ADPG formation by the ADP-specific cleavage of sucrose-reassessment of sucrose synthase

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The standardized enzyme coupling method for assaying sucrose synthase activities in the direction of sucrose cleavage was reexamined using enzyme preparations from cultured cells of sycamore (*Acer pseudoplatanus* L) and spinach leaves (*Spinacea oleracea*) Both ATP and Tris, commonly utilized in assay systems to measure sucrose synthase, were found to inhibit non-competitively the ADPG-synthesizing activities of the enzyme Upon substituting ATP by either GTP or UTP, and Tris by HEPES, we found that the sucrose synthase is capable of producing ADPG effectively, recognizing ADP as the principal substrate (K_m=5 3 μM (sycamore) and 16 8 μM (spinach)) The V_{max} value for the synthesis of ADPG clearly surpasses the V_{max} observed for the synthesis of UDPG by the enzyme It was found that UDP is not inhibitory on the synthesis of ADPG by SS, which behaves allosterically with respect to the concentration level of sucrose

ADP-glucose, Starch biosynthesis, Sucrose metabolism, Sucrose synthase, UDP-glucose

1 INTRODUCTION

The mechanism of sucrose–starch transformation, central to the partitioning of C-compounds in crop plants, is one of the classical themes of plant biochemistry We have recently reported the existence of an adenylate translocator in the envelope membranes of plastids, e.g amyloplasts [1,2] and chloroplasts [3], which is engaged in the direct import of ADPG from the cytosol and is subsequently coupled to starch biosynthesis The important missing link which must be clarified in this proposed mechanism is how ADPG is produced in the cytosol There have been reported numerous investigations concerning the role of ADP in the sucrose breakdown by SS (UDPG· D-fructose 2-glucose transferase, EC 2.4.1.13) in various plant cells [4], and we have thus assigned this enzymatic route as the principal mechanism operating therein [1–3] However, it is generally believed that SS almost exclusively uses UDP producing UDPG [5–9] Indeed, we have proposed a pathway of sucrose utilization in liquid-cultured cells of sycamore (*Acer pseudoplatanus*) growing on sucrose as a carbon source, in which SS producing UDPG occupies a predominant role rather than the classic hydrolytic invertase pathway [10] In contrast, based on the

kinetic analysis of ADPG/UDPG synthesis by SS, Silvius and Snyder [11] suggested that there may exist mechanisms of compartmentation and separation in plant cells which probably regulate sucrose cleavage In view of the crucial role of ADPG as the source of starch formation in plant cells, we have reassessed the ADPG synthesizing capacities of SS, by modifying the commonly used HK/PGI/G6PDH coupling method [5,10,12–14] We have now found that there exists an efficient machinery producing ADPG by sucrose synthase which likely may constitute an essential component of sucrose–starch transformation

2 EXPERIMENTAL

2 1 *Plant material and enzyme preparations*

In the present investigation, spinach (*Spinacia oleracea*) and cultured cells of sycamore (*Acer pseudoplatanus*) were used Protoplasts of cultured cells of sycamore and filtrates from freshly harvested spinach leaves were obtained as previously described [2,3] Spinach leaf extracts were prepared employing the method developed for sugar beet leaves [15] Desalted cell extracts of sycamore were obtained by dialysis of disrupted protoplasts against 10 mM HEPES (pH 7 0) and 1 mM EDTA

2 2 *Enzyme assays*

The basic principles of enzyme coupling methods for assaying invertase and SS are diagrammatically illustrated in Fig 1

(a) Invertase assay was carried out spectrophotometrically (340 nm) at 25°C in the direction of sucrose breakdown as shown in Fig 1a 10 μl of enzyme extracts (see above) were incubated in a reaction mixture (0 5 ml) containing 50 mM HEPES-KOH (pH 7 0), 2 mM MgCl$_2$, 1 mM EDTA, 15 mM KCl, 0 4 mM NAD, 1 mM each of either ATP, GTP or UTP and 1 unit each of HK, G6PDH and PGI The reaction was initiated by the addition of 50 mM sucrose

