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Glutamine synthetase isoforms in nitrogen-fixing soybean nodules: Distinct oligomeric structures and thiol-based regulation

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ABSTRACT

Legume root nodule glutamine synthetase (GS) catalyzes the assimilation of ammonia produced by nitrogen fixation. Two GS isoform subtypes ($GS_1\beta$ and $GS_1\gamma$) are present in soybean nodules. $GS_1\gamma$ isoforms differ from $GS_1\beta$ isoforms in terms of their susceptibility to reversible inhibition by intersubunit disulfide bond formation between C159 and C92 at the shared active site at subunit interfaces. Although nodule GS enzymes share 86% amino acid sequence identity, analytical ultracentrifugation experiments showed that $GS_1\gamma$ is a dodecamer, whereas the $GS_1\beta$ is a decamer. It is proposed that this difference contributes to the differential thiol sensitivity of each isoform, and that $GS_1\gamma1$ may be a target of thiol-based regulation.

1. Introduction

Glutamine synthetase (GS) (EC 6.3.1.2) catalyzes the formation of glutamine from glutamate and ammonia using ATP as an energy source, and is a critical enzyme for the assimilation of environmental and metabolic sources of ammonia [1]. In plants, GS is encoded as a multigene family with two major isoform classes that are distinguished by their subcellular location, with GS_1 found in the cytosol, while GS_2 resides in plastids [1,2]. Cytosolic GS_1 in legumes are encoded by a small, highly conserved gene family with three GS_1 isoform classes (designated α, β and γ) [2,3]. With respect to nitrogen-fixing soybean root nodules, transcriptional regulation of GS_1 isoforms has been observed during nodule development and in response to developmental and metabolic cues [4,5]. The $GS_1\beta$ isoforms exhibits a broad expression pattern in soybean tissues, but shows particularly high expression in mature nitrogen fixing nodules, and is inducible by high levels of ammonia. In contrast, the $GS_1\gamma$ isoforms are selectively expressed as nodulin proteins in a developmentally regulated fashion during soybean nodule formation. Expression of the GS_1 isoforms during soybean nodule development coincide with the onset of nitrogen fixation [4], consistent with their role as the major enzyme responsible for the ATP-dependent assimilation of fixed ammonia which is released from nitrogen-fixing bacteroids into the plant host cytosol.

The reason for the diversity of cytosolic GS isoforms in nitrogen-fixing nodules, and the potential metabolic function and regulation of each, is not clear. In the present study, it is shown that $GS_1\gamma$ isoforms from soybean nodules differ from the $GS_1\beta$ isoforms in sensitivity to reversible inhibition of enzymatic activity by disulfide oxidation. The potential significance of this observation with respect to posttranslational regulation of GS in nodules is discussed.

2. Materials and methods

2.1. Molecular cloning techniques

Total RNA from soybean nodules was prepared by using plant RNA reagent (Invitrogen) and cDNA was synthesized with a Superscript II reverse transcription kit (Invitrogen) according to manufacturer's instructions. cDNAs containing the open reading frames of soybean cytosolic $GS_1\beta1$ and $GS_1\gamma1$ isoforms were amplified using gene specific primers and were cloned into the pCR2.1-TOPO (Invitrogen) vector. These cDNAs were sub-cloned into the *NheI* and *NotI* restriction sites of the pET28a expression vector (Novagen) in frame with an amino terminal his-tag linker.

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PCR-based site directed mutagenesis was done by using the method of Sambrook and Russell [6]. Oligonucleotides used for molecular cloning and site-directed mutagenesis are listed in Supplementary Table S1. Analysis and verification of DNA sequences was done by automated sequencing using a Perkin-Elmer Applied Biosystems 373 DNA sequencer at the University of Tennessee Molecular Biology Resource Facility, Knoxville, TN.

2.2. GS purification and activity analyses

Expression and purification of recombinant GS_1 was performed as described previously [7]. GS activity was assayed by the determination of the release of inorganic phosphate as described by Gawronski and Benson [8] in standard activity conditions consisting of 100 mM MOPS-NaOH, 50 mM sodium glutamate, 50 mM $MgCl_2$, 10 mM ATP, 50 mM NH_4Cl, pH 7.5 at 37 °C for 5 min. Kinetic studies were performed under by varying the concentrations of each of the three substrates independently while the concentration of other two substrates was kept at the standard concentrations described above. The data were fit to the Michaelis–Menten equation assuming pseudo first order conditions.

To determine the effect of reducing and oxidizing reagents on activity, purified GS_1 isoforms were incubated with reducing agent (25 mM β-mercaptoethanol [βME] or 4 mM dithiothreitol [DTT]) or oxidizing agent (3 mM H_2O_2) in DB buffer (20 mM Tris–HCl, pH 7.5, 300 mM NaCl, 10% [v/v] glycerol) for 30 min on ice. With respect to treatment with reducing agent, identical results were obtained regardless of whether βME or DTT were used. To determine the reversibility of thiol-based oxidation/reduction, DTT-reduced GS1γ1 was incubated with 40 mM oxidized glutathione (GSSG) for 30 min prior to activity analysis. Air oxidation of GS_1 was done by dialysis of samples in DB buffer in the absence of reducing agent at 4 °C for 16 h.

