Oligomerization status, with the monomer as active species, defines catalytic efficiency of UDP-glucose pyrophosphorylase

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Barley UDP-glucose pyrophosphorylase (UGPase), a key enzyme for the synthesis of sucrose, cellulose and other saccharides, was expressed in *Escherichia coli* and purified. Using both native electrophoresis and gel filtration, the recombinant and crude leaf UGPase proteins were found to exist as a mixture of monomers, dimers and higher-order polymers. In order to understand the molecular basis for the oligomerization of UGPase, a conserved Cys residue was replaced (C99S mutant) and several amino acids were substituted (LIV to NIN, KK to LL and LLL to NNN) in a conserved hydrophobic domain (amino acids 117–138). The C99S mutant had about half the $V_{\max}$ of the wild-type and a 12-fold higher K_m for PP_i, whereas NIN and LL mutations lowered

the $V_{\max}$ by 12- and 2-fold, respectively, with relatively small effects on substrate K_m values (the NNN mutant was insoluble/inactive). The NIN mutation resulted in a low-activity oligomerized enzyme form, with very little monomer formation. Activity staining on native PAGE gels as well as gel-filtration studies demonstrated that the monomer was the sole enzymically active form. Possible implications of the oligomerization status of UGPase for post-translational regulation of the enzyme are discussed.

Key words: cytosol, site-directed mutant, sucrose metabolism, sucrose synthesis.

INTRODUCTION

UDP-glucose pyrophosphorylase (UGPase; EC 2.7.7.9) is a key activity, producing UDP-glucose, the direct or indirect precursor to oligo- and polysaccharides in all organisms [1–4]. Plant UGPase exists predominantly as a soluble cytosolic protein, but a membrane-bound UGPase has also been reported, possibly encoded by the same gene [2]. In barley seed endosperm, the reverse reaction (utilization of UDP-glucose) of UGPase is ‘coupled’ to ADP-glucose pyrophosphorylase activity, a key enzyme of starch biosynthesis, resulting in the equimolar production of ADP-glucose from UDP-glucose [2,5], the former being the sole immediate precursor of starch in all plants.

Despite the central role of UGPase in carbohydrate synthesis and metabolism, little is known about regulatory events that control UGPase activity. In animal tissues, the enzyme is responsive to hypoxia [6] and glucose availability, with UDP-glucose deficiency occurring in insulin-dependent tissues of diabetic organisms [7]. In plants, the enzyme may exist in large excess over that required for carbon flow from/to sucrose, as found by ‘antisense’ DNA approaches in transgenic potato plants [8]. On the other hand, other studies have failed to engineer potato plants with an inhibition of enzyme activity greater than 30–50%, but this level of inhibition was sufficient to decrease sugar content in stored tubers [9,10], consistent with the important role of UGPase in sugar synthesis. The activity of UGPase was also found to increase upon phosphate deficiency via regulation at the gene expression level [11], as well as sucrose feeding and cold exposure [12], with possible effects on an overall carbohydrate budget in stressed plants.

UGPase amino acid sequences are highly conserved among eukaryotes, with more than 80% identity for plant UGPases [13,14] and over 50% identity between plant and animal sequences [14,15]. Several 15–25-amino-acid-long motifs that are

strongly conserved in the protein sequence for UGPase from various eukaryotic organisms have been identified [1,16,17]. These regions contain essential amino acids that were earlier demonstrated to be involved in substrate binding and catalysis, particularly Lys-367 (based on potato sequence), essential for catalytic activity, and Lys-263 and Lys-329 that may participate in substrate binding [18,19]. Despite the overall similarity, both plant and animal UGPases differ in their oligomeric structure. The enzyme from animal tissues is believed to occur as an octamer [20], but plant UGPase was reported as a monomer or dimer [13,21]. This structural difference is even more surprising, given that both plant and animal UGPases share similar kinetic properties and that mutations at the same amino acid residues confer similar defects in activity [15].

