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Functional Expression of Two Pine Glutamine Synthetase Genes in Bacteria Reveals that they Encode Cytosolic Holoenzymes with Different Molecular and Catalytic Properties

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Two glutamine synthetase isogenes, GS1a and GS1b, isolated from pine have been functionally expressed in *E. coli* and the characteristics of individual gene products compared. When bacteria were grown at 37°C most pine GS1 protein was found in the insoluble fraction but lowering of the expression temperature increased yield of both GS1 polypeptide and activity in the soluble fraction. High levels of functionally active GS1a (309±35 nkat mg^{-1}) and GS1b (1,166±65 nkat mg^{-1}) enzymes were obtained by decreasing the expression temperature to 10°C. Purification and characterization of recombinant products showed that pine GS1 polypeptides are assembled in octameric GS holoenzymes showing structural and kinetic differences. The results are discussed with regard to the specific localization of GS1a and GS1b in different cell types of pine seedlings. The isoform GS1a may control the assimilation of the high levels of ammonium released in photosynthetic tissues, whereas GS1b enzyme could mitigate oscillations in glutamate availability providing a constant flux of glutamine for nitrogen transport in vascular cells.

Keywords: Conifers — *Pinus* — Nitrogen metabolism — Recombinant isoenzymes — Negative cooperativity.

Introduction

Plants have developed metabolic mechanisms to overcome the limitations of nitrogen availability in the environment. Extremely efficient membrane transporters and enzymes participate in the absorption of available forms of inorganic nitrogen and their incorporation into amino acids. Glutamine synthetase (GS; EC 6.3.1.2) is a key enzyme in the nitrogen assimilatory pathway and the maintenance of plant nitrogen economy. GS is involved in the assimilation of ammonium generated in the fixation of dinitrogen, nitrate reduction, or directly incorporated from soil through the radical system (primary sources). GS is also involved in the assimilation of ammonium released during variety of metabolic processes such as nucleic acid bases and amino acid degradation, phenylpropanoid metabolism and photorespiration (secondary sources) (Miflin and Lea 1980). In angiosperms, two different GS isoforms have been described: cytosolic GS1 and chloroplastic GS2 (McNally and Hirel 1983). GS2 is the predominant isoform in the leaves where is located in mesophyll cells and involved in the assimilation of ammonium released during processes such as nitrate reduction and photorespiration. GS1 is a minor component enzyme in leaves but highly abundant in the vascular elements of roots, nodules, flowers and fruits (Ireland and Lea 1999). The proposed role for GS1 is the primary assimilation of ammonium in the root and the biosynthesis of glutamine for nitrogen transport. However, very recently the localization of GS1 in mesophyll cells has also been demonstrated (García-Gutiérrez et al. 1998, Pérez-García et al. 1998) and a number of reports in the past few years supported a role for GS1 in plant development (Gallardo et al. 1999, Fuentes et al. 2001, Hirel et al. 2001) including chloroplast development (García-Gutiérrez et al. 1998, Suárez et al. 2002), senescence (Masclaux et al. 2000) and the response to biotic and abiotic stress (Bauer et al. 1997, Pérez-García et al. 1998). A post-translational regulation of GS isoforms by phosphorylation has recently been reported (Finnemann and Schjoerring 2000, Riedel et al. 2001).

In our laboratory two cytosolic GS genes from pine have been cloned and their expression patterns studied: GS1a (Cantón et al. 1993) and GS1b (Ávila et al. 2000). The chloroplastic enzyme has not been detected by using many different experimental approaches (Cánovas et al. 1998), however, the expression profile of GS1a in pine seedlings is comparable to that found for GS2 of angiosperms. GS1a mRNAs are undetectable in the seed but their relative abundance progressively increases following germination (Cantón et al. 1999). In the seedling, GS1a transcripts are highly abundant in the aerial part of the plant particularly in the cotyledons where their presence is circumscribed to cells of the chlorophyllic parenchyma (Ávila et al. 2001). GS1b mRNA levels in the embryo are high, showing a decreasing profile during the development of the cotyledons. GS1b transcripts are abundant in roots, and in the vascular bundles of hypocotyl and cotyledons, but the gene is poorly expressed in mesophyll photosynthetic cells (Ávila et al. 2001) unless the seedlings have been treated with the herbicide

