Photosynthesis Research (2005) 83: 191–217

Review

Glutamate synthase: structural, mechanistic and regulatory properties, and role in the amino acid metabolism

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Received 28 June 2004; accepted in revised form 20 September 2004

Key words: ammonium assimilation, glutamate synthase, glutamine synthetase, higher plants, nitrogen metabolism

Abstract

Ammonium ion assimilation constitutes a central metabolic pathway in many organisms, and glutamate synthase, in concert with glutamine synthetase (GS, EC 6.3.1.2), plays the primary role of ammonium ion incorporation into glutamine and glutamate. Glutamate synthase occurs in three forms that can be distinguished based on whether they use NADPH (NADPH-GOGAT, EC 1.4.1.13), NADH (NADH-GOGAT, EC 1.4.1.14) or reduced ferredoxin (Fd-GOGAT, EC 1.4.7.1) as the electron donor for the (two-electron) conversion of L-glutamine plus 2-oxoglutarate to L-glutamate. The distribution of these three forms of glutamate synthase in different tissues is quite specific to the organism in question. Gene structures have been determined for Fd-, NADH- and NADPH-dependent glutamate synthases from different organisms, as shown by searches in nucleic acid sequence data banks. Fd-glutamate synthase contains two electron-carrying prosthetic groups, the redox properties of which are discussed. A description of the ferredoxin binding by Fd-glutamate synthase is also presented. In plants, including nitrogen-fixing legumes, Fd-glutamate synthase and NADH-glutamate synthase supply glutamate during the nitrogen assimilation and translocation. The biological functions of Fd-glutamate synthase and NADH-glutamate synthase, which show a highly tissue-specific distribution pattern, are tightly related to the regulation by the light and metabolite sensing systems. Analysis of mutants and transgenic studies have provided insights into the primary individual functions of Fd-glutamate synthase and NADH-glutamate synthase. These studies also provided evidence that glutamate dehydrogenase (NADH-GDH, EC 1.4.1.2) does not represent a significant alternate route for glutamate formation in plants. Taken together, biochemical analysis and genetic and molecular data imply that Fd-glutamate synthase incorporates photorespiratory and non-photorespiratory ammonium and provides nitrogen for transport to maintain nitrogen status in plants. Fd-glutamate synthase also plays a role that is redundant, in several important aspects, to that played by NADH-glutamate synthase in ammonium assimilation and nitrogen transport.

Abbreviations: ASN – asparagine synthetase gene; CD – circular dichroism; EPR – electron paramagnetic resonance; ΔG – free energy change; ΔS – entropy change; FAD – flavin adenine dinucleotide; Fd – ferredoxin; FMN – flavin mononucleodide; Fe/S cluster – iron-sulfur cluster; FNR – ferredoxin: $NADP^+$ oxidoreductase; GAT – glutamine amidotransferase; GDH – glutamate dehydrogenase; GOGAT – glutamate synthase; *GLN1(2)* – cytosolic (chloroplastic) glutamine synthetase gene; *glsF* – ferredoxin-glutamate synthase gene; *GLT* – NADH-glutamate synthase gene; *gltB* – NADPH-glutamate synthase α subunit

gene; *gltD* – NADPH-glutamate synthase β subunit gene; GltS – glutamate synthase; *gltS* – Fd-glutamate synthase gene; *GLUI (2)* – ferredoxin-glutamate synthase 1(2) gene; GS1(2) – cytosolic (chloroplastic/plastidial) glutamine synthetase; *NAR* – nitrate reductase gene; *NIR* – nitrite reductase gene; Rubisco – ribulose-l,5-bisphosphate carboxylase/oxygenase

Introduction

Plants utilize inorganic nitrogen in the form of nitrate (NO_3^-) and ammonium ion (NH_4^+), when the latter is available in the soil or from the symbiotic fixation of atmospheric dinitrogen (N_2) into NH_4^+ in root nodules of leguminous species. Nitrate is reduced to nitrite, in a NAD(P)H-dependent reaction, catalyzed by nitrate reductase (NADH-NAR, EC 1.6.6.1; NAD(P)H-NAR, EC 1.6.6.2; NADPH-NAR, EC 1.6.6.3) in the cytosol. Nitrite is subsequently reduced to NH_4^+ in a ferredoxin (Fd)-dependent reaction catalyzed by Fd-dependent nitrite reductase (NIR, EC 1.6.6.4) in the chloroplast or plastid. Ammonium ion is the final form of inorganic nitrogen and the nitrogen present in all organic nitrogen compounds, such as amino acids and nucleic acids, is derived from NH_4^+ (Lea et al. 1990). Ammonium is released and then re-assimilated during nitrogen mobilization in germinating seeds, during the photorespiratory conversion of glycine to serine in the cells of growing leaf, and during nitrogen remobilization from sources to sinks (Ireland and Lea 1999). The assimilation of NH_4^+ into glutamine and glutamate is the crucial step in amino acid synthesis and nitrogen metabolism. Glutamine synthetase (GS, EC 6.3.1.2) catalyzes the first step of NH_4^+ incorporation into glutamate using ATP to yield glutamine in the cytosol (GS1), in chloroplasts and in plastids (GS2).

Glutamate synthase (glutamine: 2-oxoglutarate amidotransferase, henceforth abbreviated as either GOGAT or GltS) transfers the amide-nitrogen of L-glutamine to 2-oxoglutarate, providing two molecules of L-glutamate. Glutamate synthase in plants is present in two distinct forms, one that uses reduced ferredoxin as the electron donor (Fd-GOGAT/Fd-GltS, EC 1.4.7.1) and one that uses NADH as the electron donor (NADH-GOGAT/NADH-GltS, EC 1.4.1.14). A third form, which uses NADPH as the electron donor (NADPH-GOGAT/NADPH-GltS, EC 1.4.1.13) is found in bacteria (Reitzer 1996). Both the Fd-glutamate synthase and NADH-glutamate synthase are located in the chloroplast or plastid (Oliveira et al. 1997). GS and glutamate synthase occur in multiple forms encoded by distinct genes (Lam et al. 1996). Although there is some redundancy of function among the multiple enzyme forms, for the most part each form of GS and glutamate synthase plays a distinct physiological role *in vivo* during nitrogen absorption in roots, N_2-fixation in root nodules, primary NO_3^- reduction, photorespiratory nitrogen cycling and nitrogen translocation (Vance et al. 1994; Lam et al. 1996).

