as for kinetic studies contained matching concentrations of substrates (UDP-Glc and Fru-6-P) and effectors, as well as the killed enzyme. Blanks and test vials were treated simultaneously. After cooling, the absorbance at 520 nm was measured for both blank and experimental vials against water. The OD for the test vials was corrected by subtraction of the blank values. The sucrose formed was quantitated by comparison to a sucrose standard curve.

K_m, K_{act} and V_{max} values were determined from Lineweaver-Burk plot, while $S_{(0.5)}$ and $IC_{(50)}$ values were calculated from graphic plots of the data. Saturation curves of substrates or activator (in limiting or saturating concentrations) when did not follow Michaelis kinetics, the V_{max} was calculated from graphic plot of the data. Total soluble protein in aliquots of the extract was determined with the dye binding method

(Bradford, 1976) using gamma-globulin as the standard.

Mannose and Pi Treatment: Intact leaves from plants 21 DAP were excised at their base after 4-h illumination, and rapidly placed in a cuvette containing a 3 mL solution of 3 mM mannose or 50 mM P_i. They were allowed to transpire in a continuous stream of air for 1-h at 28°C and quantum irradiance of 550 μmol m-2 s-1. Intact leaves were also allowed to transpire in darkness for 6-h in 3 mM mannose or 50 mM P_i at 23°C. In a similar experiment, 35 to 40 rice leaf discs (0.75 cm diameter) from 21-day old plants were cut after 2-h darkness and floated on 200 mM mannose or 50 mM P_i solution for 8-h in the dark. They were then quickly rinsed and blotted dry prior to freezing in liquid N_2. The excised leaves and discs were then extracted and assayed for SPS activity.

Results and Discussion

The results from the methodological investigation indicate that UDP estimation is not a reliable criterion for measuring SPS activity in rice. When the reaction rates were measured via UDP formation at high levels of Fru-6-P, UDP-Glc and Glc-6-P, rice cultivars, IR-30 and Gulfmont, not only differed in maximum extractable activity, but the rates were appreciably lower as compared to when sucrose production was estimated by the resorcinol method (Table 1). When representative amounts of UDP were added at the beginning of incubation period, the recovery ranged from 12 to 18% and when UDP was added after termination of SPS assay, the recovery was around 80% (Table 2). In contrast, sucrose recovery measured by the resorcinol method did not vary regardless of whether sucrose was added at the start or the end of incubation, and recovery ranged from 82 to 100%. It is possible that extracts from rice cultivars may contain a phosphatase that degrades UDP. However, this needs further investigation. Results obtained by Crafts-Brander & Salvucci (1989) are in accord with these findings for rice. They evaluated the conventional SPS assay methods based on UDP or sucrose formation for different species and reported 100% UDP recovery for spinach and maize, whereas, for wheat and soyben nearly all the UDP was depleted from the assay medium. In tobacco, loss of UDP was variable. Consequently, these findings together with the rice data suggest that the UDP method may greatly

Table 1. Comparison of UDP and resorcinol methods for measuring SPS activity in rice cultivars.

Method	SPS activity (nmol mg^{-1} prot. min^{-1})	
	IR-30	Gulfmont
UDP	4.7 ± 0.9	17.6 ± 1.1
Resorcinol	23.9 ± 1.7	31.3 ± 1.0

SPS was assayed with 12 mM UDP-Glc, 4 mM Fru-6-P, and 20 mM Glc-6-P at 3o°C and pH 7.5. Results are the mean ± SE of six replications.

Table 2. Percent recovery of UDP and sucrose in the SPS assay using rice cultivar IR-30.

SPS Assay	Time of addition	Product added	Concentrations (mM)	Recovery (%)
UDP method	Start of reaction	UDP	2	12.0 ± 2.2
			4	16.0 ± 1.1
			6	18.0 ± 1.4
	After terminating	UDP	2	83.2 ± 0.9
			4	74.8 ± 2.7
			6	76.1 ± 2.3
			8	81.8 ± 1.3
Resor-cinol method	Start of reaction	Sucrose	2	82.3 ± 4.2
			4	98.0 ± 3.8
			6	101.7 ± 1.8
	After terminating reaction	Sucrose	2	89.1 ± 3.7
			4	95.0 ± 4.8
			6	98.6 ± 6.0

Desalted extracts were assayed with 12 mM UDP-Glc, 4 mM Fru-6-P, nad 20 mM Glc-6-P at 30°C and pH 7.5 and known concentrations of UDP or sucrose added (i) at the start of the reaction, (ii) after the termination of the reaction. Data are the means ± SE of four to five replications.

underestimate the SPS activity in some species, and its use should be carefully considered.