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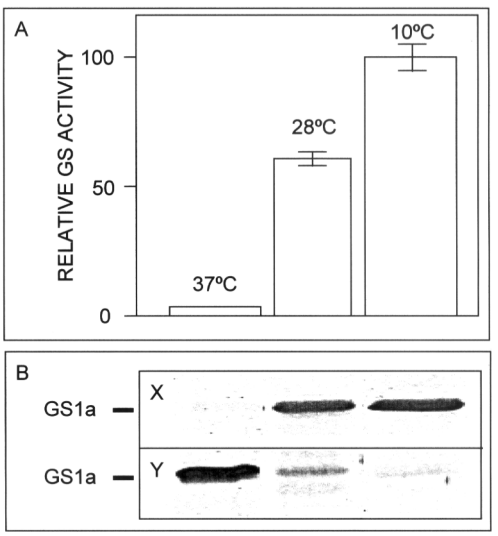

Fig. 1 Influence of temperature in the overproduction of active recombinant GS1 enzyme. (A) Bacteria overexpressing pine GS1a were grown at 37°C, 28°C and 10°C for 5 h, 10 h and 14 h respectively. Proteins were extracted and GS activity determined in the soluble fraction by the transferase assay. The values are given as means ± SD of at least three independent experiments assayed twice. Maximal GS activity corresponded to 309±35 nkat mg^{-1}. (B) Proteins (50 μg) from soluble (X) and insoluble (Y) fractions were separated by SDS-PAGE, electrotransferred to nitrocellulose filters and the abundance of the recombinant polypeptide analyzed by using specific GS antiserum (Cantón et al. 1996).

phosphinothricin (Suárez et al. 2002). Thus GS1b, mainly found in the vascular cells of heterotrophic tissues, would be an enzyme comparable to other cytosolic GS of angiosperms with similar physiological roles. Regulation of gene expression has also been investigated in our laboratory by the functional characterization of GS1a and GS1b promoters (Ávila et al. 2001, Gómez-Maldonado, J., Ávila, C. and Cánovas, F.M. unpublished). In contrast to what is known on gene structure and regulation of expression, the available knowledge on the protein gene products and how they are assembled into functional holoenzymes is much more limited. In addition, we are interested to know how these two different isoenzymes are specialized in specific physiological functions in the cells of conifers. In this paper we describe the generation of GS1a and GS1b as recombinant holoenzymes in *E. coli*, and then the structural and catalytic properties of each enzyme are compared.

Results

Overexpression of active pine GS1 enzymes

In a previous paper we reported a high-level expression of the recombinant protein for pine GS1a in *E. coli*, however, most of the protein was found as insoluble aggregates (Cantón et al. 1996) and therefore no functional studies of the enzyme were performed. In order to overcome this inconvenience, an objective of this work was to increase the protein yield in the soluble fraction of the cell. We have found that the expression temperature affects the solubility of the expressed enzyme. As shown in Fig. 1A, a relatively low level of GS activity was detectable in protein extracts derived from bacterial cultures grown at 37°C, however, a substantial increase in the steady-state levels of active enzyme was found in the extracts derived from cells grown at a lower temperature. The Western blot analysis of soluble and insoluble fractions derived from bacterial cultures grown at the different temperatures showed that the observed increase in enzyme activity is supported by a progressive shift of the GS polypeptide towards the soluble fraction (Fig. 1B). The time course of GS induction was first studied by determination of GS activity in protein extracts derived from cell cultures at several periods after isopropyl-β-D-thiogalactoside (IPTG) addition, and the observed increase in activity was accompanied by a parallel accumulation of the GS polypeptide (results not shown). It was also found that gentle shaking produced active recombinant enzyme (results not shown), although obviously the bacteria needed much more time to reach the late log-phase. In these conditions the rate of cellular division was low, which was patent because the necessary time to reach saturation of the culture was tripled. According to these results we decided to follow the same experimental protocol to overexpress GS1b in *E. coli*. As shown in Fig. 2, high levels of GS activity were found in soluble extracts derived from recombinant cells transformed with the pET3c-GS1b construct after IPTG induction. The observed specific activity was about 1,000 nkat mg^{-1}, a value threefold higher than that found in the extracts derived from cells transformed with the pET3c-GS1a construct. The Western blots of these extracts confirmed a parallel accumulation of the GS1b polypeptide with regard to non-induced or untransformed BL21 cells (Fig 2, Western).