The apparent discrepancies between structural properties of animal and plant UGPases have prompted us to re-examine oligomerization status of plant UGPase, using heterologously expressed barley UGPase as a model. This protein exists in both photosynthetic and non-photosynthetic tissues of barley [14] and is known to contain all critical amino acid motifs that were earlier found to be present in UGPases of both plant and animal origin. In the present study, we demonstrate directly that the barley enzyme exists as a mixture of oligomers, with the monomer as the active species, and we describe mutations affecting oligomerization status and/or catalysis/substrate binding of UGPase.

MATERIALS AND METHODS

Cloning and site-directed mutagenesis

The full coding sequence of barley UGPase (GenBank accession no. X91347) [14] was amplified by PCR with forward primer 5'-T GTA CAT GCC ATG GCC GCC GCC GCC GTC-3' and

Abbreviations used: Ni-NTA, Ni²⁺-nitrilotriacetate; UGPase, UDP-glucose pyrophosphorylase; wt, wild type.

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reverse primer 5'-A CCC AAG CTT GGG AAT ATC CTC TGG GCC G-3', containing *NcoI* and *HindIII* restriction sites, respectively (underlined; the ATG translation start codon and last UGPase codon, for isoleucine, are indicated in bold). The *NcoI*–*HindIII* complete coding region (1.4 kb) was subcloned into the pET23d expression vector (Novagen) in fusion with a poly-His epitope at the C-terminus. Site-directed mutagenesis of the UGPase coding sequence was performed using the Quik-change Site-Directed Mutagenesis kit (Stratagene). Site-specific mutations were introduced by extension and amplification of overlapping nucleotide primers using PCR and *Pfu turbo* polymerase (Stratagene), according to the instructions of the manufacturer. All sequences were confirmed in both strands using the Dyanamic Sequencing kit (Amersham Biosciences).

Heterologous expression and purification of UGPase

Escherichia coli BL21 cells were transformed with the plasmids containing the coding sequences of UGPase. The transformation and subsequent isopropyl β -D-thiogalactoside induction of the UGPase protein were carried out according to standard procedures [22]. Following cell lysis and centrifugation to separate soluble and insoluble proteins, the His-tagged protein was purified from the soluble fraction under native conditions on Ni^{2+} -nitrilotriacetate (Ni-NTA) spin columns according to the manufacturer's instructions (Qiagen). Bound protein was eluted from the column with elution buffer (0.1 M sodium phosphate, pH 8.0, 300 mM NaCl and 250 mM imidazole), and 25 % (final concentration) glycerol was added before storage at -20°C . Protein content was quantified according to the Bradford method using the Bio-Rad Assay kit (Bio-Rad) and with BSA as protein standard.

SDS and native PAGE

SDS/PAGE was run on slab gels containing 10 % acrylamide according to Laemmli [23]. Following electrophoresis, the gels were stained with Coomassie Blue. Native 8.5 % polyacrylamide gels (1.5 mm thick) were run at 4°C according to the same procedure, but SDS (and β -mercaptoethanol) was omitted from samples and buffers. The relative positioning of UGPase oligomers on native PAGE gels was inferred from positioning of reference proteins [albumin monomer (66 kDa) and dimer (132 kDa), urease monomer (272 kDa) and dimer (545 kDa)] run on the same gels.

Immunoblots

Following PAGE, the resolved proteins were transferred to nitrocellulose membrane and the UGPase protein was detected with rabbit antibodies raised against potato tuber UGPase ($0.6\ \mu\text{g}/\text{ml}$) [21] followed by goat anti-rabbit IgG coupled to peroxidase (Amersham Biosciences). Specific labelling was detected with the enhanced chemiluminescence kit (Amersham Biosciences).