Sucrose formation: Preliminary investigation of sucrose formation via SPS catalyzed reaction, as a function of time, was essential to demonstrate that the enzyme activity does not alter during assays. When assayed via resorcinol method, except for first 2-3 minutes, sucrose accumulation was linear (r^2 = .98) over 30 min time period (Fig.1). This indicates that there is some delay before sucrose accumulation commences. Stitt *et al.*, (1988) using resorcinol method, also reported a similar lag of 3-4 minutes before a linear accumulation of sucrose began and in contrast SPS activity detected via UDP formation was linear with time. The authors suggested that the initial delay of sucrose accumulation was not the consequence of non-linearity of reaction catalyzed by SPS rather it could be due to a delay before sucrose starts to accumulate, perhaps because an adequate pool of sucrose must first be built-up.

Substrate saturation kinetics: Substrate kinetic studies were performed with desalted crude extracts prepared from 21 day-old rice leaves. When UDP-Glc was the variable substrate, the response was hyperbolic (Fig.2). The apparent K_m(UDP-Glc) was 2.7 mM. In the absence of the putative activator Glc-6-P, and in the presence of a high concentration (15 mM) of the second substrate Fru-6-P, the UDP-Glc response curve

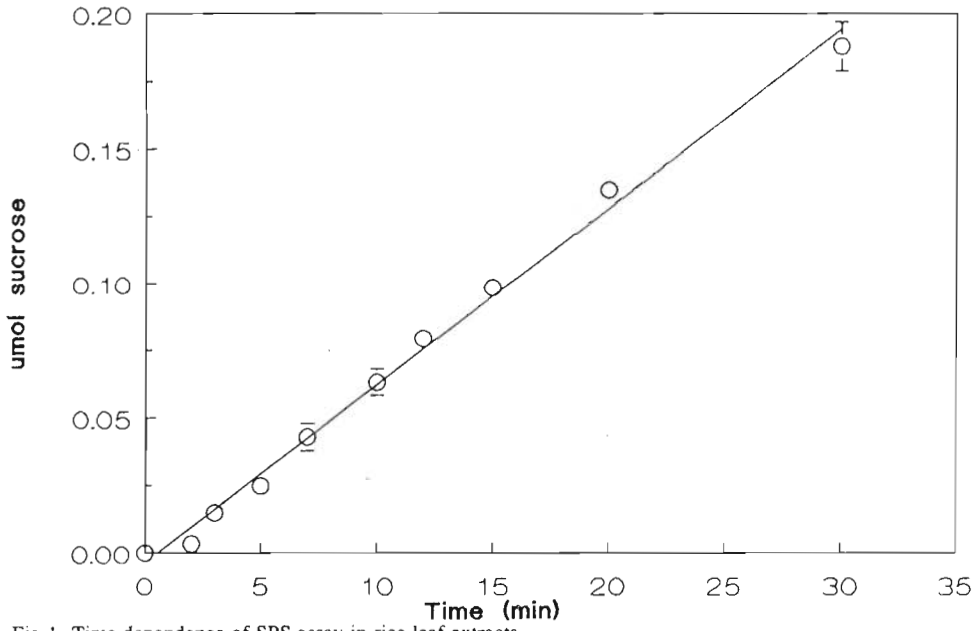

Fig.1. Time dependence of SPS assay in rice leaf extracts.
At different time intervals, aliquots were removed from an assay of SPS and alkalinized to terminate the reaction and assayed for sucrose. The results are the mean ± SE of four separate measurements, the error bars being omitted when they are smallar than the symbol.