Identification of the recombinant GS1a and GS1b holoenzymes

As described in a previous paper (Ávila et al. 1998), GS activity in the cotyledons of phosphinothricin-treated pine seedlings can be clearly resolved by ion-exchange chromatography into two peaks, the first one (native GS1a) eluting at 0.15 M KCl and the second peak (native GS1b) eluting from the column at higher ionic strength (0.5 M KCl). In the Fig. 3A, the ion-exchange chromatography profiles of bacterial extracts overexpressing pET3c-GS1a and pET3c-GS1b constructs are compared to native isoenzymes from pine cotyledons. In order to further characterize the recombinant holoenzymes, subunit composition was analyzed by two-dimensional gel electrophoresis (2D-PAGE) and Western blotting (Fig. 3B) and the results compared to pine native isoenzymes. As shown in Fig. 3B, subunits of the GS isoenzymes are of similar size (40–41 kDa) but different in charge; GS1a holoenzyme has identical subunits with an estimated pI of 6.5 whereas GS1b has identical subunits of about pI 5.6. These data indicate that GS1 isoenzymes are composed of different subunits in perfect

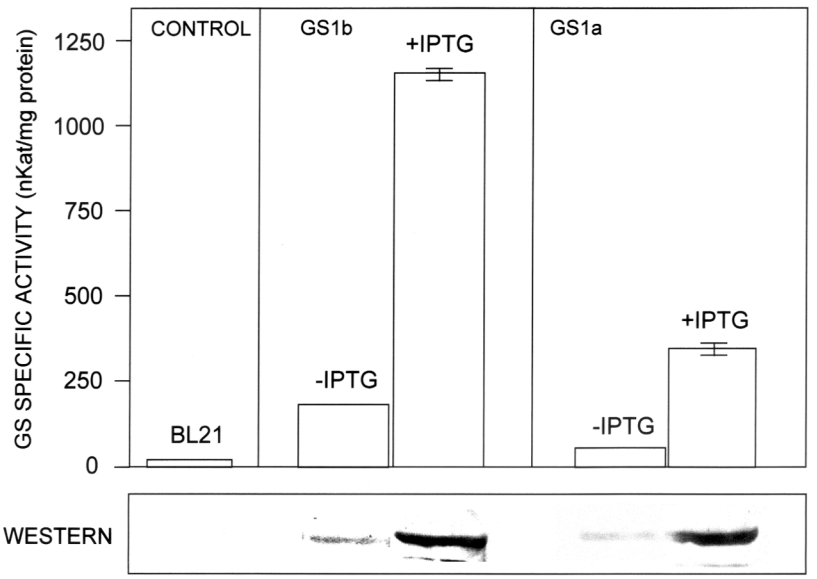

Fig. 2 Levels of GS in *E. coli* cells overexpressing pine genes. Cells harbouring pET3c-GS1a or pET3c-GS1b plasmids were grown at 10°C for 14 h either in the presence or the absence of IPTG. Soluble proteins were extracted and samples processed for either determination of GS transferase activity or Western blotting analysis. The values of GS activity are given as means ± SD of at least three independent experiments assayed twice.