An alternative pathway for the formation of glutamate involves the reductive amination of 2-oxoglutarate by NH_4^+, catalyzed by mitochondrial glutamate dehydrogenase (NADH-GDH, EC 1.4.1.2). However, the role of GDH in plant cells remains controversial (Fox et al. 1995; Melo-Olivera et al. 1996; Miflin and Habash 2002). Molecular genetic and biochemical studies using ^{13}N- or ^{15}N-radiorabeled tracers, enzyme inhibitors, and mutants, as well as studies using transgenic plants affected in GS, glutamate synthase or GDH all indicate that the GS/glutamate synthase cycle is the primary pathway for NH_4^+ assimilation (Ratcliffe and Shachar-Hill 2001; Lea and Miflin 2004). Also, expression analysis revealed that the plants display cell-specific and organ-specific patterns for expression of GS and glutamate synthase genes by sensing the light and metabolite signals in the regulation of *in vivo* function of GS and glutamate synthase isoforms (Edwards et al. 1990; Thum et al. 2003). The amino-nitrogen of glutamate, incorporated into the carbon skeleton by the sequential reaction of GS and glutamate synthase, then serves as the source of the amino groups of aspartate and alanine, formed by the transamination of oxaloacetate and pyruvate, respectively (Reitzer 1996). As amino acid synthesis is controlled by availability of carbon skeletons, nitrogen assimilation is tightly coupled to carbon metabolism. Glutamate,

aspartate and alanine then provide the nitrogen required for the formation of other amino acids. The amide-nitrogen of glutamine is used for the biosynthesis of amino acids, including the formation of asparagine from aspartate (Ireland and Lea 1999). Glutamine, asparagine, glutamate and aspartate are the major amino acids in leaves and roots and are transported in the vascular tissues to control the nitrogen status during growth and development of plants (Pate and Layzell 1990).

In this review, we will analyze the current information on the distribution of different types of glutamate synthase in prokaryotes and eukaryotes. As reaction mechanisms and structural aspects of glutamate synthase will be discussed in the accompanying article by Vanoni et al., we will present instead a summary of the current state of knowledge of the oxidation-reduction properties of Fd-glutamate synthase and of its mode of interaction with ferredoxin. We will also describe the regulatory properties of glutamate synthase in higher plants with regard to defining the biological role of the enzyme in the nitrogen assimilation and nitrogen translocation in higher plants.

Occurrence of glutamate synthases

Two distinct classes of glutamate synthase are distinguished in higher plants: Fd-glutamate synthase and NADH-glutamate synthase, both of which are located in the chloroplast or plastid. Biochemical and molecular analyses detected both Fd-glutamate synthase and NADH-glutamate synthase in germinating seeds (Grevarec et al. 2004), roots (Redinbaugh and Campbell 1993), root nodules (Chen and Cullimore 1988; Anderson et al. 1989; Vance et al. 1995; Cordovilla et al. 2000), cotyledons (Turano and Muhitch 1999), etiolated shoots and leaves (Sakakibara et al. 1992a; Yamaya et al. 1992), green leaves (Sakakibara et al. 1992b), cultured tobacco cells (Suzuki et al. 1982; Hayakawa et al. 1992). It has been reported that purified NADH-glutamate synthase is either inactive with NADPH as an electron donor (Hayakawa et al. 1992) or displays very low activity (0.8–2.6% of the NADH-dependent activity) (Chen and Cullimore 1988). NADPH-linked glutamate synthase activity has been detected in seeds (Murray and Kennedy 1980), endosperms (Oaks et al. 1979), roots (Oaks et al. 1979), root nodules (Chen and Cullimore 1988) and cultured soybean cells (Chiu and Shargool 1979). Although the results of these biochemical studies suggest the presence of NADPH-glutamate synthase in these tissues, NADPH-glutamate synthase protein has not yet been unambiguously identified in either photosynthetic or non-photosynthetic tissues of any higher plant. It should also be pointed out that no open reading frame coding for NADPH-glutamate synthase has been detected in the *Arabidopsis* genome database.

Fd-glutamate synthase and NADH-glutamate synthase from the green alga *Chlamydomonas reinhardtii* have both been characterized (Galván et al. 1984; Márquez et al. 1984). Fd-glutamate synthase has been detected in the chloroplast of green alga *Caulerpa simpliciuscula* (McKenzie et al. 1979). The plastid genome of red algae contains a structural gene of *glsF* (or *gltB* according to the authors) for Fd-glutamate synthase in *Antithamnion* sp. (Valentin et al. 1993) and *gltB* in *Porphyra purpurea* (Reith and Munholland 1993). Fd-glutamate synthase activity has been detected in the cyanobacterium *Synechococcus* sp. PCC 6301 (Marqués et al. 1992). It was reported that unicellular cyanobacterium *Synechocystis* sp. PCC 6803 contains two putative Fd-glutamate synthase genes, *gltB* and *glsF* (later renamed *gltS* after it was confirmed that it encodes a Fd-glutamate synthase) (Navarro et al. 1995). However, the genome of *Synechocystis* sp. PCC 6803 contains a sequence similar to the *gltD* encoding the β-like subunit of bacterial NADPH-glutamate synthase (Kaneko et al. 1996). Therefore, it may be that the gene originally thought to encode a second Fd-glutamate synthase in *Synechocysitis* sp. PCC 6803 actually codes for the α subunit of αβ heterodimeric glutamate synthase, which is active with NADH as the electron donor rather than with NADPH (Navarro et al. 2000). Both the *glsF* for Fd-glutamate synthase, and the *gltB* and *gltD* have been cloned from the cyanobacterium *Plectonema boryanum* (Okuhara et al. 1999). The *gltB* and *gltD* encode the dissimilar α and β subunits, respectively, of NADH-glutamate synthase (Okuhara et al. 1999). The complete genome of cyanobacterium *Anabaena* sp. PCC 7120 has been sequenced, showing that *glsF* encoding Fd-glutamate synthase is the unique glutamate synthase gene (Martin-Figueroa et al.

2000), and to date genes for NADH-glutamate synthase have not been found in this cyanobacterium. In contrast, only NADH-glutamate synthase has been detected in fungi and yeast such as *Neurospora crassa* (Hummelt and Mora 1980), *Saccharomyces cerevisiae* (Cogoni et al. 1995; Filetici et al. 1996) and *Kluyveromyces lactis* (Romero et al. 2000), where it appears to be present as a monomeric protein of high molecular weight.