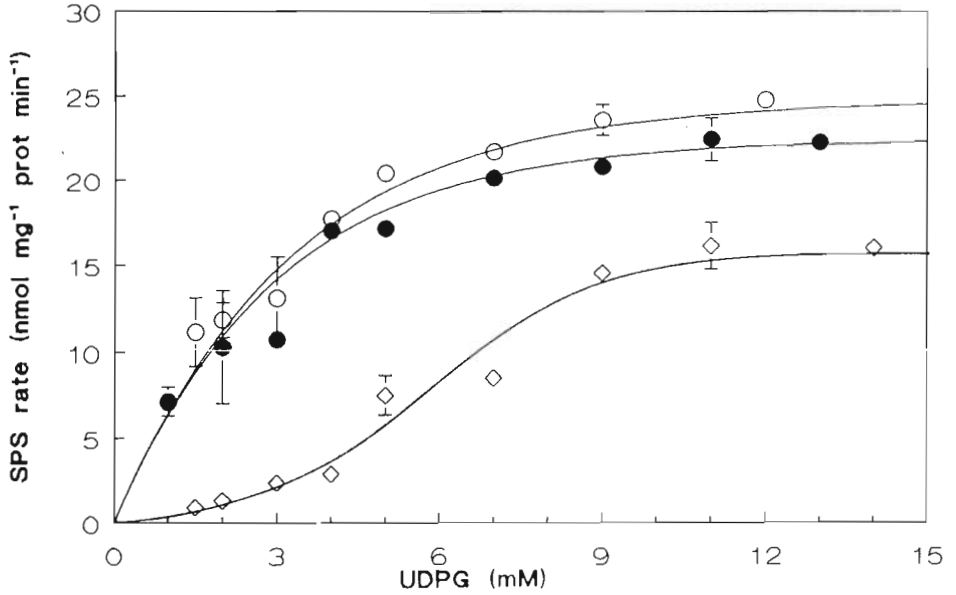

Fig.2. Response of rice SPS activity to varying concentrations of UDP-Glc substrate.
Desalted extracts of enzyme from 21 days old leaves were assayed at pH 7.5 and 30°C with: 4 mM Fru-6-P and 20 mM Glc-6-P (solid circles); 15 mM Fru-6-P and in the absence of Glc-6-P (open diamonds). The points are means ± SE of three determinations.

was essentially the same (Fig.2) and apparent K_m(UDP-Glc) was 2.3 mM. It appears that in rice, saturating concentrations of substrate Fru-6-P eliminate the need for Glc-6-P as an activator of the SPS reaction.

The UDP-Glc response curve under limiting substrate (Fru-6-P) and activator (Glc-6-P) concentrations, and in the presence of 10 mM P_i was sigmoidal (Fig.2) and did not follow Michaelis-Menten kinetics. The V_{max} was decreased by 26% and the UDP-Glc concentration required for half maximum velocity ($S_{0.5}$) was 8 mM. The sigmoidal curve for UDP-Glc may be due to the existance of various forms of SPS, not all of which exhibit Michaelis kinetics (Salerno & Pontis, 1978). Sub-saturating concentrations of Fru-6-P, Glc-6-P as well as the presence of P_i are more reflective of physiological conditions than when saturating amounts are used. The lower SPS activity under sub-saturating conditions probably more closely depict the *in vivo* activity.

The response profiles of Fru-6-P from rice leaf SPS activity differ markedly in the presence or absence of activator Glc-6-P. In the presence of 20 mM Glc-6-P (UDP-Glc held at 12 mM) the response was strictly hyperbolic (Fig.3) but did not follow Michaelis-Menten kinetics. The $S_{0.5}$ (Fru-6-P) was 1.2 mM. In the absence of Glc-6-P a sigmoidal pattern was observed and $S_{0.5}$(Fur-6-P) increased to 7 mM. These findings confirm that Glc-6-P is an activator of rice SPS, with its major effect being a 5.8-fold decrease in the $S_{0.5}$(Fru-6-P) value, and to a lesser extent an increase in V_{max}. This is consistent with the findings of Loewe *et al.*, (1996).

To further characterize the Fru-6-P response kinetics, Fru-6-P and Glc-6-P concentrations were varied while maintaining a 1:5 ratio, which is close to their thermodynamic equilibrium in the cytosol (Ap-Rees 1980). At a low concentration of Fru-6-P (below 1 mM), the SPS rate was low, and produced a sigmoidal response (Fig.4). However, as the Fru-6-P concentration was increased (with a concomitant increase in Glc-6-P) the curve assumed a hyperbolic function, and the V_{max} was attained at 4 mM Fru-6-P. The $S_{0.5}$(Fru-6-P) value was 2 mM.

The sigmoidal nature of the Fru-6-P curve in the present study, using crude enzyme preparations, is consistent with the results obtained for the partially purified, phosphoglucose isomerase free enzyme isolated from wheat germ (Preiss & Greenberg 1969), barley and rape (Murata, 1972), and spinach leaves (Amir & Preiss, 1982). Therefore, it appears that the sigmoidal response to Fru-6-P concentration is an inherent property of SPS possibly due to the presence of multiple and interactng sites for Fru-6-P (Amir & Preiss, 1982), which may have some physiological significance *in vivo*.

The Fru-6-P response curve under more limiting substrate and effector concentrations (2 mM UDP-Glc, 10 mM Glc-6-P) and in the presence of 10 mM P_i was hyperbolic (Fig.4) but did not follow Michaelis-Menten kinetics. The $S_{0.5}$(Fru-6-P) increased by 2-fold and V_{max} decreased by 36% when compared with the response under saturating conditions respectively, suggesting that low concentrations of metabolites could exert a fine control on SPS activity.