(b) SS assay was carried out in the direction of sucrose cleavage, in which UDP/ADP-dependent formation of UDPG/ADPG and fruc-
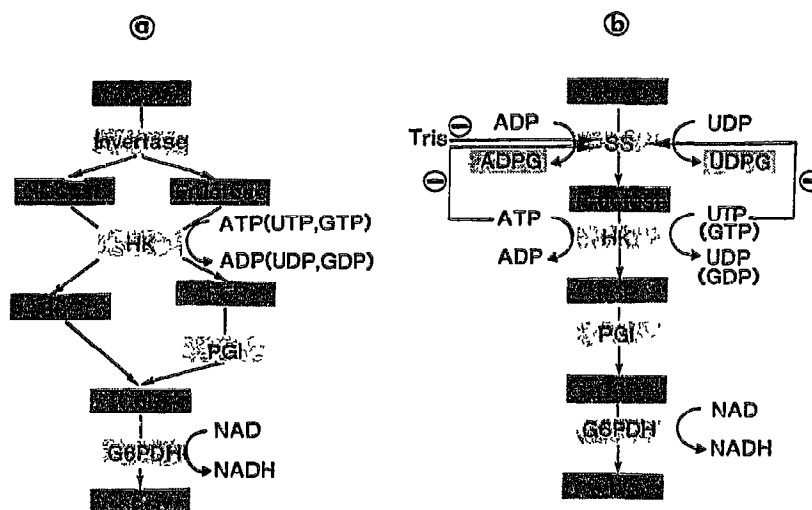
Fig 1 Schematic diagram of enzyme coupling method assaying two enzyme reactions engaged in sucrose cleavage (a) invertase and (b) SS In SS, inhibitory effects of ATP and Tris on sucrose + ADP $\rightleftharpoons$ ADPG + fructose and that of UTP on the SS reaction sucrose + UDP $\rightleftharpoons$ UDPG + fructose are indicated by arrows

tose was measured spectrophotometrically (Fig 1b) Assay procedures were essentially identical with those of invertase except that the reaction was initiated by the addition of UDP or ADP (2–600 μM) For kinetic analysis of SS as a function of varying sucrose concentrations, ADP (300 μM) was included in the assay mixture and the reaction was initiated by the addition of sucrose (2–100 mM) The true SS activities were then estimated after subtracting the invertase activities measured without addition of NDP (see above)

In experiments testing the effect of ATP, AMP, ADPG and UDP as well as Tris on the formation of ADPG by SS, each one of these reagents at their indicated concentrations was included in the reaction mixture (see below) In order to make sure that these reagents added do not affect HK, G6PDH or PGI activities, reduction of NAD (absorbance increments at 340 nm) was compared under each experimental condition after the addition of fructose (800 μM) Since commercially available HK and G6PDH are slightly contaminated by invertase [14], control systems without extracts were run in parallel with the complete reaction system Boiled enzyme extracts were used as negative controls

2 3 Separation of SS by isoelectrofocusing

Various samples of sycamore extracts were isoelectrofocused in a LKB 2117 Multiphor II Electrophoresis Unit (Sweden) At the end of electrophoresis, strips of gel were cut and incubated in 5 ml of reaction mixture containing NBT (20 mg/ml) and phenazinemethosulfate (5 mg/ml) [16] The NADH liberated during the course of reactions of invertase and SS will reduce the NBT which precipitates forming an intense blue deposition in the area where the enzyme proteins are located

2 4 Reagents

NAD, ATP, ADP, AMP, ADPG, UDP, UTP, GTP, raffinose and phenazine methosulfate were purchased from Sigma (USA) HK (H4502), G6PDH (G5885) and PGI (P5381) were purchase from Sigma (USA) NBT was purchased from Wako Pure Chemical Industries Ltd (Japan) The carrier Ampholine for isoelectrofocusing experiments, pH range of 3 5–9 5, was purchased from Pharmacia (Sweden)