Bacterial NADPH-glutamate synthases are heterodimeric proteins. The structural *gltB* and *gltD* genes, coding for the large α subunit and small β subunit, respectively, have been cloned from *Escherichia coli* (Oliver et al. 1987; Castaño et al. 1992), *Azospirillum brasilense* (Pelanda et al. 1993), *Salmonella typhimurium* (Madonna et al. 1985), *Thiobacillus ferrooxidans* (Deane and Rawling 1996), *Rhizobium etli* (Castillo et al. 2000). A different nomenclature has been used for the genes of *Bacillus subtilis*, where the names *gltA* and *gltB* have been used to designate the genes encoding the α subunit and β subunit, respectively (Belitsky et al. 1995). NADPH-glutamate synthase is also a αβ heterodimeric protein in *Aerobacter aerogenes* (Geary and Meister 1977), *Bacillus magaterium* (Hemmilä and Mäntsälä 1978), *Rhodospirillum rubrun* (Carlberg and Nordlund 1991), and other bacteria (Brenchley et al. 1975; Okon et al. 1976; Smith et al. 1977; Ely et al. 1978; Vanoni et al. 1990). However, NADH-glutamate synthase activity has been detected in some nitrogen-fixing bacteria (Nagatani et al. 1971) and in three species of *Chromatiaceae* (Bast 1977). The genomes of the archaeal bacteria *Methanococcus jannaschii* (Bult et al. 1996) and *Archaeoglobus fulgidus* (Klenk et al. 1997) both contain a sequence similar to the *gltB* for a bacterial NADPH-glutamate synthase α subunit. These archaeal sequences of approximately 500 amino acid residues resemble the FMN-binding region and C-terminal cysteine-rich region of the *gltB*-encoded α subunit of NADPH-glutamate synthase and *gltS*-encoded Fd-glutamate synthase (Vanoni and Curti 1999). Genomic DNA sequences similar to the *gltB* and *gltS* have been identified in *Methanococcus thermoautotrophicum* (Smith et al. 1997). Another archaeal glutamate synthase has been cloned from *Pyrococcus* sp. KOD1, and this glutamate synthase protein has been expressed in E. *coli*. Homologies between the *Pyrococcus* sp. KODI glutamate synthase and the β subunit of the bacterial NADPH-glutamate synthase have been reported, but no genes with any significant homology to *gltB* have been detected in *Pyrococcus* sp. KOD1 (Jongsareejit et al. 1997). The *Pyroccocus horikoshii* OT3 genome contains a sequence homologous to *gltD* (Kawarabayashi et al. 1998). The presence of one of the *gltB* and *gltD* genes and the corresponding subunits in different archaea suggests that NAD(P)H-glutamate synthase of eubacteria and eukaryotes could have originated from genes of the two different species (Dincturk and Knaff 2000). NADH-glutamate synthase activity is found in animal cells (Seshachalam et al. 1992; Hirayama et al. 1998; Doverskog et al. 2000). A monomeric NADH-glutamate synthase of high molecular mass of 190–195 kDa has been shown to be present in the body fat of the silkworm *Bombyx mori* (Hirayama et al. 1998).

Gene and primary protein structure of glutamate synthases

DNA sequences for genes and for cDNA have been characterized for Fd-glutamate synthase and NADH-glutamate synthase from several plant species, and those of *Arabidopsis* glutamate synthase are available from the complete sequence of *Arabidopsis* genome. Fd-glutamate synthase genes (*GLU*, Fd-*gltS*) are homologous to *gltB*, which codes for the α subunit of bacterial glutamate synthase. In contrast, no homologies exist between Fd-glutamate synthase genes and *gltD*, the gene encoding the β subunit in the αβ protomer of bacterial NADPH-glutamate synthase (Table 1). In *Arabidopsis*, Fd-glutamate synthase is encoded by two genes; *GLU1* and *GLU2*, which are located on chromosome 5 and 2, respectively. The *GLU1* cDNA has an ORF encoding a 1648-amino acid precursor protein (180.1 kDa). It consists of a 131 - amino acid transit peptide (14.6 kDa) and a 1517-amino acid mature peptide (165.5 kDa) (Suzuki and Rothstein 1997). Another reported cloning of *GLU1* cDNA revealed a nearly identical sequence except for the presence of a 75 bp sequence (nucleotides 277–351, located between amino acids 94 and 122) that is likely to be an intron (Coschigano et al. 1998). By alignment with the cDNA sequence of the *GLU1* isoform, it has been determined that the *Arabidopsis GLU1* gene is transcribed as a 8590-

Table 1. Genes encoding glutamate synthases. Denomination of the gene and the protein of gene product corresponds to the term employed in the literature and in the GenBank database. Sequence information was obtained from the GenBank database using the accession number or loci indicated

Gene (acronym)	Protein (acronym)
Higher plants	
GLU/*glu*/*gluS*/*gltS*	Fd-glutamate synthase/Fd-GOGAT Green leaves, etiolated leaves/shoots, roots, N_2-fixing nodules (M59190, Y09667, U03006, U39287, AY189525, AF039851, U39288, At5g04140, At2g41220)
GLT	NADH-glutamate synthase/NADH-GOGAT Green leaves, etiolated leaves/shoots, roots, N_2-fixing nodules (L01660, L37606, AB008845: AB001916, AK110476, At5g53460)
Algae	
glsF or *gltB*	Fd-glutamate synthase/Fd-GOGAT *Chlamydomonas reinhardtii* (AF135592), *Antithamnion* sp. (Z21705, Z75242), *Caulerpa simpliciuscula*
gltB	*Porphyra purpurea* (U38804)
Cyanobacteria	
glsF or *gltS*	Fd-glutamate synthase/Fd-GOGAT *Synechocystis* sp. PCC 6803 (X92480), *Plectonema boryanum* (D85735), *Anabaena* sp. PCC 7120 (AJ249913)
gltB and *gltD*	NADH-glutamate synthase/NADH-GOGAT *Plectonema boryanum* (D85230), *Synechocystis* sp. PCC 6803 (X80485)
Fungi	
*gltB*D	NADH-glutamate synthase/NADH-GOGAT *Neurospora crassa* (XM328182, AL356815)
Yeasts	
*GltB*D, *GLT1*	NADH-glutamate synthase/NADH-GOGAT *Saccharomyces cerevisiae* (X89221)
Bacteria	
gltB and *gltD*	NADH-glutamate synthase/NADPH-GOGAT/NADPH-GltS *Escherichia coli* (M18747, L20253, M68876), *Azospirillum brasilense* (AF192408, X71090), *Pseudomonas aeruginosa* PA01 (AE004916), *Preudomonas aeruginosa* (U81261, AE004916), *Salmonella typhimurium* (AE008853), *Thiobacillus ferrooxidans* (U36427), *Rhizobium etli* (AF107264), *Chromatiaceae*
gltA and *gltB*	NADPH-glutamate synthase/NADPH-GOGAT/NADPH-GltS *Bacillus subtilis* (M28509)
Archaeal bacteria	
gltB	NADPH-glutamate synthase/NADPH-GOGAT/NADPH-GltS *Methanococcus jannaschii* (U67575), *Archaeoglobus fulgidus* (AE001038), *Methanococcus thermo-autotrophicum* (AE000800),
gltD or *gltA*	*Pyrococcus* sp. KOD1, *Pyroccocus horikoshii* OT3 (PH1873)
Insect	
*gltB*D	NADH-glutamate synthase/NADH-GOGAT/NADH-GltS silkworm *Bombyx mor*