Estimated cytosolic concentrations of UDP-Glc and Fru-6-P (spinach leaf) are in the range of 1.4 - 2.3 mM and 1.4 - 3.0 mM, respectively, (Gerhardt *et al.*, 1987). Thus, the concentrations of UDP-Glc and Fru-6-P in the cytoplasm are close to the K_m and $S_{(0.5)}$ values reported in the present study. Consequently, the SPS reaction rate might be expected to be a linear function with respect to UDP-Glc and Fru-6-P

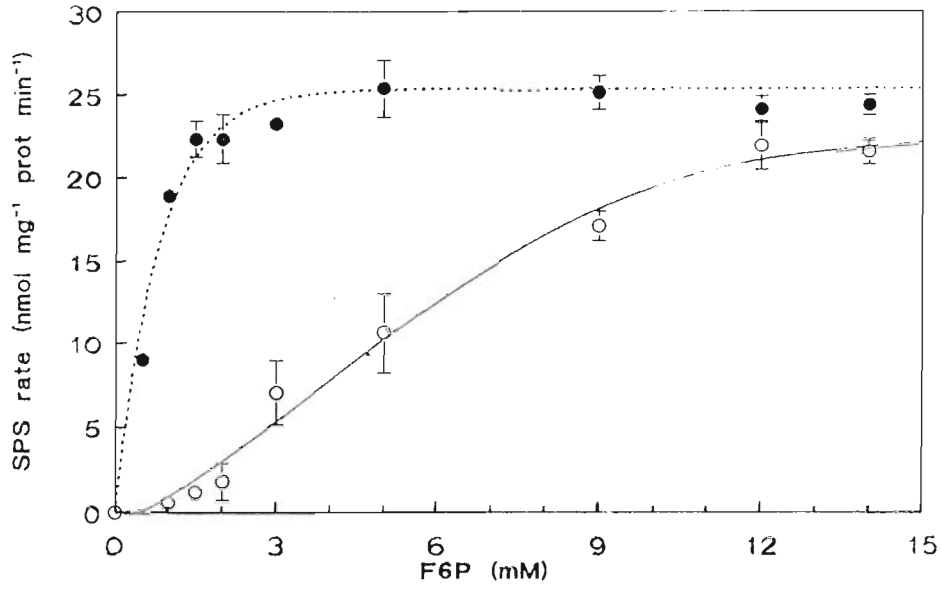

Fig.3. Response of rice SPS activity to varying concentrations of Fru-6-P substrate.
Desalted extracts of enzyme from 21 days old leaves were assayed at pH 7.5 and 30°C with: 12 mM UDP-Glc and 20 mM Glc-6-P (solid circles); and 12 mM UDP-Glc and in the absence of Glc-6-P (open circles). The points are means ± SE of three determinations.

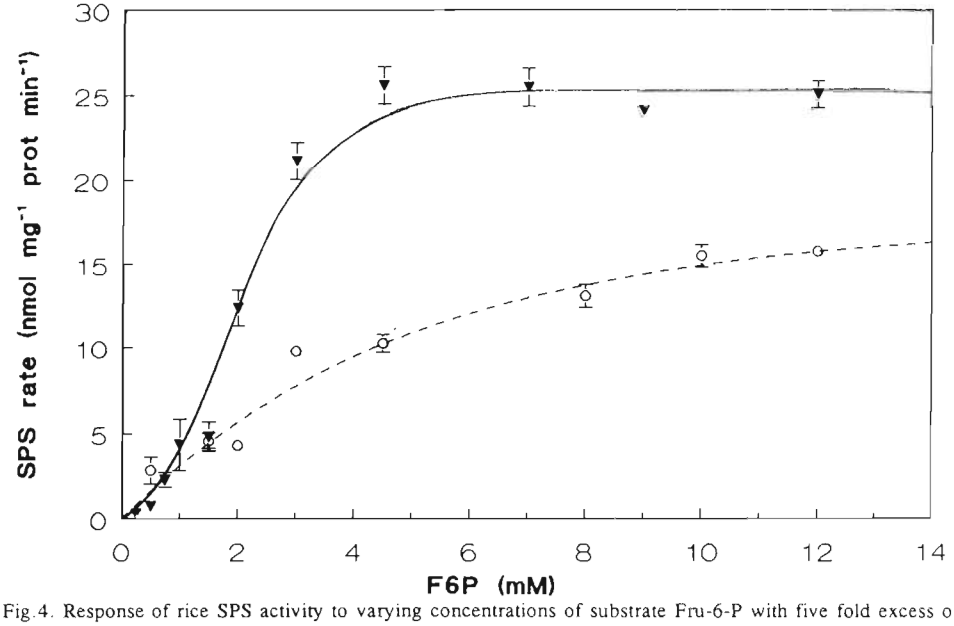

Fig.4. Response of rice SPS activity to varying concentrations of substrate Fru-6-P with five fold excess of Glc-6-P.
Desalted extracts of enzyme from 21 days old leaves were assayed at pH 7.5 and 30°C with: 12 mM UDP-Glc and Fru-6-P to Glc-6-P ration of 1:5 (solid triangles); and 2 mM UDP-Glc, 10 mM Glc-6-P and 10 mM P_i (open circles). The points are means ± SE of three determinations.