3 RESULTS

3.1. Enzyme coupling method

Two enzymic pathways, catalyzed by invertase and

SS, are known to operate in the breakdown of sucrose in the plant cell. It was thus necessary for us to establish a standardized assay method which can discriminate between these enzyme reactions The spectrophotometric enzyme coupling method as outlined in Fig 1b is commonly used to measure the SS activities present in various plants [5,10,12–14] Although different NTP, i e ATP, CTP, UTP, ITP and GTP are shown to be effective phosphate donors of HK, ATP was always used In principle, the same enzyme coupling method can be applicable to the invertase assay (Fig. 1a) However, it must be taken into account that the inclusion of ATP may possibly interfere with SS sucrose + ADP $\rightleftharpoons$ ADPG + fructose Caution must be taken also in the general use of Tris as extracting buffer or as a component in the SS assay system [5,6,8,10,14,17,19–22]. The potent inhibitory effects exhibited by these two compounds is a key finding in the present investigation, and following the standard assay system as described in the Experimental section, we have determined invertase and SS activities

3 2. Invertase

Since sucrose but not raffinose was specifically hydrolyzed by the sycamore extracts (data not shown), it is concluded that sycamore invertase is a β-D-fructofuranosidase Typical Michaelis-Menten kinetics were observed when the concentration of sucrose was varied (Fig. 2A) V_m= 19–23 mU/mg protein and $K_{m(sucrose)}$= 15–22 mM were calculated from three independent experiments, in which 1 mM ATP was replaced by 1 mM GTP or UTP as the phosphate donor of HK in the assay method. The K_m value is comparable to that previously observed in soybean nodules (K_m= 10 mM) [23].

3.3. SS

The kinetic properties of sucrose cleavage by the sycamore enzyme were determined using ADP (300 μM) as the glucose acceptor employing the standard assay system as described, in which ATP was replaced by either UTP or GTP A typical sigmoidal saturation curve was observed with respect to sucrose concentrations ($n=2$, $S_{0.5}=$ 24 mM) (Fig 2B) (see [17,18]). The $V_{m(ADP)}=$ 147±16 1 mU/mg protein ($n=5$) exceeds several times that obtained in experiments using UDP (500 μM) as the glucose acceptor and ATP or GTP as the phosphate donors for the HK reaction ($V_{m(UDP)}=$17±3 2 mU/mg protein, ($n=4$)) [10] (data not shown)

3.4. Formation of ADPG from sucrose cleavage catalyzed by SS

The kinetics of the sucrose cleavage by SS from both sycamore and spinach as a function of ADP concentration is hyperbolic (Fig 3A,B) (see [5,17,18,20]) In contrast to the reported $K_{m(UDP)}$ values observed for SS from various plant sources, e.g. 320 μM (*Jerusalem artichoke*) [5], 190 μM (*Phaseolus aureus*) [8], 2 4 mM (*Beta vulgaris*) [11], 130 μM (sweet potato) [17], 44 μM (*Leleba oldhami*) [18], 77 μM (sugar beet) [19] and 800

μM (rice) [20], much smaller $K_{m(ADP)}$ values were obtained in the present study 5.3±2 3 μM ($n=4$) (spinach) (Fig. 3A) and 16 8±4.4 μM ($n=4$) (sycamore) (Fig 3B).