Higher plants/M59190: *Zea mays* Fd-glutamate synthase mRNA; L01660: *Medicago sativa* NADH-glutamate synthase mRNA; L37606: *Medicago sativa* NADH-glutamate synthase gene; U03006: *Spinacia aleracea* Fd-glutamate synthase mRNA; Y09667: *Arabidopsis* Fd-glutamate synthase *GLU1* mRNA; U39287: *Arabidopsis* Fd-glutamate synthase *GLU1* mRNA; AY189525: *Arabidopsis* Fd-glutamate synthase *GLU1* gene; At5g04140: *Arabidopsis* Fd-glutamate synthase *GLU1* gene; AF039851: *Glycine max*: Fd-glutamate synthase *glu* mRNA; U39288: *Arabidopsis* Fd-glutamate synthase *GLU2* mRNA; At2g41220: *Arabidopsis* Fd-glutamate synthase *GLU2* gene; At5g53460: *Arabidopsis* NADH-glutamate synthase *GLT1* gene; AB008845: *Oryza sativa* NADH-glutamate synthase mRNA; AB001916: *Oryza sativa* NADH-glutamatee synthase gene; AK110476: *Oryza sativa* cDNA clone (002-166-H10) – Algae/AF135592: *Chlamydomonas reinhardtii* putative Fd-glutamate synthase gene; Z75242: *Antitamnion* sp. glutamate synthase *gltB* gene; Z21705: *Antitamnion* sp. chloroplast Fd-glutamate synthase *gltB* gene – Cyanobacteria/X92480: *Synechocystis* sp. Fd-glutamate synthase *gltS* gene; X80485: *Synechocystis* sp. Fd-glutamate synthase *gltB* gene; D78371: *Synechocystis* sp. putative Fd-glutamate

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synthase gene; D85735: *Plectonema boryanum* Fd-glutamate synthase *glsF* gene; D85230: *Plectonema boryanum* URF141 NADH-glutamate synthase *gltB* and *gltD* genes; AJ249913: *Anabaena* sp. PCC 7120 Fd-glutamate synthase *gltS* gene – Fungi/XM328182: *Neurospora crassa* OR74A putative glutamate synthase mRNA; AL356815: *Neurospora crassa* putative NADPH-glutamate synthase *glt1* gene (BAC clone B24H17) – Yeast/X89221: *Saccharomyces cerevisiae* glutamate synthase gene – Bacteria/M18747: *Escherichia coli* glutamate synthase *gltB* and *gltD* genes; L20253: *Escherichia coli* glutamate synthase *gltB* gene; M68876: *Escherichia coli* glutamate synthase *gltB* gene; AF192408: *Azospirillum brasilense* NADPH-glutamate synthase *gltB* and *gltD* genes; X71090: *Azospirillum brasilense* NADPH-glutamate synthase *gltB* gene; U81261: *Preudomonas aeruginosa* NADPH-glutamate synthase *gltB* and *gltD* genes; AE004916: *Preudomonas aeruginosa* PA01 NADPH-glutamate synthase *gltD* gene; AE008853: *Saimonella typhimurium* LT2 glutamate synthase *gltD* gene (complete genome, section 157 of 220); U36327: *Thiobacillus ferrooxidants* glutamate synthase *gltB* and. *gltD* genes; AF107264: *Rhizobium etli* glutamate synthase *gltB* and *gltD* genes; M28509: *Bacillus subtilis* glutamate synthase *gltA* gene – Archaeal bacteria/U67575: *Methanococcus jannaschii* NADPH-glutamate synthase *gltB* gene; AE001038: *Archaeoglobus fulgidus* DSM 4304 glutamate synthase *gltB* gene; AE000800: *Methanobacterium thermoautotrophicum* genome glutamate synthase *gltB* gene; PH 1873: *Pyrococcus horikoshii* glutamate synthase *gltD* gene.

nucleotide mRNA, which consists of a 5′-untranslated region (5′-UTR) (179 nucleotides), a coding sequence (8154 nucleotides) and a 3′-UTR (257 nucleotides) (unpublished data and At5g04140). The transcribed region of *GLU1* is composed of 33 exons interrupted by 32 introns (Figure 1). The cDNA of the *GLU2* isoform has an ORF which encodes a 1629-amino acid precursor peptide (177.8 kDa) (Coschigano et al. 1998). The *GLU2* gene of 10728 nucleotides contains a 5′-UTR (274 nucleotides) and the coding sequence includes 33 exons and 32 introns (Figure 1). The predicted amino acid sequences of *GLU1* and *GLU2* isoforms are 80% identical. The cDNA for maize Fd-glutamate synthase has an ORF encoding a 1616-amino acid precursor peptide (174.7 kDa). The mature protein consists of 1519 amino acids with a molecular mass of 165.3 kDa (Sakakibara et al. 1991). The mature form of spinach Fd-glutamate synthase has been shown to contain 1504 amino acids and the mature forms of the maize and spinach Fd-glutamate synthases are 83% identical at the amino acid level (Nalbantoglu et al. 1994; Dincturk and Knaff 2000). These Fd-glutamate synthase peptides share a significant similarity (40–42%) with the *gltB*-encoded α subunit (~166 kDa), but contain no regions similar to the *gltD*-encoded β subunit (~52 kDa) of the $(\alpha\beta)_x$ heterodimeric form of bac-