concentrations in the cytoplasm. The rising concentration of these metabolites could modulate SPS activity to divert more photosynthate into sucrose than starch (Galtier *et al.*, 1993).

The Glc-6-P response curve was hyperbolic in the presence of 12 mM UDP-Glc and 4 mM Fru-6-P (Fig.5) and apparent K_{act}(Glc-6-P) was 3.2 mM. The inclusion of the P_i with limiting Fru-6-P and UDP-Glc concentrations suggested that P_i antagonized Glc-6-P activation and altered the shape of Glc-6-P response from hyperbolic to sigmoidal (Fig.5). Therefore, it is intutively obvious that in rice SPS activity is also a function of Glc-6-P:P_i ratio, and that the ratio act as a control mechanism of enzyme activity *in vivo*. This sensitive regulation of SPS activity has important implications in allowing sucrose synthesis to be coordinated with other aspects of cell metabolism (Still *et al.*, 1987; Lunn & Hatch, 1997).

P_i Inhibition: From the above results it is apparent that the presence of P_i in the assay medium, especially under limiting substrate and effector concentrations, modifies the shape of the curve. Consequently experiments were conducted to study the sensitivity of SPS activity to P_i. In the presence of increasing amount of P_i, SPS activity decreased, even under saturating conditions of substrates and activator. The degree of inhibition by P_i was progressively increased as the UDP-Glc was decreased from 4 to 2 mM (Fig.6), suggesting that P_i may possibly be a competitive inhibitor with respect to UDP-Glc. $IC_{(50)}$ under saturating and limiting conditions occurred at 24 mM and 10 mM P_i concentrations respectively. These results indicate that the degree of P_i inhibition changes with the substrate concentration. The effect of P_i on SPS activity also depends on prior incubation of the leaves in the light or dark, the assay method, and the source of the enzyme (Crafts-Brandner & Salvucci, 1989). For example, P_i strongly inhibited SPS activity in spinach (Still *et al.*, 1988) and wheat (Salerno & Pontis, 1978) but the effect was much less in soybean and tobacco (Crafts-Brandner & Salvucci, 1989).

Diel SPS activity: Changes in SPS activity from leaves incubated in the light and dark, when measured under saturating conditions indicated that SPS activity increased by 31% in light and reached a maximum after 4-h irradiance (Fig.7). In the dark, activity dropped, and this was followed by a slight recovery. In order to simulate the *in vivo* response of SPS to light/dark transitions, limiting substrate and effector concentrations were used for measurement. In the dark the enzyme activity decreased with the minimum activity observed at 0300 h in the middle of the dark period, at which point the activity was 58% less than in the light. Subsequently, in the light activity recovered and essentially remained unchanged throughout the light period. These findings indicate that in rice the SPS activity undergoes diel fluctuations. It seems that rice belongs to the Group I classification of Huber *et al.*, (1989b), as species falling into this grouping are those where light activation involves an increase in the V_{max} of the enzyme. Light does not affect SPS activity directly. There is now compelling evidence that light modulation of SPS activity involves protein phosphorylation (Huber and Huber, 1990; Jones and Ort, 1997). These workers suggested that illumination of the leaves results in dephosphorylation of SPS and thus its activation. Conversely, darkening of the leaves results in phosphorylation (inactivation). The signals responsible for diel changes in rice SPS activity are not fully known at the present. The results of the present study indicate that the sensitivity to P_i could be one factor. These findings are consistent with the work