Enzyme assays and activity staining on native PAGE gels

UGPase activity was routinely measured in 1 ml assays containing 0.1 M Hepes (pH 7.6), 1 mM UDP-glucose, 1 mM PP_i , 0.8 mM NAD, 1 mM MgCl_2 , 2 units of phosphoglucomutase (Boehringer Mannheim), 5 units of glucose-6-phosphate dehydrogenase (Boehringer Mannheim) and 6–30 ng of purified protein. For K_m determination, one substrate was maintained at 1 mM and the other varied from 0.02 to 1 mM (the K_m with UDP-glucose) or from 0.02 to 0.2 mM [the K_m with PP_i , with the exception of K_m determination for the Cys-99 $\rightarrow$ Ser (C99S)

mutant, where PP_i was varied from 0.04 to 1 mM]. The change in absorbance was monitored spectrophotometrically (340 nm) for 5 min (25°C) and its initial linear portion was used to determine UGPase activity. One unit of UGPase activity was defined as the amount of the enzyme required to reduce $1\ \mu\text{mol}$ of NAD/min. Colorimetric assays of UGPase activity, following native PAGE, were done as in [24] (for assay of UDP-glucose synthesis reaction) and in [25] (pyrophosphorolytic direction of the reaction), using enzyme samples supplemented with 0.05 % Tween-20.

Molecular sieving

Purified recombinant UGPase (0.25 mg of protein) was size-fractionated on a Sephacryl S-200 column in a buffer containing 0.1 M phosphate and 140 mM NaCl (pH 8.0). The flow rate was 0.2 ml/min, and fractions of 1 ml were collected. Ovalbumin monomer (43 kDa) and dimer (86 kDa) were used as protein molecular-mass markers.

Preparation of barley leaf extracts

Etiolated leaves from 7-day-old barley (*Hordeum vulgare*) plants were ground in 2 vol. of extraction buffer (0.1 M sodium phosphate, pH 8.0, 5 mM MgCl_2 and 1 mM EDTA) and centrifuged for 3 min at $10000\ g$ (4°C). The supernatant was supplemented with 0.05 % Tween-20 and 25 % glycerol and analysed immediately by native PAGE.

RESULTS AND DISCUSSION

Purification of recombinant UGPase and its oligomerization status

The coding region of barley UGPase [14] was fused to a poly-His epitope at the C-terminus by subcloning into the pET23d vector, and the fusion protein was expressed in *E. coli* BL21 cells. The recombinant non-mutated UGPase, referred to from hereon as the wild-type (wt), was almost fully soluble in *E. coli* (see below) and could be purified from bacterial soluble fraction using Ni-NTA spin columns (Qiagen). The purified protein displayed the expected molecular mass for the fusion protein (53 kDa; Figure 1A) and the identity of the protein was immunologically confirmed by Western blots (Figure 1B). The same molecular mass was obtained regardless of whether the protein was pretreated or

Figure 1 Purification of recombinant barley UGPase from *E. coli*

The *E. coli*-expressed barley UGPase was analysed by SDS/PAGE followed by (A) Coomassie Blue staining and (B) immunoblot analysis. Lanes 1, *E. coli* soluble proteins ($6\ \mu\text{g}$) before loading on Ni-NTA spin columns; lanes 2, flow-through ($6\ \mu\text{g}$) obtained after Ni-NTA spin column; lanes 3, eluted protein ($0.6\ \mu\text{g}$). Numbers on the left refer to positions of PAGE protein molecular-mass markers (in kDa).

Figure 2 Oligomerization status of barley UGPase

(A) Western-blot analysis after native PAGE of the purified recombinant enzyme (left-hand lane, 1.2 μ g of protein loaded) and UGPase from crude leaf extracts (right-hand lane, 60 μ g of total protein); and (B) Sephacryl S-200 gel filtration of recombinant purified enzyme. $\circ$, UGPase activity. For both (A) and (B), positions of monomers (M), dimers (D) and higher-order oligomers (H), based on positioning of native PAGE and gel-filtration protein markers, are indicated with arrows.

not with dithiothreitol (or β -mercaptoethanol) prior to SDS/PAGE (results not shown). The protein was at least 98 % pure as judged from SDS/PAGE analysis.