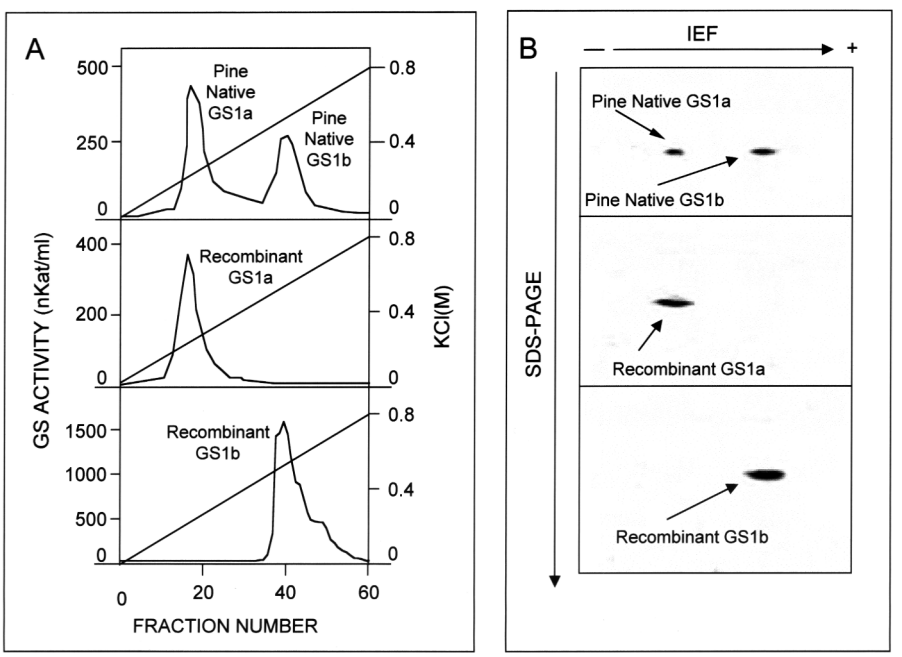

Fig. 3 Correspondence of pine GS1 isoenzymes with the GS1 isogenes. (A) Separation of GS isoforms from phosphinothricin-treated pine seedlings (Ávila et al. 1998) and bacterial cells overexpressing recombinant GS1a or GS1b. GS activity was determined by the transferase assay. (B) Separation of GS1 polypeptides by 2D-PAGE and Western blot analysis. 2-D analysis of native enzymes is shown in the same panel for comparative purposes. Native GS1a and GS1b peaks were shown to be constituted by single spots of the expected pI value.

agreement with the predictions derived from the cDNA sequence analysis (Ávila et al. 2000). Furthermore, these results show a correspondence between the pine native GS1 and the overexpressed gene products and demonstrate that GS1a and GS1b genes encoded separate functional holoenzymes.

In order to determine whether or not the recombinant enzymes exhibit similar characteristics to the pine native enzymes, GS1a and GS1b active fractions from ion-exchange chromatography were pooled separately, concentrated by ammonium sulfate precipitation, and the molecular mass of the

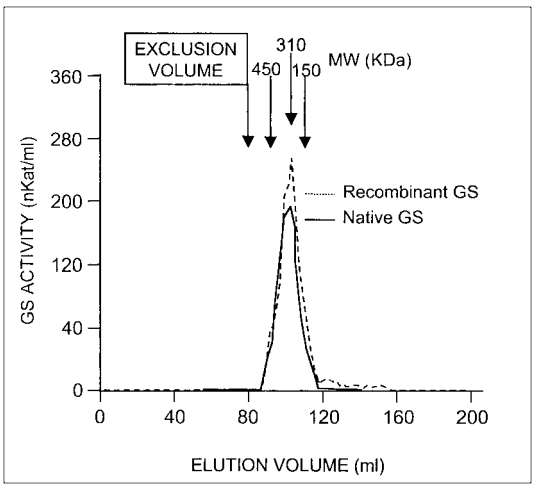

Fig. 4 Molecular mass estimation of native and recombinant GS isoenzymes. Samples (50 μg) of partially purified enzymes after DEAE-Sephacel step were subjected to Sephacryl-S-300 chromatography. The elution volume of GS was determined by the transferase assay. The proteins used to calibrate the column were: Ferritin (450 kDa), Catalase (240 kDa), Alcohol dehydrogenase (150 kDa), Bovine serum albumin (68 kDa), Cytochrome c (12 kDa). The elution profile correspond to GS1a. A similar pattern was observed for GS1b.

holoenzymes estimated by gel filtration through a calibrated column with protein markers of a known size (Fig. 4). Recombinant and native GS1a and GS1b enzymes showed a similar size of about 320 kDa, which is consistent with octameric holoenzymes composed of subunits of 40–41 kDa (Fig. 3). Taken together, all these data indicate that pine GS genes encode distinct GS polypeptides that are assembled into homo-octameric functional holoenzymes and therefore no hetero-oligomers were found.