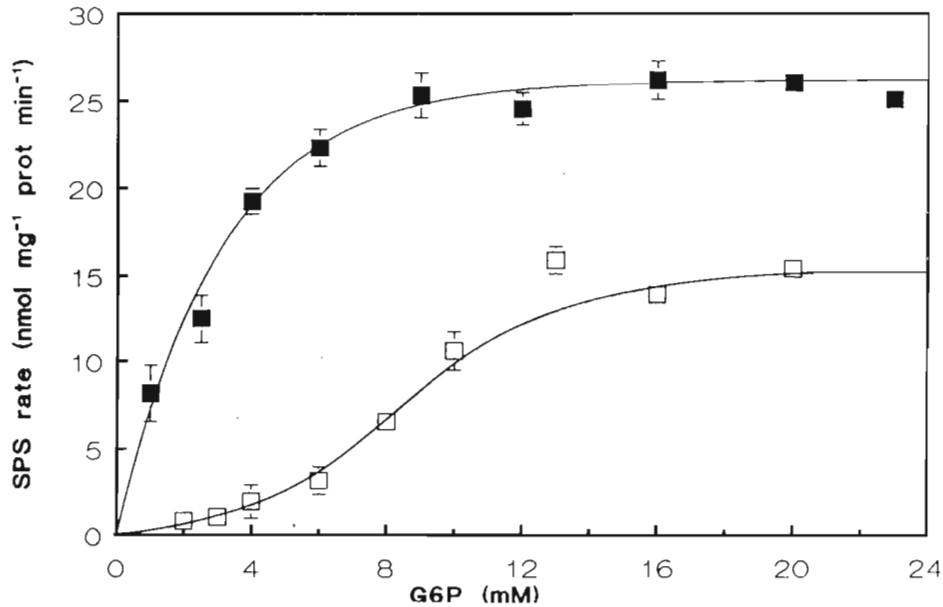

Fig.5. Resposne of rice SPS activity to varying concentrations of Glc-6-P activator.
Desalted extracts of enzyme from 21 days old leaves were assayed at pH 7.5 and 30°C with: 12 mM UDP-Glc and 4 mM Fru-6-P (solid Squares); and 2 mM UDP-Glc, 2 mM Fru-6-P and 10 mM P_i (open squares). The points are means ± SE of three determinations.

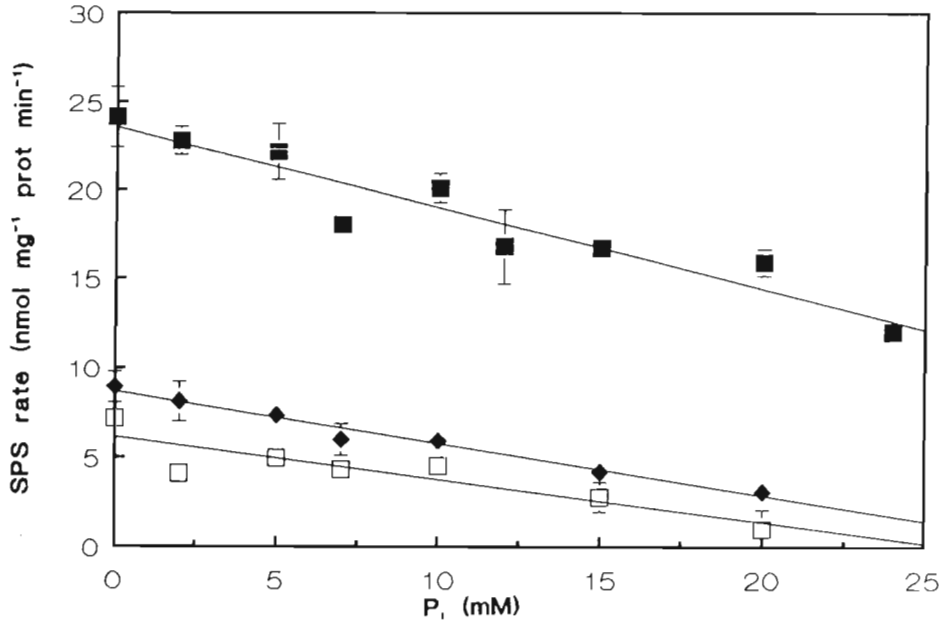

Fig.6. Inhibition of rice SPS activity by inorganic phosphate (P_i).
The activity of SPS in desalted extracts of 21 days old leaves was assayed at pH 7.5 and 30°C under: saturating conditions of 12 mM UDP-Glc, 4 mM Fru-6-P and 20 mM Glc-6-P (solid squares); and limiting conditions of either 4 mM UDP-Glc, 2 mM Fru-6-P and 10 mM Glc-6-P (solid diamonds), or 2 mM UDP-Glc, 2 mM Fru-6-P and 10 mM Glc-6-P (open sauares). The values are mans ± SE of three determinations.

of Stitt *et al.*, (1988) who showed that the diurnal rhythms in spinach SPS activity were due to changes in kinetic properties which involved change in sensitivity to inhibition by P_i.