2.3. Analytical ultracentrifugation (AUC)

AUC experiments were performed with an Optima XL-I Beckman Coulter analytical ultracentrifuge equipped with an AN50Ti rotor. For sedimentation velocity experiments, 0.5 mg/ml protein samples in 20 mM Tris–HCl, pH 7.5, 20 mM imidazole, 10% [v/v] glycerol, 300 mM NaCl were centrifuged at 30000 rpm at 22 °C and absorbance optics were measured at 280 nm. Two hundred scans were recorded in 1 min intervals and data were analyzed using the continuous $c(M)$ distribution model described by the Lamm equation (Eq. (1)) with the SEDFIT software (http://www.AnalyticalUltracentrifugation.com) [9,10].

$$\min_{c(M)}\left\{\sum_{i,j}[a(r_i,t_j)-\int c(M)\mathrm{L}(M,r_i,t_j)dM]^2\right\} \quad (1)$$

L(M,r,t) denotes the sedimentation profile of a monodisperse species of size M at radius r and time t. $a(r, t)$ denotes the experimentally observed signal. The analysis covered a molecular weight range of 0–2000kDa with a resolution of 100 and a confidence level (F-ratio) of 0.95. The best fit was assumed when Z values below 40 and rmsd values below 0.01 absorbance units were obtained. The buffer density, viscosity, and partial specific volume were determined using the program SEDNTERP (http://sednterp.unh.edu/).

Sedimentation equilibrium experiments were performed using different concentrations of GS_1 protein (0.68 mg/ml, 0.34 mg/ml and 0.23 mg/ml in 20 mM Tris–HCl, pH 7.5, 300 mM NaCl) at 10 °C at 3800, 6000 and 7500 rpm. Absorbance optics was collected at 6 h intervals by averaging 20 scans at 282 nm until equilibrium was achieved (between 30 and 48 h). Molecular weights of each isoform were calculated by global analysis using the SEDPHAT software (http://www.AnalyticalUltracentrifugation.com) with the best fit of the data done by using the discrete species model [11]. A good fit was obtained using the discrete species model in SEDPHAT with rmsd values below 0.007 and chi-squared values near 1.

2.4. Homology modeling

Homology models for soybean GS_1 were prepared by using the molecular operating environment (MOE) software package (MOE 2008.10; Chemical Computing Group Inc) by the general approach described in [12]. The atomic structure of maize glutamine synthetase GS1a (pdb 2d3a) was used as a structural template [13]. Ten models were generated using the AMBER99 force field with medium model refinement. The quality of the models was checked for disallowed ϕ, ψ angles using the Ramchandran plot function in the MOE software. The model chosen showed a backbone rmsd < 1 Å with the GS structural template, and showed a single residue (Asp137, a solvent exposed residue in a non-essential loop region distal to the active site) with a disallowed angle.

2.5. Other analytical methods

Q-PCR was performed by the comparative threshold cycle (Ct) method as previously described [14]. The *GmCRK* gene was used as an internal reference for standardization as described in [15]. All the primers used for Q-PCR analysis are shown in Supplementary Table S1.

GS_1 proteins (5 μg) were analyzed using reducing and non-reducing SDS–PAGE to determine the effect of oxidation by using the buffer system of Laemmli [16] under reducing (100 mM DTT) or non-reducing (no DTT) conditions. For native PAGE analysis, protein samples (5 μg) in 25% [v/v] glycerol, 0.25 M Tris–HCl, pH 6.8, 0.015% [w/v] bromophenol blue were resolved on 6% [w/v] polyacrylamide gels. Electrophoresis was performed with 50 mM Tris–HCl, pH 8.9, 70 mM glycine as the cathode buffer and 100 mM Tris–HCl, pH 7.8 as the anode buffer at a constant current of 25 mA at 4 °C. The presence or absence of reducing agent (DTT) did not affect the electrophoretic mobility profile of GS_1 proteins on native gels.

The concentration of free cysteine residues in oxidized and reduced $GS_1\gamma1$ was determined by using Ellman's reagent [17]. The number of free cysteine residues per monomer was calculated from the protein concentration using the monomeric subunit molecular weight of recombinant $GS_1\gamma1$ (41.63kDa). Protein concentrations were determined by the Bradford assay [18].

3. Results

3.1. Comparison of the expression and enzymatic properties of soybean nodule $GS_1\beta$ and $GS_1\gamma$ isoforms

In mature soybean nodules, two representatives each of the β ($GS_1\beta1$, $GS_1\beta2$) and γ ($GS_1\gamma1$, and $GS_1\gamma2$) isoform subclasses of GS_1 are expressed (Table 1). Q-PCR analysis shows that $GS_1\beta$ isoforms are the predominant transcript present (86%) while the $GS_1\gamma$ isoforms represent a nodule-specific component which is a smaller fraction of the total nodule GS_1 transcript pool (14%), (Table 1). To investigate the comparative enzymatic and functional properties of β and γ isoform subclasses, recombinant $GS_1\beta1$ and $GS_1\gamma1$ were expressed and purified from *Escherichia coli*.

It has been previously observed from the investigation of *Arabidopsis* cytosolic glutamine synthetases that minor differences in amino acid sequence can cause large changes (>250-fold) in the K_m for the critical substrate ammonia [19]. Comparison of the k_{cat} and the K_m for ammonia, glutamate and ATP showed that unlike their *Arabidopsis* counterparts, the soybean nodule GS_1 isoforms