Thermal stability of recombinant GS1 enzymes

The differential behavior showed by the two recombinant enzymes overexpressed in bacteria, suggested that each holoenzyme exhibited different properties as molecular catalysts. As thermal inactivation profiles have been extensively used for the differentiation of GS isoenzymes in higher plants we decided to investigate the heat stabilities of GS1a and GS1b (Fig. 5). Both enzymes were equally stable when incubated at 37°C, the assay temperature. However, GS1a was more sensitive to heat than GS1b at higher temperatures, retaining for example less than 10% of the initial activity after 15 min incubation at 45°C and being immediately inactivated at higher temperatures. In contrast, GS1b was relatively heat stable retaining more than 80% of its original activity after 20 min incubation at 53°C and still about 40% of activity after 10 min at 60°C.

Catalytic properties of the recombinant GS1 isoenzymes

We were also interested to know whether or not GS1a and GS1b isoenzymes differ in their kinetic parameters against substrates because this would be a direct evidence for different metabolic roles. Kinetic properties were determined by assaying biosynthetic activity as described in the Materials and Methods section and using GS fractions purified by DEAE-Sephacel chromatography and Sephacryl S-300 gel filtration. As shown in Table 1 GS1 isoforms differed in their kinetic behavior for ammonium, glutamate, and ATP. GS1b exhibited higher affinity for the ammonium ion (K_m value 80 μM) than GS1a (K_m value 200 μM). GS1b enzyme has an apparently strong affinity for glutamate when the concentrations of the substrate are low, but this affinity is progressively decreased when the concentration of glutamate is increased. This kinetic behavior that was analyzed by the Lineweaver-Burk and Eadie-Hofstee plots (Fig. 6A, B) was interpreted as a typical negative cooperativity (Kennet 1983) whose extent was estimated according to the Hill plot (nH 0.25) (Fig. 6C).

Discussion

Pine glutamine synthetase enzymes were overexpressed in bacteria to prevent the inconveniences that interfere with protein extraction procedures from woody plants, particularly from gymnosperm species. Conifer tissues contain abundant phenolic compounds, even in seedlings, and other secondary metabolites that greatly hinder the preparation of protein extracts suitable for normal enzymatic determinations. In this paper we present the production of recombinant GS1a and GS1b enzymes in bacteria, and a comparative analysis of their structural and kinetic properties. Our final goal is to highlight the physiological roles that each one could play, according to the differential expression profile of the two genes. This question has an especial interest due the redundancy of the genes encoding isoforms catalyzing the same metabolic reaction.

The growth of cells at low temperature is crucial to overproduce active pine GS in *E. coli*. The production of active recombinant enzyme was not possible under standard conditions at 37°C because the polypeptide was present as inclusion bodies in the bacteria (Cantón et al. 1996). These protein

Table 1 Kinetic parameters of GS1a and GS1b pine recombinant enzymes

	Ammonium	Glutamate	ATP
GS1a	K_m = 200 μM	K_m = 13 mM	K_m = 0.5 mM
GS1b	K_m = 80 μM	Negative cooperativity Hill index = 0.25	K_m = 2.7 mM

The biosynthetic assay was used (Cánovas et al. 1984).

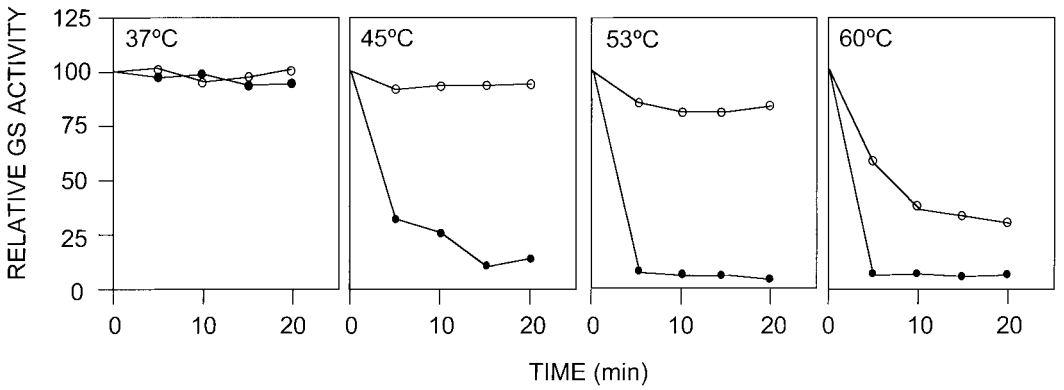

Fig. 5 Thermal stability of recombinant GS1a and GS1b. The holoenzymes were purified by ion-exchange chromatography, concentrated with ammonium sulfate and extensively dialyzed. Aliquots (12–15 µl) from fractions of GS1a (closed circle) and GS1b (open circle) were incubated at different temperatures: 37°C, 45°C, 53°C, and 60°C. At the indicated periods of incubation (5, 10, 15 and 20 min), samples were removed from the bath and stored on ice until GS activity was determined by the transferase assay. The 100% of GS1a and GS1b activities was 30 nkat and 15 nkat respectively.