Purified barley UGPase was run on native PAGE followed by Western blotting. Surprisingly, the protein appeared as several oligomers of different sizes (Figure 2A). Quantitative analysis of band intensities on ECL films revealed that the monomeric form comprised over half of UGPase protein. Preincubation with magnesium (1–5 mM) or glycerol (10–30 %) had no effect on the oligomerization state observed. The same oligomerization pattern was observed for the purified UGPase before and after desalting (Sephadex G-25 column) into 0.1 M sodium phosphate (pH 8.0; results not shown), ruling out any effect of salt or imidazole, present in the elution buffer, on the polymerization process. The results obtained for purified recombinant UGPase were confirmed for the enzyme from crude leaf preparations. For the latter only one band was detected after SDS/PAGE (results not shown), but two bands with similar migration patterns to the recombinant UGPase monomer and dimer were detected after native PAGE (Figure 2A), confirming the results observed with the recombinant UGPase and ruling out a possible role of the His tag in the polymerization process. However, higher polymers were not as prominent for crude leaf UGPase as for recombinant enzyme (Figure 2A).

The oligomeric structure of barley UGPase differs from the monomeric or dimeric structure reported for potato UGPase [13,21], but is similar to that of the liver enzyme [15]. The evidence for potato UGPase came mostly from gel-filtration experiments, where conditions of the fractionation may have an effect on

Figure 3 Expression of wt and mutated barley UGPase in *E. coli*

(A) Alignment of selected UGPase protein sequences, corresponding to protein fragment (amino acids 95–138) of barley UGPase, and description of the barley enzyme mutations. Bold letters denote conserved amino acids. Stars denote amino acids that were modified in the present study. GenBank accession numbers for the sequences are X91347 (barley), P19595 (potato), Q64459 (pear) and Q16851 (human). Proteins were expressed in *E. coli* cells upon 3 h of isopropyl β -D-thiogalactoside induction, and (B) soluble and (C) insoluble bacterial protein fractions were analysed by SDS/PAGE. Protein aliquots of approximately 10–15 μ g were loaded per lane. Numbers on the left refer to positions of SDS/PAGE protein molecular-mass markers (in kDa), and arrows indicate the position of UGPase. n.i., non-induced cells.

subunit separation. Indeed, in our hands, when barley UGPase was fractionated on a Sepharose S-200 column, the monomer fraction was more prominent than after native PAGE; however, dimer and higher oligomer fractions were still relatively easy to distinguish (Figure 2B). Activity assays of different fractions eluted from the size-exclusion Sepharose S-200 column have indicated that the monomer is the most likely active species of the UGPase (see also Figure 5, below). There was some activity that was associated with dimer and higher-order oligomer fractions, but it was two orders of magnitude lower than that of the monomer.

Generation of UGPase mutants

We modified the hydrophobicity of wt UGPase by site-directed mutagenesis in a conserved hydrophobic domain that extends from amino acid 117 to 138 (Figure 3A). This domain covers two exons, based on comparisons of genomic DNA and cDNA from potato [13,26], and has not been studied by mutagenesis before. To decrease the hydrophobicity, Leu-117 and Val-119 as well as three Leu residues at positions 135–137 were changed to Asn to give the NIN and NNN mutants, respectively. Increased hydrophobicity was obtained by mutating two Lys residues at positions 127 and 128 to Leu (the LL mutant; Figure 3A). In addition to mutants likely affecting the hydrophobic interactions, we have also mutated Cys-99 (conserved in all eukaryotic UGPases) to Ser (the C99S mutant). This Cys residue lies in a distinct exon domain from other mutants generated in this study, and was

previously reported to be important for substrate binding in human liver UGPase [17]. The C99S mutation did not affect the solubility of UGPase, whereas the NIN and LL mutants were partially insoluble (Figures 3B and 3C). These three mutated proteins were purified from the soluble bacterial fraction using the same procedure as that used for wt UGPase. The NNN mutation resulted in an exclusively insoluble/inactive protein (Figure 3C), preventing its purification under native conditions. Whereas the LIV residues are probably localized on the protein surface (in a mildly hydrophobic region) and are most likely involved in an α -helix structure, according to secondary-structure analyses (results not shown), the LLL residues are found in a highly hydrophobic region (probably β -sheet) that is likely involved in the hydrophobic core of the enzyme; this possibly explains the insolubility of the NNN mutant. In contrast, the mutated KK residues (LL mutant) are in a hydrophilic region which is most likely a loop located on the protein's surface.