Mannose and Pi Pre-treatment: In order to understand the *in vivo* SPS kinetics in rice, the effect of mannose and P_i pre-treatment of the leaves on the enzyme activity was investigated. Mannose treatment of the intact leaves in the light, to sequester the cytosolic P_i, activated SPS by 2.6-fold when measured under limiting substrate and effector concentrations (Table 3). Conversely, P_i treatment induced a 25% decrease in activity. In darkened tissue a 4.1-fold activation by mannose, and a 57% decrease by P_i was recorded. Mannose and P_i treatment also influenced the light:dark SPS activity ratio. The ligh:dark ratio was low (1.9) when leaves were treated with mannose and high (5.3) for P_i-fed leaves. Under saturating conditions, SPS activation and inhibition by Mannose and P_i, respectively, were less pronounced and there was essentially no difference in the light:dark ratios.

This comparison of SPS activity in light and dark with mannose or P_i pre-treatment further indicated that the enzyme activity when measured under limiting substrate and effector concentrations, was considerably lower in dark-treated leaves. The differences

Table 3. Changes in SPS activity in rice leaf material pretreated with manose or P_i.

Plant material	Pre-treatment	Light	Dark	Light; dark SPS activity ratio.
		SPS rate (nmol mg^{-1} prot. min^{-1})		
		Saturating substrate concentration		
Intaact	3 mM mannose	32.8 ± 0.2	26.6 ± 0.4	1.2
leaves	50 mM P_i	20.8 ± 0.3	15.9 ± 0.3	1.3
	Control	28.0 ± 0.2	16.2 ± 0.1	1.7
Leaf	200 mM mannose		24.5 ± 0.4	
discs	50 mM P_i		9.3 ± 0.1	
	Control		14.1 ± 0.2	
		Limiting substrate concentration		
Intact	3 mM mannose	18.1 ± 1.0	9.5 ± 0.1	1.9
leaves	50 mM P_i	5.3 ± 0.6	1.0 ± 0.1	5.3
	Control	6.9 ± 0.1	2.3 ± 0.2	3.0
Leaf	200 mM mannose		11.2 ± 0.1	1.9
discs	50 mM P_i		1.1 ± 0.1	5.3
	Control		2.8 ± 0.3	3.0

Data of leaf discs in light not available.

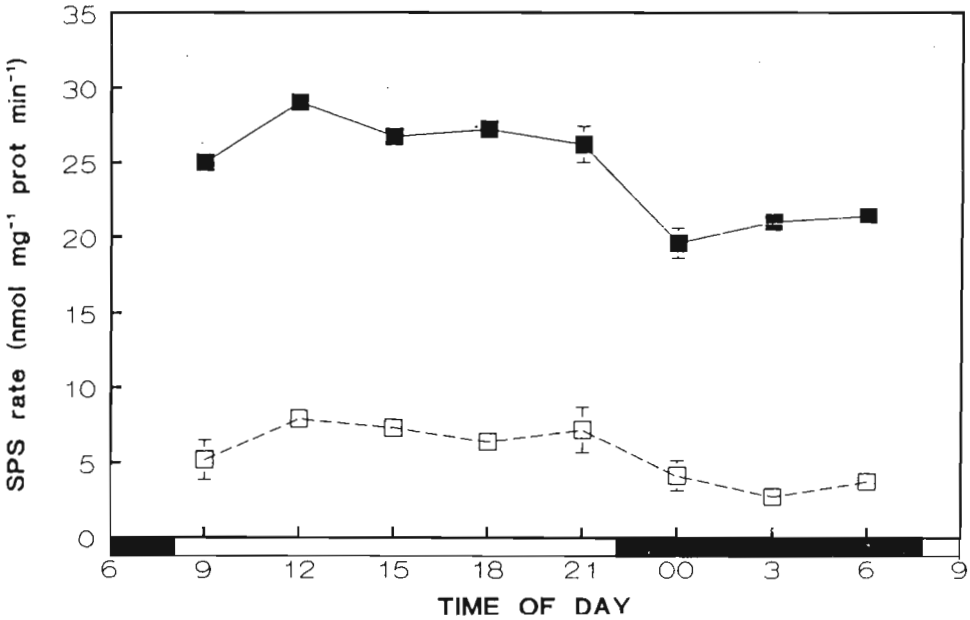

Fig.7. Diel SPS activity in rice leaves grown in a 14 h light/10 h dark cycle at 21 days after planting.
SPS in desalted extracts was assayed at pH 7.5 and 30°C in two conditions: saturating concnetrations of 12 mM UDP-Glc, 4 mM Fru-6-P and 20 mM Glc-6-P (solid squares); and limiting concentrations of 2 mM UDP-Glc, 2 mM Fru-6-P, 10 mM Glc-6-P(Open sauares). The results are given as the mean ± SE of three independent samples, the error bars being omitted when they are smaller than the symbol.