3 5 Inhibitory effects of ATP and Tris on ADPG formation by SS

An important finding was the fact that the addition of ATP strongly inhibits non-competitively the sucrose cleavage when ADP is used as the glucose acceptor (Fig 3A,B) This inhibitory effect was shown to be significantly stronger in the spinach than in the sycamore extracts Moreover, ADPG (product of sucrose cleavage) as well as AMP were shown to inhibit the ADPG synthesis by SS in the spinach system (Fig. 3B), although their inhibitory effects were not pronounced in the sycamore system (Fig 3A) It was also observed that Tris is a potent non-competitive inhibitor in the formation of ADPG by both spinach and sycamore SS, requiring only 0.6 mM Tris for 50% inhibition (Fig 3A). It will be noted that Tris barely inhibits the formation of UDPG by SS (data not shown) (see also [5,8,14, 17–22]). Consistent with our previous report [10], Tris exerted an inhibitory effect on the sycamore invertase (15 mM Tris for 50% inhibition) A strong non-competitive inhibition of the invertase activity by Tris has been also described in soybean nodules [23].

In agreement with previous reports showing the inhibitory effect of UTP on the UDPG synthesis by SS [21],
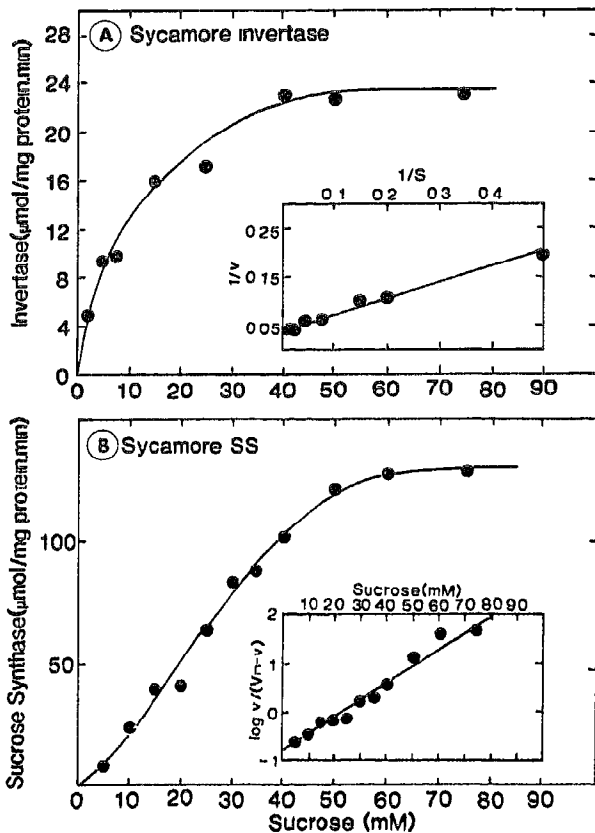

Fig 2. (A) Substrate saturation curve of sycamore invertase with respect to sucrose concentrations (B) Substrate saturation curve of sycamore SS with respect to sucrose concentrations (inset Hill plots) Details of assay conditions are given in the text
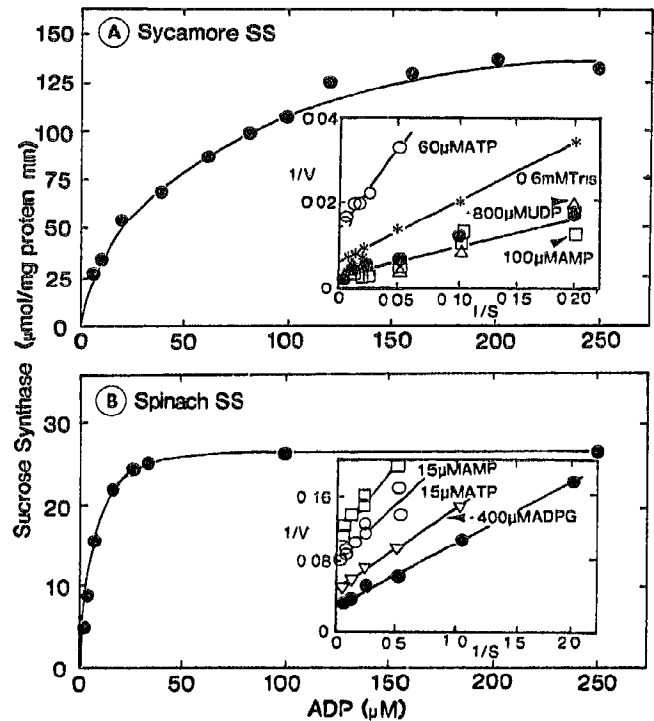