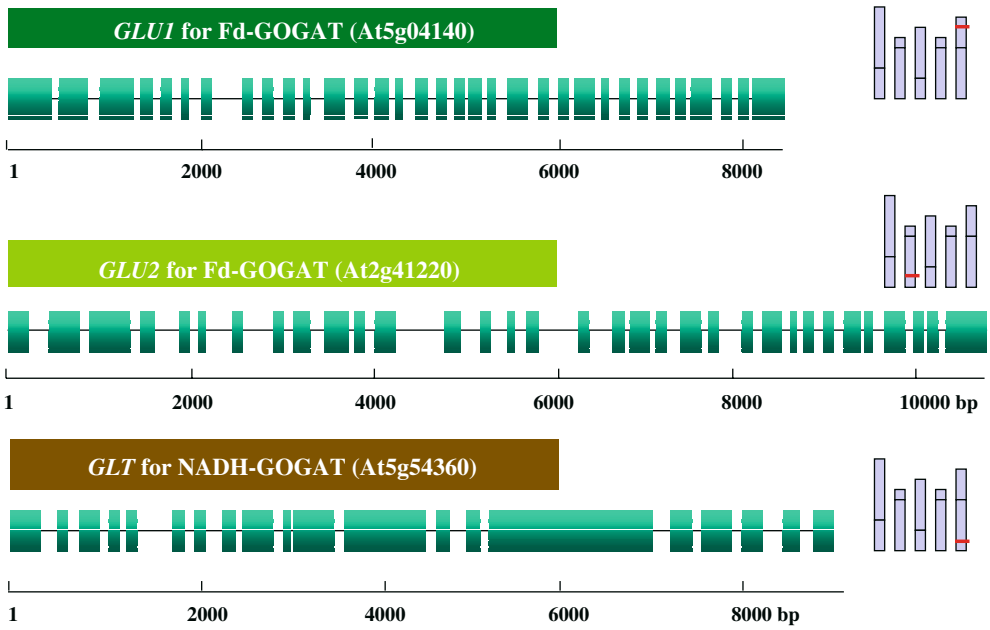

Figure 1. Diagrammatic structure of the genes for ferredoxin-glutamate synthase (*GLU1* and *GLU2*) and NADH-glutamate synthase (*GLT*) in *Arabidopsis thaliana*. Exons are represented by boxes and introns are indicated by lines. Numbers start from the first nucleotide of the coding sequence. Chromosomal map position of the glutamate synthase genes is denoted by line at right of the structural presentation.

terial NADPH-glutamate synthase (Table 1). In the red alga *Antithamninon* sp., *glsF* (*gltF*) encodes a Fd-glutamate synthase of 1537 amino acids, corresponding to the *gltB*-encoded α subunit of bacterial enzymes (Valentin et al. 1993). Fd-glutamate synthase from cyanobacterium *Synechococcus* sp. PCC 6301 has a molecular mass of 160 kDa (Marqués et al. 1992). The *gltB* and *gltS* of the cyanobacterium *Synechocystis* sp. PCC 6803 encode an ORF of 1550 amino acids (169.0 kDa) and 1557 amino acids (170 kDa), respectively. The amino acid sequence analyses showed that two glutamate synthase polypeptides contain a conserved peptide loop insert unique to Fd-glutamate synthase (Vanoni and Curti 1999). Both the *glsF* for Fd-glutamate synthase, and the *gltB* and *gltD* for NADH-glutamate synthase have been cloned from the cyanobacterium *Plectonema boryanum* (Okuhara et al. 1999). The *glsF* encodes an ORF of 1551 amino acids (169 kDa). The complete nucleotide sequence of 9038 bp of *Plectonema boryanum* genome was characterized (Okuhara et al. 1999), and it contains two ORF encoding polypeptides of 1530 amino acids (168 kDa) (GltB) and 492 amino acids (54 kDa) (GltD) with 106 bp apart. Despite the lack of similarity of the NH_2-terminal signal peptides of different Fd-glutamate synthases (105 amino acids/*Arabidopsis GLU1*; 107 amino acids/*Arabidopsis GLU2*; 97 amino acids/maize *GLU*), they have the common characteristics of chloroplast transit peptides, showing high contents of basic amino acids, hydroxylated amino acids and small hydrophobic amino acids such as alanine and valine and a high serine/arginine ratio (Sakakibara et al. 1991; Suzuki and Rothstein 1997). The predicted molecular masses of mature proteins, which start with a cysteine residue, are similar to the values calculated from the mobility of the polypeptide by SDS-PAGE (Sakakibara et al. 1991; Suzuki and Rothstein 1997; Dincturk and Knaff 2000). Partial Fd-glutamate synthase cDNAs have also been determined from other plant species including tobacco (Zehnacker et al. 1992), barley (Avila et al. 1993), spinach (Nalbantoglu et al. 1994), alfalfa nodules (Vance et al. 1995), pine (García-Gutiérrez et al. 1995), grapevine (Loulakakis and Roubelakis-Angelakis 1997) and soybean (Turano and Muhitch 1999). Although definite evidence is not yet available, Fd-glutamate synthase may be encoded by two genes in tobacco (*Nicotiana tabacum*, amphidiploid) (Zehnacker et al. 1992), barley (on chromosome 2) (Avila et al. 1993) and grapevine (Loulakakis and Roubelakis-Angelakis 1997). On the other hand, a single Fd-glutamate synthase gene is identified in maize (Sakakibara et al. 1991) and spinach (Nalbantoglu et al. 1994).

Plant NADH-glutamate synthase genes (*GLT*, NADH-*gltS*) are single open reading frames, with 5′ ends that show homology to *gltB* and 3′ ends that are homologous to the *gltD* of bacterial NADPH-glutamate synthase. The *GLT* gene in alfalfa nodules has a transcribed region (12214 nucleotides composed of 22 exons and 21 introns), which encodes 2194 amino acids (240.4 kDa) and includes a 291-nucleotide 5′UTR and a 101-amino acid presequence (Gregerson et al. 1993; Vance et al. 1995). The rice NADH-glutamate synthase *GLT* is composed of a 5′UTR (261 nucleotides), a coding sequence (6498 nucleotides) and a 3′UTR (285 nucleotides) (Goto et al. 1998). It contains 23 exons and 22 introns, and an ORF encodes 2166 amino acids (236.7 kDa), preceded by a 99-amino acid presequence. The exon/intron organization of the rice gene is similar to that found in the transcribed portion of the alfalfa NADH-glutamate synthase gene, but the alfalfa gene appears to contain an additional exon lacking in the rice gene. A partially transcribed region of the *Arabidopsis* NADH-glutamate synthase gene (9112 nucleotides) is composed of 20 exons and 19 introns (Figure 1). The exons encode a precursor peptide predicted to contain 2208 amino acids (241.9 kDa). The exon/intron organization is similar to that of the *GLT* genes of alfalfa and rice. Most of the corresponding introns of rice and alfalfa NADH-glutamate synthase genes show 20–40% similarity (Goto et al. 1998). NADH-glutamate synthase *GLT* contains the conserved sequences of *gltB* and *gltD* found in prokaryotic NADPH-glutamate synthase genes, and a β subunit-like polypeptide has been fused at the C-terminus of the α subunit-like polypeptide. NADH-glutamate synthase has also been shown to be a monomeric polypeptide in fungi (Hummlet and Mora 1980), yeasts (Cogoni et al. 1995; Filetici et al. 1996; Romero et al. 2000) and insects (Hirayama et al. 1998). A region linking heterodimeric αβ subunit-like polypeptides of NADH-glutamate synthase contains hydrophilic and charged amino acids (histidine, lysine, arginine, glutamate) (Gregerson et al. 1993; Goto et al. 1998). *Arabidopsis* NADH-glutamate synthase has characteristics of a plastid-targeting 166 amino acid