aggregates are one of the classical problems found when enzymes are overexpressed in bacteria. The denatured protein chains accumulate as inclusion bodies, being recovered in the insoluble fraction of bacterial extracts. The final yield of native recombinant protein is function of a competition between aggregation and folding tendency, which is conditioned by the rate of recombinant protein synthesis. An intermediate state, as thermolabile partially folded product, could evolve toward two competitive possibilities: protein correctly folded or insoluble aggregate (King et al. 1996). Several factors influence the way finally followed but aggregation of recombinant polypeptides is favored by their tendency to hydrophobic association (Georgiou et al. 1994). The temperature seems to be a crucial factor: Zwanzig (1995) has considered a theoretical thermodynamic model of protein folding, concluding that lower temperatures favor correct native conformations. The technical problem has been treated by Weickert et al. (1997), who observed that the levels of soluble recombinant protein were enhanced by reducing the synthesis of recombinant protein. In addition, when a protein reaches its correct folded state it could be stable enough to remain soluble. Thus it is appropriate to lengthen the time of overproduction to avoid aggregation. Bennett and Cullimore (1990) have also reported a substantial production of active *Phaseolus vulgaris* GS when the bacteria were cultured at lower temperatures.

In the context of our results, it might be considered that the rate of recombinant GS1a synthesis could control the amount of folded product and therefore the availability of GS subunits in good shape to be assembled into the holoenzyme. In this way when the temperature of expression was lowered, the growth of cultures was retarded and the production of pine recombinant GS1a polypeptide diminished. These events would avoid protein aggregation and improve the conditions for a correct folding. This assumption is supported by a progressive shift of the GS1a protein from the insoluble to the soluble fraction and a parallel yield in GS activity (Fig. 1). We conclude that lowering the temperature and shaking slowly are a good combination to overproduce active recombinant enzymes.

The overexpression of functionally active GS enables us

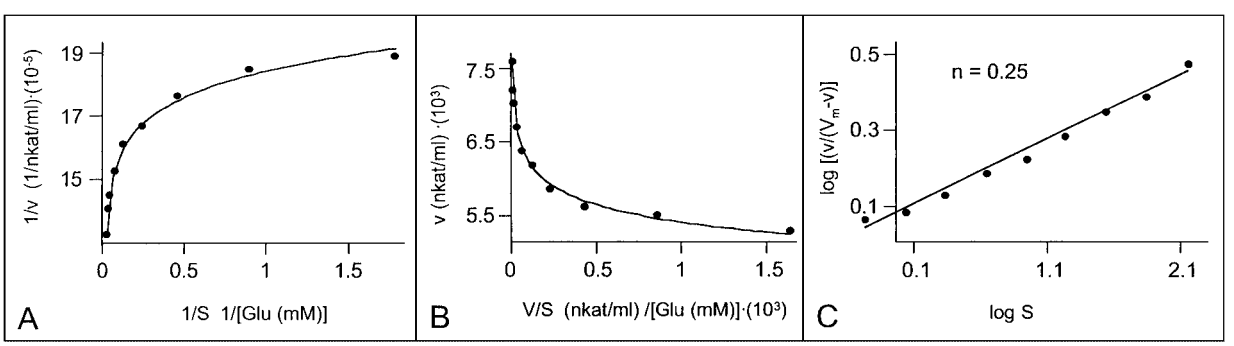

Fig. 6 Representation of kinetic characteristics of GS1b for the substrate glutamate. (A) Lineweaver-Burk plot. (B) Eadie-Hofstee plot. (C) Hill plot.