Oligomerization abilities of the mutant proteins were tested by native PAGE under the same conditions as the wt UGPase in Figure 2. Most importantly, a higher polymerization capacity was observed for the NIN mutant, which produced very little of the monomer form (Figure 4). The C99S and LL mutants behaved similarly to wt on native PAGE gels, regardless of conditions. However, the exchange of two Lys to two Leu in the LL mutant led to a modification in the net charge and a decrease in the pI (4.93 compared with 5.07 for the wt), reflected by a higher protein mobility during native PAGE.

The NIN mutation impairs depolymerization, with effects on catalysis

The purified barley wt UGPase had a $V_{\max}$ value of 1200 units/mg of protein (Table 1), which was equivalent to that measured for the UGPase purified from potato tubers [13,18]. Compared with the wt UGPase, the NIN and LL mutants displayed reduced $V_{\max}$ values, with 8 and 41 % of the wt activity for the NIN and LL proteins, respectively (Table 1). For wt UGPase, the K_m values for PP_i and UDP-glucose were 34 and 65 μ M, respectively (Table 1). These values were lower than those described previously for UGPase from barley malt [27] (0.17 and 0.19 mM for PP_i and UDP-glucose, respectively); however, the malt enzyme was later found to be proteolytically modified at the N-terminus [14], which possibly accounts for its higher K_m values. Alternatively, the presence of a His tag in the recombinant wt protein could perhaps increase affinity for both substrates. For both NIN and LL mutants, the K_m values with both substrates were consistently lower by about 50 %, compared with the wt protein, suggesting that the mutations slightly improved the accessibility of substrates to their respective binding sites on the enzyme. However, the NIN mutant produced very little monomer (Figure 4), and had by far the lowest $V_{\max}$ of the mutants obtained in the present study. The high order of oligomerization and very low apparent $V_{\max}$ of the NIN mutant suggest that depolymerization of UGPase has a critical impact upon catalysis of the enzyme.

Based on comparison of cDNA and genomic sequences of potato [13,26] and rice (accession no. AP001383) UGPases, the NIN mutation is located in an α -helix protein domain encoded by a small exon (exon 5; amino acids 107–126 and 92–113 in potato and rice, respectively) that is separate from those of other mutants generated in this study [C99S, exon 4; LL (and NNN), exon 6], which had no effect on the oligomerization status of UGPase (Figure 4). Thus, it is tempting to speculate that the protein domain encoded by exon 5 is involved in subunit interactions during the oligomerization process, whereas the

Figure 4 Effects of mutations on oligomerization of UGPase

Western-blot analysis after native PAGE of purified wt, C99S, NIN and LL mutants of UGPase (2 μ g each). Positions of monomers (M), dimers (D) and higher oligomers (H) are indicated with arrows.

Table 1 Apparent $V_{\max}$ and K_m values of wt and mutated UGPase

Apparent $V_{\max}$ was determined in the presence of 1 mM PP_i and 1 mM UDP-glucose. 100 % activity corresponded to 1200 units/mg of protein. Values are the means from three assays and the S.D. was always less than 10 %.

Parameter	Protein ...	wt	C99S	NIN	LL
Apparent $V_{\max}$ (%)		100	54	8	41
K_m (μ M)					
PP_i		34	407	16	20
UDP-glucose		65	101	30	36

adjoining domains, especially that encoded by exon 4 in potato and rice, are important for substrate binding (see below).