presequence (a high serine/arginine ratio, and high asparagine and glutamate contents) while the alfalfa NADH-glutamate synthase leader sequence predicts a mitochondrial localization (Gregerson et al. 1993). It has been proposed that the pre-protein of unusual amino acid composition undergoes a multiple processing prior to the import of the mature protein into the plastid: a first cleavage upon translocation across plastid membrane and a second cleavage to produce the mature protein starting with the cysteine residue, which is also found as the first amino acid of bacterial glutamate synthases (Gregerson et al. 1993). Several lines of evidence indicate that dual targeting of nuclear gene-encoded pre-proteins, including the GS2 precursor to the chloroplasts and to the mitochondria, can occur (Taira et al. 2004). All the available evidence indicates that the alfalfa NADH-glutamate synthase is located in the amyloplast (Trepp et al. 1999b). Yeast NADH-glutamate synthase is preceded by a transit peptide of 53 amino acids. This presequence is supposed to serve as a reserve of inactive glutamate synthase that could be activated by the cleavage and the consequent exposure of N-terminal cysteine of the mature protein in the cell (Filetici et al. 1996). NADH-glutamate synthase may be encoded by a small gene family in Alfalfa nodules (Gregerson et al. 1993), while it is encoded by a single gene in *Arabidopsis* (Lam et al. 1996) and rice (Goto et al. 1998).

Prokayotic NADPH-glutamate synthase is encoded by the *gltB* and *gltD* genes for the large α subunit (≈150 kDa) and small β subunit (≈50 kDa), respectively (Table 1). The structural genes of *gltB* and *gltD* were initially cloned from the genomic DNA of *E. coli* (Oliver et al. 1987), *Azospirillum brasilense* (Pelanda et al. 1993) and other bacteria (as noted above, *gltA* has been used to denote the gene coding for the α subunit and *gltB* to denote the gene coding for the β subunit in the *Bacillus subtilis* protein (Belitsky et al. 1995). In *E. coli*, the terminal codon TAA of the *gltB* for the α subunit and the translation initiation site of the *gltD* genes for the β subunit are separated by a 12 nucleotide-intercistronic DNA region and form an operon with *gltF* encoding a putative kinase (Oliver et al. 1987; Castaño et al. 1992). The *gltB* gene is upstream of the *gltD* gene in the *glt* operon (*gltBDF*) in *E. coli,* but the organization of the *glt* locus in *Azosprillum brasilense* is the opposite (Madonna et al. 1985; Oliver et al. 1987; Castaño et al. 1992; Pelanda et al. 1993). NADPH-glutamate synthase is catalytically active as an αβ protomer of approximately 200 kDa, and the NADPH-glutamate synthase holoenzyme appears to be an $(\alpha\beta)_4$ tetramer (Stabile et al. 2000; Petoukhov et al. 2003). Kinetic and mechanistic properties were extensively studied for bacterial NADPH-glutamate synthase and cyanobacterial and plant Fd-glutamate synthases (Vanoni et al. 2005).

Amidotransferase and synthase reactions of glutamate synthases

Fd-glutamate synthase is active as a bacterial α subunit-like single polypeptide in which noncovalently bound reduced Fd provides the electrons for the formation of L-glutamate from L-glutamine and 2-oxoglutarate. NADPH-glutamate synthase is active as an $(\alpha\beta)_x$ heterodimer in which NADPH binds to the β subunit and delivers the electrons for the reductive formation of l-glutamate in the α subunit. Structural studies, largely by X-ray crystallography, have provided the three-dimensional structures of the NADPH-glutamate synthase α subunit from *Azospirillum brasilense* (Binda et al. 2000) and Fd-glutamate synthase from *Synechocystis* sp. PCC 6803 (van den Heuvel et al. 2002, 2003; Vanoni et al. 2005). Structure-based biochemical analyses have characterized the catalytic mechanisms of the complex iron-sulfur flavoproteins of Fd-glutamate synthase and NADPH-glutamate synthase. L-Glutamine-dependent amidotransferase activity takes place on the N-terminal glutamine amidotransferase (GAT) domain. This amidotransferase domain belongs to the Pur-F-type amidotransferases class, now defined as NH_2-terminal nucleophile (Ntn)-type class, and corresponds to the N-terminal 450 residues of Fd-glutamate synthase, NADH-glutamate synthase and NADPH-glutamate synthase (Oliver et al. 1987;Gregerson et al. 1993; Pelanda et al. 1993; Navarro et al. 1995; Suzuki and Rothstein 1997; Coschigano et al. 1998; Vanoni and Curti 1999). The cysteine 1 residue of the GAT domain is conserved as the first amino acid of the mature form of Fd-glutamate synthase and of NADH-glutamate synthase, and this domain is involved in the release of glutamine-amide group and formation of an enzyme-γ-glutamyl thioester intermediate prior to glutamate formation in the α subunit. On addition of ammonia from L-gluta-