Fig 3 Substrate saturation curve of ADP-specific SS in sycamore (A) and spinach (B) with respect to ADP concentrations and effects of ATP, ADPG, AMP, UDP and Tris (0 6 mM) Assay conditions are given in the text

it was observed that UTP (5 mM) totally inhibits the UDPG synthesis by sycamore SS (data not shown) It should be emphasized, however, that the addition of UTP does not interfere with the ADPG synthesis by SS, thus allowing us to examine the possible inhibitory effect of UDP As can be clearly seen in Fig 3A, as much as 800 μM UDP did not inhibit the ADPG formation by SS The overall findings thus strongly indicate that there are at least two different types of SS (one highly specific for ADP and the other less specific for other NDPs) which may take part a role in the enzymic formation of sugar nucleotides from the cleavage of sucrose.

3.6. Characterization of ADP-specific SS

It became essential for us to characterize the SS molecule Sycamore extracts were subjected to separation by isoelectrofocusing and after electrophoresis invertase and SS were detected by precipitation of reduced NBT (Fig 4). Since the formation of NADH takes place solely on the gel surface, deposited blue bands are inevitably blurred (pI=ca 5 5) As yet we could clearly detect the most intense band in the presence of sucrose and ADP, inclusion of ATP and Tris in the assay mixture caused the reduction of band intensities. The addition of UDP gave a barely visible positive band. These zymogram patterns are essentially in conformity with the results of the kinetic analysis obtained by the coupling enzymic assay method described above (see Fig 3)

4. DISCUSSION

It is a general belief that among different NDPs, UDP serves as the principal glucose acceptor in the sucrose cleavage reaction catalyzed by SS [4,8,9,13,17–22] Thus, UDPG, which can be synthesized additionally by UDPG pyrophosphorylase, is thought to be the key sugar nucleotide in the plant cell, from which various types of cell wall polysaccharides are derived [24]. However, we tend to think that this view must now be revised. Our present investigation strongly indicates that the enzymic formation of ADPG catalyed by SS might represent a far more important role in the sucrose utilzation Overall results strengthen our view that, in contrast to another commonly accepted scheme of 'starch biosynthesis controlled by ADPG pyrophosphorylase' [9,25], most of the ADPG biosynthesis takes place in the extraplastidic compartment, in which ADP-specific SS is the crucial player [1–3]

The allosteric modulation of SS activities by the concentration of sucrose as shown in Fig. 2B is thought to be another interesting facet of this enzyme which plays a key regulatory role in the sucrose to starch transition [1–3]. ADPG produced in the extrachloroplastic compartment by SS at the higher sucrose levels will be imported into plastids by adenylate translocator and then
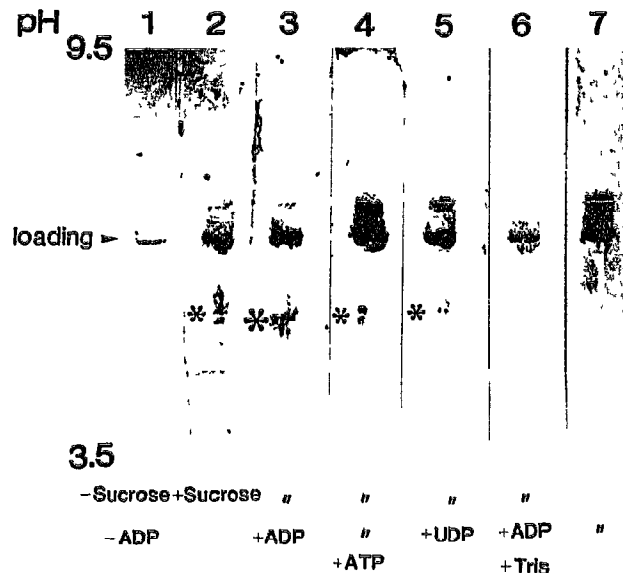