The C99S mutation affects PP_i binding

In addition to mutants likely affecting the hydrophobic interactions, we also engineered a C99S mutation located in a conserved domain of UGPase, encoded by exon 4 [26]. The mutation had no effect on the oligomerization of the protein, but it had $\approx$ 50 % lower $V_{\max}$ and a 12-fold higher K_m with PP_i , when compared with wt, whereas its K_m with UDP-glucose was higher by only 50 % (Table 1). This suggests that Cys-99 is located at or near the PP_i -binding site (or near a site that affects substrate binding), but is not essential for activity. A similar K_m modification for PP_i (an increase by 7-fold) in an analogous Cys mutant of UGPase from human liver was described previously [17]. A G115D mutant of mammalian UGPase (position 92 in the barley sequence, located in the same conserved domain as Cys-99) was reported to strongly impair the enzymic activity, likely by affecting the glucose 1-phosphate-binding site [1]. Several residues essential for catalytic activity were identified in potato UGPase, particularly a number of conserved Lys residues [18,19,28], but none of them are located in the domains that were mutated in this report.

Figure 5 UGPase activity of oligomers

UGPase activity in (A) synthesis and (B) pyrophosphorolysis reactions was detected after native PAGE of purified wt as well as the C99S, NIN and LL mutant UGPases. Aliquots of 3–6 μ g of purified protein were loaded per lane. After the activity staining, the proteins were transferred to PVDF membrane and UGPase was detected by immunoblotting. Positions of monomers (M), dimers (D) and higher-order oligomers (H) are indicated with arrows. Note that the immunoblots were overexposed due to the high amount of protein loaded, and are shown only to link activity patterns to given oligomeric forms.

Monomer is the active form of UGPase

Enzymic activity of the different oligomers of the wt and mutated proteins was investigated by activity staining following native PAGE. Staining for UDP-glucose synthesis as well as pyrophosphorolysis activities gave the same result (Figure 5): the activity was detected mainly for the monomer, with very little or no staining for the dimer. No activity was visible for larger oligomers. These activity staining assays have directly confirmed the results obtained for gel filtration of UGPase (Figure 2B), where only the monomer was active, but they challenge the belief derived from earlier studies on animal UGPase, where the octameric form of the enzyme was assumed to be the active species [20]. Given the high similarity between plant and animal UGPases [14,15], it would be surprising if the structure/function properties of the enzyme differed so drastically in these two groups of organisms.

On the possible role of oligomerization for UGPase regulation

The inability of the NIN mutant to form monomers and its very low $V_{\max}$ suggests that equilibration between monomers and higher-order oligomers may affect the catalytic efficiency of the enzyme. In fact, careful enzymic studies on both potato and liver UGPases have implied the existence of different interconvertible forms of the enzyme, consistent with different oligomerization and/or conformational states [21,29]. Based on the present study, UGPase activity will be profoundly affected by any factor which affects equilibrium between oligomerization and deoligomerization (monomer formation) of the protein. This may have

important implications for regulation of the enzyme *in vivo*. Several enzymes of the sucrose and glucose pathways in plants are believed to form multi-enzyme complexes *in vivo*, and are regulated via post-translational modification and/or by binding regulatory proteins [30–32]. Thus, given that UGPase indeed represents a limiting step in sucrose conversions in some tissues and under certain conditions [9,10], its conformation and oligomerization status may affect interactions with other proteins (or interaction with other proteins may regulate the oligomerization of UGPase). Interestingly, activity of UGPase from spinach leaves was already reported to be regulated by 14-3-3 proteins and phosphorylation [33], implying a role for protein–protein interactions in the control of UGPase activity. UGPase was also reported to bind to intracellular membranes [2]; such a binding most probably involves a hydrophobic interaction that may depend on oligomerization status of the native enzyme.

Besides the oligomerization ability of barley UGPase, there are two important outcomes of this study: evidence that the monomer is the only active species, and the generation of mutants, especially NIN and C99S, that affect oligomerization and/or catalysis/substrate-binding efficiency of UGPase. These mutations, as well as those described earlier for the enzyme from potato, human liver and muscle and from other organisms [1,17–19,28,29], provide tools for attempts at defining structure/function properties of the protein. Unfortunately, no X-ray crystal structure models of UGPase are available, so we do not have a firm molecular blueprint for verification. Whatever the exact molecular determinants of UGPase subunit structure, the oligomerization status of UGPase needs to be considered seriously in any studies concerned with regulation of the enzyme.