to complete a detailed study of their subunit composition and the comparison with native pine isoenzymes. The identity of each functional isoenzyme was determined as the corresponding in vivo expression product of a particular GS1 isogene. Our results demonstrate that GS1a and GS1b polypeptides are assembled into individual GS1a and GS1b homo-octameric enzymes in *E. coli* and pine cotyledons (Fig. 3, 4). This appears also to be the case in most plant tissues examined because GS1 isogenes showed non-overlapping expression patterns and individual GS1a and GS1b mRNAs were localized in different cellular types (Ávila et al. 2001), although the possible existence of hetero-octameric holoenzymes cannot be completely ruled out. These findings are in contrast to those found in some herbaceous plants, such as maize (Sakakibara et al. 1996) and *Medicago truncatula* (Carvalho et al. 1997) where expression products for members of the GS1 family are assembled into hetero-octameric holoenzymes.

The overexpression of pine GS1 also provided a good source of the isoenzymes in unlimited amounts for purification procedures and biochemical studies allowing us to perform comparative studies of the enzyme properties for each isoform. Taken together the results reported here suggest that the two pine enzymes present differences in stability. This differential feature became apparent even during the expression process in bacteria, thus, when overexpressed in the same experimental conditions at 10°C the final amount (in terms of specific activity) of recombinant GS1b was 3-fold greater than the GS1a isoform; moreover, recombinant GS1a was recovered at the insoluble fraction when synthesized at 37°C in the bacteria whereas substantial GS1b activity was found in the same conditions (results not shown). Differences in stability are additionally supported by the differential response to thermal inactivation of the holoenzymes (Fig. 5). GS1a was much more sensitive to heat than GS1b suggesting a higher degree of stability for the three-dimensional structure of this late isoform. The temperature inactivation behavior was similar to those reported for GS2 and GS1 isoforms in angiosperms (McNally and Hirel 1983).

Catalytic studies carried out with partial purified preparations of the two enzymes showed that they have different kinetic properties confirming they are different enzymes and these data are of interest for a better understanding of the respective physiological role of the enzymes in pine trees. Recently, it has been demonstrated that GS1a and GS1b genes have distinct expression patterns during plant development and their corresponding transcripts have been located in different cell types by in situ hybridization (Ávila et al. 2001, Suárez et al. 2002). The expression of GS1a mRNA is highly abundant in mesophyll cells of cotyledons, while the presence of GS1b transcripts is restricted to the vascular bundles. Recently, it has been reported that asparagine is the form of nitrogen that is transported from cotyledons to other parts of the pine seedling (King and Gifford 1997) and asparagine synthetase (AS) expression has been reported in the vascular bundles of rice (Nakano et al. 2000). Furthermore, an asparagine synthetase gene (psAS1) with a pattern of expression close to GS1b has been characterized in pine seedlings (Cañas, R., Cantón, F.R., Cánovas, F.M., unpublished). Therefore the available biochemical and molecular data suggest the existence of two metabolic cycles in developing pine seedlings: (1) a GS1a/GOGAT pathway implicated in ammonium assimilation and glutamate biosynthesis in mesophyll cells where a high demand of nitrogen compounds would be required for cell growth and proliferation; and (2) a GS1b/AS pathway involved in the biosynthesis of amides in vascular tissues for nitrogen transport to the growing apices.

Accordingly, it would be of interest to consider the results on kinetics (Table 1) presented here at the light of these recent findings. Negative cooperativity refers to the phenomenon in oligomeric enzymes in which the binding activities of ligands decrease as a function of ligand saturation. This kinetic behavior desensitizes the enzyme preventing fluctuations in the levels of the substrate being reflected in the final flux of the product (Levitzki and Koshland 1976). Thus, in the case of pine GS1b the consequence will be the desensitization of the enzyme for glutamate in such a way that enzyme activity would remain relatively constant in the presence of great cellular fluctuations of the substrate. Therefore, GS1b could act as a buffer enzyme able to provide a constant flux of glutamine in the cellular environment where it is located, the vascular bundles. This sustained supply of glutamine would be required for asparagine biosynthesis and nitrogen transport to sink tissues. This hypothesis would also be supported by the high affinity of the enzyme (K_m value of 80 μm) for ammonium. However, GS1a showed higher K_m values for ammonium and glutamate than GS1b (Table 1), and these results are consistent with the functional role of the enzyme in photosynthetic cells where high levels of ammonium ions should be incorporated to carbon skeletons for net glutamate biosynthesis.