mine amide group, 2-oxoglutarate is converted to 2-iminog1utarate intermediate at the FMN/FeS synthase site. In *GLU1*-encoded Fd-glutamate synthase of *Arabidopsis*, a region of 52 amino acids (leucine 1079–threonine 1130 of the mature protein) belonging to the FMN binding domain is located on the exons 20 and 21 (Suzuki and Rothstein 1997 and unpublished data). Aspartate 1100 and lysine 1104, which bind to the ribityl side chain of FMN are conserved in many Fd-glutamate synthases, NADH-glutamate synthases and NADPH-glutamate synthases (Oliver et al. 1987; Sakakibara et al. 1991; Gregerson et al. 1993; Pelanda et al. 1993; Navarro et al. 1995; Suzuki and Rothstein 1997). *Arabidopsis* Fd-glutamate synthase contains three cysteines (cysteine 1132, cysteine 1138 and cysteine 1143) (Suzuki and Rothstein 1997), which are located on the exon 21. This cysteine cluster exhibits (CX_5CX_4C) spacing and aligns with the similar cysteine residues of all Fd-glutamate synthases, NADPH-glutamate synthases and NADH-glutamate synthases, with the three cysteine residues serving as ligands to the single [3Fe-4S] $^{1+,0}$ center present in the enzyme (Knaff et al. 1991; Binda et al. 2000; van den Heuvel et al. 2002). According to the structure-based studies, the FMN/FeS synthase site mediates the transfer of the reducing equivalents through an intramolecular electron transfer chain connecting the initial electron donor and synthase site, and leads to the 2-iminoglutarate reduction and L-glutamate formation (Hirasawa et al. 1996; Vanoni and Curti 1999; van der Heuvel et al. 2004). Fd-glutamate synthases have a short polypeptide-insert conserved in the synthase domain (Vanoni and Curti 1999), and it has been suggested that a single Fd binds to the enzyme surface in the vicinity of the [3Fe-4S] cluster and the FMN cofactor (see below) and that sequential one-electron transfers from first one reduced ferredoxin and then from a second reduced ferredoxin provide, via these prosthetic groups, the two electrons needed to reduce 2-iminoglutarate (van den Heuvel et al. 2003). The C-terminal region of Fd-glutamate synthase contains one glycine-rich region (running from glycine 1389 through glycine 1434 of *GLU1*-encoded Fd-glutamate synthase from *Arabidopsis*), which showed a limited sequence similarity to the consensus sequence for the formation of an adenylate binding site (Pelanda et al. 1993; Navarro et al. 1995; Suzuki and Rothstein 1997). As FAD is not present in the Fd-glutamate synthase (Hirasawa et al. 1996) and recombinant NADPH-glutamate synthase α subunit (Vanoni et al. 1998), it has been suggested that this putative ADP-binding fold may serve for the binding of a regulatory adenylate-containing nucleotide. Recently, three-dimensional structural analysis of NADPH-glutamate synthase α subunit (Binda et al. 2000) and of Fd-glutamate synthase (van den Heuvel et al. 2002) clearly showed that this region is part of the C-terminal β-helical domain of glutamate synthase which appears to serve a structural rather than a catalytic or ligand binding role. Two regions matching the consensus sequence for the formation of adenylate-binding folds are found in the C-terminal part of NADH-glutamate synthase. Five conserved glycine residues (glycine 1974, glycine 1976, glycine 1979, glycine 1990 and glutamate 1998) are on exon 20 of alfalfa NADH-glutamate synthase (Vance et al. 1995) or the equivalent five residues on exon 21 of rice NADH-glutamate synthase (Goto et al. 1998). This region is involved in NADH-binding and it finds no counterpart in the eukaryotic Fd-glutamate synthase.

Redox properties

Oxidation-reduction titrations of spinach Fd-glutamate synthase, using absorbance changes in the visible region to monitor the redox state of the FMN group and changes in electron paramagnetic resonance (EPR) spectra to monitor the redox state of the [3Fe-4S]$^{0,+1}$ cluster allowed measurement of the oxidation-reduction midpoint potential (E_m) of both prosthetic groups at pH 7.7, the pH-optimum for the enzyme. Titrations of the [3Fe-4S] cluster gave an excellent fit to the Nernst Equation for a one-electron redox couple with an E_m value of -170 ± 10 mV and the FMN titration gave an excellent fit to the Nernst Equation for a two-electron redox couple with an E_m value of -180 ± 10 mV (Hirasawa et al. 1992). Thus, within the experimental uncertainties of the measurements, the two prosthetic groups of the enzyme are isopotential. Absorbance spectra in the visible region, taken over the course of the FMN titration, showed no evidence for detectable amounts of the one-electron reduced FMN semiquinone (Hirasawa et al. 1992). This observation,

and the fact that no free radical signals attributable to a FMN semiquinone were detected in the course of the EPR measurements (Hirasawa et al. 1992), are both consistent with two-electron character of the titration curve. A cyclic voltammetry investigation of the redox properties of spinach glutamate synthase also demonstrated that the two prosthetic groups of the enzyme are isopotential, although this technique gave a somewhat more negative E_m value of −225 mV for both groups (Hirasawa et al. 1996). Thus, it was not possible to predict the likely sequence of electron transfer events between the two prosthetic groups of spinach Fd-glutamate synthase on thermodynamic grounds alone. It is known that complex formed between spinach ferredoxin and spinach FNR causes shifts in the E_m values of both the [2Fe-2S] cluster of ferredoxin and the FAD group of FNR (Knaff 1996). Thus, although no evidence is currently available for any such shifts in redox potentials arising from the interaction between ferredoxin and Fd-glutamate synthase, the E_m values measured for Fd-glutamate synthase alone may perhaps be different from the values operating within a Fd-glutamate synthase complex with ferredoxin.

Oxidation-reduction titrations of the FMN in *Synechocystis* sp. PCC 6803 Fd-glutamate synthase gave a good fit to the two-electron Nernst Equation with $E_m = -200 \pm 25$ mV at pH 7.5 (Navarro et al. 2000). As was the case for the spinach enzyme, no evidence for the presence of a flavin semiquinone was observed in the course of titrations of the FMN group of *Synechocystis* sp. PCC 6803 Fd-glutamate synthase, suggesting that the two one-electron E_m values for the oxidized/semiquinone and semiquinone/fully-reduced FMN couples differ by at least 100 mV (Ravasio et al. 2002). An estimation of the E_m value of the $[3Fe\text{-}4S]^{0,+1}$ cluster in *Synechocystis* sp. PCC 6803 Fd-glutamate synthase from absorbance changes in the visible region during an anaerobic titration of the enzyme with sodium dithionite suggested that the E_m value of the cluster was 40–50 mV more positive than that of the enzyme's FMN group, putting the E_m of the $[3Fe\text{-}4S]^{0,+1}$ cluster in *Synechocystis* sp. PCC 6803 Fd-glutamate synthase at the rather positive value of −150 to −160 mV (Ravasio et al. 2002). This result was somewhat surprising, given the fact that the two prosthetic groups are not only isopotential in spinach Fd-glutamate synthase, but are also approximately isopotential (i.e., with E_m values of −240 mV for the FMN and −260 mV for the [3Fe-4S] cluster) in the α-subunit of the NADPH-dependent glutamate synthase from *A. brasilense* (Ravasio et al. 2001, 2002). However, given the experimental uncertainties in these measurements and the fact that E_m values are quite sensitive to experimental conditions (such as pH, temperature and ionic strength), it is perhaps more important to focus on the similarities in these E_m values, rather than on relatively small differences. It should also be mentioned that titration of *Synechocystis* sp. PCC 6803 Fd-glutamate synthase with L-glutamate under anaerobic conditions caused an initial reduction of the [3Fe-4S] cluster followed by a subsequent reduction of FMN (Ravasio et al. 2002), consistent with a more positive E_m value for the [3Fe-4S] cluster. Similar results were found with *A. brasilense* NADPH-dependent glutamate synthase α subunit, while only FMN was reduced during L-glutamate equilibrium titrations of the NADPH-glutamate synthase $(\alpha\beta)_x$ holoenzyme (Ravasio et al. 2001, 2002). More detailed descriptions of the redox properties of *Synechocystis* sp. PCC 6803 Fd-glutamate synthase and of the *A. brasilense* NADPH-glutamate synthase are presented in the article by Vanoni et al. in this issue.