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REFERENCES

- Flores-Díaz, M., Alape-Girón, A., Persson, B., Pollesello, P., Moss, M., Eichel-Streiber, C., Thelestam, M. and Florin, I. (1997) Cellular UDP-glucose deficiency caused by a single point mutation in the UDP-glucose pyrophosphorylase. *J. Biol. Chem.* **272**, 23784–23791.
- Kleczkowski, L. A. (1994) Glucose activation and metabolism through UDP-glucose pyrophosphorylase in plants. *Phytochemistry* **37**, 1507–1515.
- Miernyk, J. A. (1999) Protein folding in the plant cell. *Plant Physiol.* **121**, 695–703.
- Johansson, H., Sterky, F., Amini, B., Lundberg, J. and Kleczkowski, L. A. (2002) Molecular cloning and characterization of a cDNA encoding poplar UDP-glucose dehydrogenase, a key enzyme in hemicellulose/pectin formation. *Biochim. Biophys. Acta* **1576**, 53–58.
- Kleczkowski, L. A. (1996) Back to the drawing board: redefining starch synthesis in cereals. *Trends Plant Sci.* **1**, 363–364.
- Aw, T. Y. and Jones, D. P. (1984) Control of glucuronidation during hypoxia. Limitation by UDP-glucose pyrophosphorylase. *Biochem. J.* **219**, 707–712.
- Robinson, K., Weinstein, M. L., Lindenmayer, G. E. and Buse, M. G. (1995) Effects of diabetes and hyperglycemia on the hexosamine synthesis pathway in rat muscle and liver. *Diabetes* **44**, 1438–1446.
- Zrenner, R., Willmitzer, L. and Sonnewald, U. (1993) Analysis of the expression of potato uridinediphosphate-glucose pyrophosphorylase and its inhibition by antisense RNA. *Planta* **190**, 247–252.
- Spychalla, J. P., Scheffler, B. E., Sowokinos, J. R. and Bevan, M. W. (1994) Cloning, antisense RNA inhibition, and the coordinated expression of UDP-glucose pyrophosphorylase with starch biosynthetic genes in potato tubers. *J. Plant Physiol.* **144**, 444–453.
- Borovkov, A. Y., McClean, P. E., Sowokinos, J. R., Ruud, S. H. and Secor, G. A. (1996) Effect of expression of UDP-glucose pyrophosphorylase ribozyme and antisense RNAs on the enzyme activity and carbohydrate composition of field-grown transgenic potato plants. *J. Plant Physiol.* **147**, 644–652.