Fig 4 Zymogram by NBT stain of SS and invertase (Lane 1) control (without sucrose and ADP), (lane 2) 50 mM sucrose, (lane 3) 50 mM sucrose and 300 μM ADP, (lane 4) 50 mM sucrose, 300 μM ADP and 100 μM ATP, (lane 5) 50 mM sucrose and 800 μM UDP, (lane 6) 50 mM sucrose, 300 μM ADP and 10 mM Tris, (lane 7) 300 μM ADP 80 μg protein was applied in each lane Deposition of reduced NBT due to SS and invertase are shown by asterisks

transformed to starch in the stroma Work attempting to elucidate the subcellular localization of the SS described in this communication as well as its molecular properties is now in progress

REFERENCES

[1] Akazawa, T , Pozueta-Romero, J and Ardila, F (1991) in Molecular Approaches to Compartmentation and Metabolic Regulation (Huang, A C and Taiz, L eds) Am Soc Plant Physiol , Rockville, MD (in press)

[2] Pozueta-Romero, J , Frehner, M , Viale, A M and Akazawa, T (1991) Proc Natl Acad Sci USA 88, 5769–5773

[3] Pozueta-Romero, J , Ardila, F and Akazawa, T (1991) Plant Physiol (in press)

[4] Akazawa, T and Okamoto, K (1980) in The Biochemistry of Plants vol 3 (Preiss, J ed) pp 199–220 Academic Press, New York

[5] Avigad, G (1964) J Biol Chem 239, 3613–3618

[6] Delmer, D P and Albersheim, P (1970) Plant Physiol 45, 782–786

[7] Grimes, W J , Jones, B L and Albersheim, P (1970) J Biol Chem 245, 188–197

[8] Delmer, D P (1972) J Biol Chem 247, 3822–3828

[9] Preiss, J (1982) Annu Rev Plant Physiol 33, 431–454

[10] Huber, S C and Akazawa, T (1986) Plant Physiol 81, 1008–1013

[11] Silvius, J E and Snyder, F W (1979) Plant Physiol 64, 1070–1073

[12] Hubbard, N L, Pharr, D M and Huber S C (1990) Plant Physiol 94, 201–208

[13] Nguyen-Quoc, B, Krivitzky, M, Huber, S C and Lecharmy, A (1990) Plant Physiol 94, 516–523

[14] Avigad, G and Milner, Y (1966) Methods Enzymol 8, 341–345,

[15] Rorem, E S, Walker, H G and McCready, R M (1960) Plant Physiol 35, 269–272

[16] Work, T S and Work E (1969) in Laboratory Techniques in Biochemistry and Molecular Biology, vol 1, pp 535–536, Elsevier, Amsterdam

[17] Murata, T (1971) Agr Biol Chem 35, 1441–1448

[18] Su, J -C, Wu, J -L and Yang, C -L (1977) Plant Physiol 60, 17–21

[19] Milner Y and Avigad, G (1965) Nature 22, 825

[20] Nomura, T and Akazawa, T (1973) Arch Biochem Biophys 156, 644–652

[21] Su, J -C and Preiss, J (1978) Plant Physiol 61, 389–393

[22] Echt, C S and Chourey, P S (1985) Plant Physiol 79, 530–536

[23] Morell M and Copeland, L (1984) Plant Physiol 74, 1030–1034

[24] Feingold, D S and Avigad, G (1980) in The Biochemistry of Plants, vol 3 (Preiss, J ed) pp 101–169 Academic Press, New York

[25] Preiss, J (1988) in The Biochemistry of Plants, vol 14 (Preiss, J ed) pp 181–254, Academic Press, New York