Materials and Methods

General techniques

Chemicals, reagents and chromatographic resins were obtained from Sigma, St Louis (U.S.A.). Enzymes for molecular biology were from Roche Diagnostics, Barcelona (Spain). Media for bacterial culture were from DIFCO, Detroit (U.S.A.). Standard molecular biology manipulations, such as restriction digestions, ligations, bacterial cultures, plasmid preparations, agarose analytical electrophoresis, and restriction analysis were performed according to Sambrook and Russell (2001). The SDS-PAGE, 2D-PAGE and Western blot analysis were carried out as previously described (Ávila et al. 2001).

Construction of plasmids for bacterial expression of pine GS1 isoenzymes

The construction of plasmid to overexpress GS1a (pET3c-GS1a) was described elsewhere (Cantón et al. 1996). To construct pET3c-GS1b plasmid the original GS1b cDNA clone (pGSP15) (Ávila et al. 2000) was amplified by PCR using the forward 5′-CCC**ATTAA**-GTCTCTACTGACGGATTTGATC-3′ and reverse 5′-CCC**ATTAAT**-

CGGCATCTTTATTCATTGAG-3′ primers which corresponded to stretches from the 5′ and 3′ non-coding ends respectively, of the cDNA sequence. Amplifications were made with *Pfu* DNA Polymerase from Stratagene (La Jolla, CA, U.S.A.). The amplified DNA was digested with the restriction enzyme *Asn*I (bold in the oligonucleotide sequences), and then subcloned in the *Nde*I site of the pET3c vector, placing the ATG of the GS1b cDNA under transcriptional control of the ϕ-10 promoter of the T7 phage. The insertion was easily made because of the compatibility of the sticky ends generated by *Asn*I and *Nde*I DNA digestion. The constructions were transformed in the *E. coli* strain BL21(DE3). The definitive constructions with the correct orientation of inserts were selected by restriction analysis.

Expression of recombinant enzymes

Transformed BL21(DE3) bacteria with pET3c-GS1a or pET3c-GS1b were grown at 37°C in 3 liter of LB medium supplemented with ampicillin (100 μg ml^{-1}) and chloramphenicol (170 μg ml^{-1}). When the OD of cultures was 0.5 at 600 nm, the temperature was lowered at 28°C or 10°C and then 0.1 mM IPTG was supplied to induce the expression of the recombinant proteins. The incubation was prolonged for 12–14 h more by shaking at 200 rpm at the same temperature.

Extraction of proteins from bacteria

Cells were collected by centrifugation and resuspended in 5 volumes of buffer A (50 mM Tris pH 8, 5 mM 2-mercaptoethanol, 1 mM $MnCl_2$) for 1 volume of pellet. The bacteria were lysed by ultrasonication with a microprobe emitting 20 pulses of 3 s and identical intervals, at the intensity level 2 of a sonifier-250 (Branson Ultrasonics, U.S.A.). The soluble fraction was clarified by centrifugation (22,000×*g*, for 30 min).

Purification of recombinant enzymes

All experiments were carried out at 4°C. Proteins from the soluble fraction of bacterial extracts were precipitated with 20–60% $(NH_4)_2SO_4$, centrifuged at 22,000×*g* for 30 min, resuspended in Buffer A, and then dialyzed twice in the same buffer. Twenty mg of dialyzed protein were loaded onto an ion-exchange column (DEAE-Sephacel, 2.5×25 cm) equilibrated in buffer A (the buffer used for recombinant GS1a was 10 mM instead of 50 mM Tris). The enzymes were eluted by a linear 0–0.8 M KCl gradient. Fractions showing GS activities were pooled, concentrated by ammonium sulfate precipitation, dialyzed in buffer A, and stored in 20% (v/v) glycerol for use in kinetic measurements. Molecular masses of GS1a and GS1b holoenzymes (native and recombinant) were calculated by gel filtration through a Sephacryl S-300 column (1.6 cm ×1.0 m) equilibrated in buffer A and calibrated with proteins of known molecular mass.

Determination of GS activity and protein quantification

The protein concentration was estimated by the method of Bradford (1976). GS activity was determined by the transferase assay and catalytic properties were studied by means of the biosynthetic assay (Cánovas et al. 1984).

Acknowledgments

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