- 11 Cierieszko, I., Johansson, H., Hurry, V. and Kleczkowski, L. A. (2001) Phosphate status affects the gene expression, protein content and enzymatic activity of UDP-glucose pyrophosphorylase in wild-type and *pho* mutants of *Arabidopsis*. *Planta* **212**, 598–605
- 12 Cierieszko, I., Johansson, H. and Kleczkowski, L. A. (2001) Sucrose and light regulation of a cold-inducible UDP-glucose pyrophosphorylase gene via a hexokinase-independent and abscisic acid-insensitive pathway in *Arabidopsis*. *Biochem. J.* **354**, 67–72
- 13 Nakano, K., Omura, Y., Tagaya, M. and Fukui, T. (1989) UDP-glucose pyrophosphorylase from potato tuber: purification and characterization. *J. Biochem. (Tokyo)* **106**, 528–532
- 14 Eimert, K., Villand, P., Kilian, A. and Kleczkowski, L. A. (1996) Cloning and characterization of several cDNAs for UDP-glucose pyrophosphorylase from barley (*Hordeum vulgare*) tissues. *Gene* **170**, 227–232
- 15 Konishi, Y., Tanizawa, K., Muroya, S. and Fukui, T. (1993) Molecular cloning, nucleotide sequencing and affinity labelling of bovine liver UDP-glucose pyrophosphorylase. *J. Biochem. (Tokyo)* **114**, 61–68
- 16 Daran, J. M., Dallies, N., Thines-Sempoux, D., Paquet, V. and François, J. (1995) Genetic and biochemical characterization of the *UGP1* gene encoding the UDP-glucose pyrophosphorylase from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **233**, 520–530
- 17 Chang, H.-Y., Peng, H.-L., Chao, Y. C. and Duggleby, R. G. (1996) The importance of conserved residues in human liver UDP-glucose pyrophosphorylase. *Eur. J. Biochem.* **236**, 723–728
- 18 Katsube, T., Kazuta, Y., Tanizawa, K. and Fukui, T. (1991) Expression in *Escherichia coli* of UDP-glucose pyrophosphorylase cDNA from potato tuber and functional assessment of the five lysyl residues located at the substrate-binding site. *Biochemistry* **30**, 8546–8551
- 19 Fukui, T., Kazuta, Y., Katsube, T., Tagaya, M. and Tanizawa, K. (1993) Exploring the active site in UDP-glucose pyrophosphorylase by affinity labelling and site-directed mutagenesis. *Biotechnol. Appl. Biochem.* **18**, 209–216
- 20 Levine, S., Gillett, T. A. and Hageman, E. (1969) Uridine diphosphate glucose pyrophosphorylase. II. Polymeric and subunit structure. *J. Biol. Chem.* **244**, 5729–5734
- 21 Sowokinos, J. R., Spychalla, J. P. and Desborough, S. L. (1993) Pyrophosphorylases in *Solanum tuberosum*. IV. Purification, tissue localization, and physicochemical properties of UDP-glucose pyrophosphorylase. *Plant Physiol.* **101**, 1073–1080
- 22 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn., Cold Spring Harbor Press, Cold Spring Harbor, NY
- 23 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 24 Weaver, S. H., Glover, D. V. and Tsai, C. V. (1972) Nucleoside diphosphate glucose pyrophosphorylase isoenzymes of developing normal, *brittle-2*, and *shrunk-2* endosperms of *Zea mays* L. *Crop Sci.* **12**, 510–514
- 25 Manchenko, G. P. (1994) *Handbook of Detection of Enzymes on Electrophoretic Gels* (Manchenko, G. P., ed.), pp. 20–21, CRC Press, Boca Raton, FL, U.S.A.
- 26 Borovkov, A. Y., McClean, P. E. and Secor, G. A. (1997) Organization and transcription of the gene encoding potato UDP-glucose pyrophosphorylase. *Gene* **186**, 293–297
- 27 Elling, L. (1996) Kinetic characterization of UDP-glucose pyrophosphorylase from germinated barley (malt). *Phytochemistry* **42**, 955–960
- 28 Kazuta, Y., Omura, Y., Tagaya, M., Nakano, K. and Fukui, T. (1991) Identification of lysyl residues located at the substrate-binding site in UDP-glucose pyrophosphorylase from potato tuber: affinity labeling with uridine di- and triphosphopyridoxals. *Biochemistry* **30**, 8541–8545
- 29 Duggleby, R. G., Chao, Y. C., Huang, J. G., Peng, H.-L. and Chang, H.-Y. (1996) Sequence differences between human muscle and liver cDNAs for UDP-glucose pyrophosphorylase and kinetic properties of the recombinant enzymes expressed in *Escherichia coli*. *Eur. J. Biochem.* **235**, 173–179
- 30 Kleczkowski, L. A. (1994) Inhibitors of photosynthetic enzymes/carriers and metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45**, 339–367
- 31 Givan, C. V. (1999) Evolving concepts in plant glycolysis: two centuries of progress. *Biol. Rev.* **74**, 277–309
- 32 Winter, H. and Huber, S. C. (2000) Regulation of sucrose metabolism in higher plants. Localization and regulation of activity of key enzymes. *Crit. Rev. Plant Sci.* **19**, 31–67
- 33 Huber, S. C., Winter, H., Toroser, D. and Athwal, G. S. (2000) Metabolic regulation of nitrate and sucrose metabolism. *Plant Cell Physiol.* **41S**, 8

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