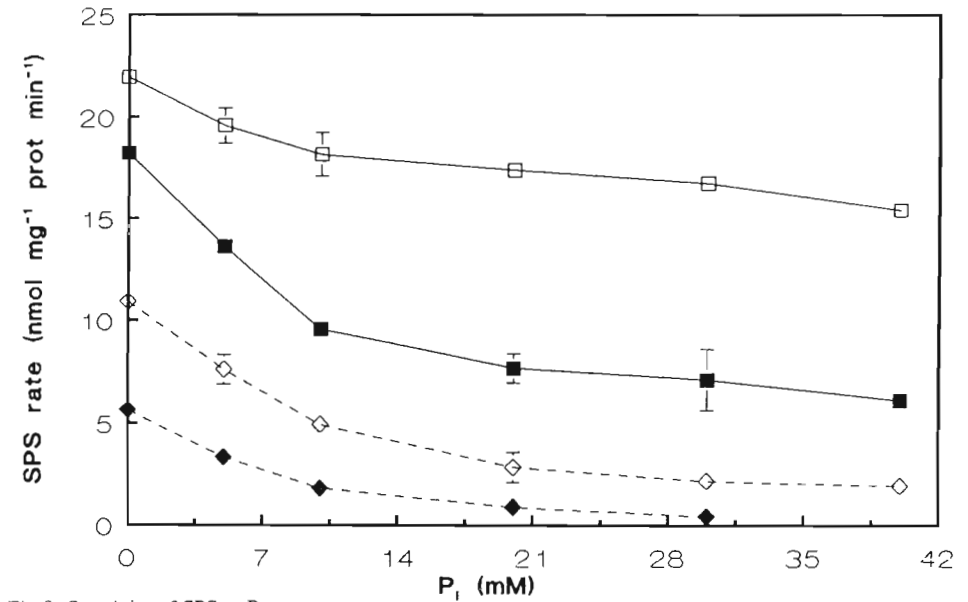

Fig.8. Senstivity of SPS to P_i.
SPS was extracted from rice leaves 21 days after planting which were pre-treated with mannose in light (open squares) or dark (solid squares), and P_i in light (open diamonds) or dark (solid diamonds). SPS was assayed with 2 mM UDP-Glc, 2 mM Fru-6-P, 10 mM Glc-6-P and with different concentrations of P_i at pH 7.5 and 30°C. The results are given as the mean ±SE of three independent replications.

were less apparent under saturating assay conditions. In a similar experiment, pre-treatment of leaf discs, instead of intact leaves, with mannose and P_i in the dark produced similar results to the intact leaves.

These results led to the consideration as to whether the variations in SPS activity, as a result of mannose and P_i treatment, were due to changes in the amount of active enzyme or to changes in the kinetic properties. Consequently, it was of interest to examine the inhibition pattern of SPS activity in the mannose and P_i treated leaves. SPS activity from the leaves pre-treated with P_i in the dark decreased dramatically in response to P_i in the assay medium (Fig.8), and was almost completely inhibited at 20 mM P_i. In contrast to this result, mannose-fed leaves in the light were only 18% inhibited by the same concentration of P_i. Of interest were also the results of dark-fed mannose and light-fed P_i leaves. The latter resulted in far more down-regulation of SPS activity across the 0 to 40 mM P_i concentration range in the assay medium. The changes in SPS sensitivity to P_i, found in the extracts of treated leaves were stable since they were retained after desalting through G-25 Sephadex column and probably reflect alterations in the kinetic properties of the enzyme.

The results of the present study suggest that rice SPS exists in different kinetic forms with different P_i sensitivities. The data indicate that the P_i-sensitive form is more active in the dark, which could explain the decrease in SPS activity upon darkening. Stitt *et al.*, (1988) proposed that interconversion of the different kinetic forms was the major reason for diel changes in SPS activity seen in spinach. In the present study it was not investigated whether the changes of SPS activity to P_i-sensitivity also involved alterations in the substrate affinity, which would provide further evidence for the inter-conversion of SPS forms in light and dark. To confirm this hypothesis for rice SPS would require isolation of the two forms of the enzyme.

The kinetic data indicate that rice SPS is an allosteric enzyme which is regulated *in vivo* by fluctuations in the levels of effectors such as Glc-6-P, an activator, and P_i, an inhibitor (metabolic fine control). Moreover, light/dark changes in SPS activity which apparently involve different kinetic forms that exhibit different P_i sensitivities, suggest a coarse control of regulation, possibly mediated through protein modification.

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