Complex formation with ferredoxin

A large body of evidence supports the hypothesis that ferredoxin forms electrostatically-stabilized complexes with enzymes that use ferredoxin as the electron donor, with ferredoxin supplying most of the negative charges involved in complex formation and the target enzyme supplying most of the positive charges (Knaff 1996). Spectral perturbations, gel filtration chromatography, membrane ultrafiltration and chemical cross-linking experiments are all consistent with the hypothesis that ferredoxin and Fd-glutamate synthase form such a complex (Knaff 1996). Changes in the UV/visible spectra of spinach ferredoxin and/or spinach Fd-glutamate synthase, that occur when the two proteins are mixed and which arise from complex formation between the two proteins, only occur at low ionic strength, consistent with the idea that electrostatic forces play a significant role in stabilizing the spinach chloroplast ferredoxin/glutamate synthase complex (Hirasawa et al. 1986). The observation that the two spinach chloroplast pro-

teins co-migrate during gel filtration chromatography at low ionic strength but not at high ionic strength is also consistent with the formation of an electrostatically-stabilized complex between spinach ferredoxin and spinach Fd-glutamate synthase (Hirasawa et al. 1986). Analysis of the hyperbolic plots of the magnitude of the absorbance changes arising from complex formation between spinach ferredoxin and spinach Fd-glutamate synthase *versus* ferredoxin concentration indicated a single binding process with a K_d of 14.5 μM at low ionic strength (Hirasawa et al. 1986). It should also be mentioned that the rates of the ferredoxin-dependent reactions catalyzed by both *Synechocystis* sp. PCC 6803 Fd-glutamate synthase (Schmitz et al. 1996) and spinach Fd-glutamate synthase (M. Hirasawa, unpublished observations) decline markedly with increasing ionic strength, consistent with the idea that electrostatic interactions between the proteins play an important role at some point in the reaction catalyzed by Fd-glutamate synthase.

Chemical modification of spinach Fd-glutamate synthase with either the lysine-modifying reagent, N-acetylsuccinimide, or the arginine-modifying reagent, phenylglyoxal, results in inhibition of enzyme activity when reduced ferredoxin serves as the electron donor but had no inhibitory effect on enzyme activity with the non-physiological electron donor, reduced methyl viologen (Hirasawa and Knaff 1993). These observations, and the fact that formation of the ferredoxin/glutamate synthase complex prior to addition of either chemical modifier completely protected the enzyme from inhibition are consistent with a role for lysine and arginine residues in ferredoxin-binding by the enzyme (Hirasawa and Knaff 1993).

If the ferredoxin-binding site of spinach Fd-glutamate synthase does indeed contain positively-charged arginine and lysine residues, one would expect that ferredoxin would contribute negatively-charged residues to electrostatic interactions involved in complex formation. In fact, it has been shown that chemical modification of carboxylic acid side chains of spinach ferredoxin with glycine ethyl ester and a water-soluble carbodiimide (a treatment that elements the negative charges on glutamate and aspartate residues) decreases the binding affinity of ferredoxin for spinach Fd-glutamate synthase (Hirasawa et al. 1986). The wild-type ferredoxin from vegetative cells of the cyanobacterium *Anabaena* sp. PCC 7120 has kinetic and binding parameters for spinach Fd-glutamate synthase very similar to those measured for spinach ferredoxin and the availability of a number of site-specific variants of this very well-characterized cyanobacterial ferredoxin, for which a high-resolution X-ray crystal structure is available (Rypniewski et al. 1991; Holden et al. 1994; Hurley et al. 1997), led to its use in such studies (Hirasawa et al. 1998). Replacement of a highly-conserved ferredoxin glutamate residue near the C-terminus of the protein (Glu94 in *Anabaena* sp. PCC 7120 ferredoxin) by either lysine or glutamine produced a very large decrease in the ability of the ferredoxin to serve as an efficient electron donor to spinach Fd-glutamate synthase (Hirasawa et al. 1998). In contrast, similar replacements at the adjacent glutamate, Glu95, had essentially no effect on ferredoxin-dependent activity of the reaction catalyzed by spinach Fd-glutamate synthase (Hirasawa et al. 1998). This sort of high positional specificity, when comparing effects of modifying these two adjacent glutamate residues, had been observed previously in several studies of the interaction between ferredoxin and FNR and between ferredoxin and ferredoxin-dependent nitrite reductases (Knaff 1996). Similar site-directed mutagenesis studies indicate that Glu94 in *Anabaena* sp. PCC 7120 ferredoxin plays an important role in the interaction between ferredoxin and the *Synechocystis* sp. PCC Fd-glutamate synthase but, as site-specific replacements of the adjacent Glu95 were not a part of this study, no conclusions could be drawn about any positional specificity (Schmitz et al. 1996). In contrast to the differences observed in replacing of Glu94 *versus* replacing Glu95 in *Anabaena* sp. PCC 7120 ferredoxin on the ability of reduced ferredoxin to serve as an electron donor for spinach Fd-glutamate synthase, a study with *C. reinhardtii* Fd-glutamate synthase showed that elimination of the negative charge on Glu92 of *C. reinhardtii* ferredoxin (the residue that corresponds to Glu95 in *Anabaena* sp. PCC 7120 ferredoxin) by site-specific mutagenesis produces a significant decrease in the rate of the reaction catalyzed by *C. reinhardtii* Fd-glutamate synthase, although the extent of inhibition (compared to the rate obtained with wild-type *C. reinhardtii* ferredoxin) was somewhat less than that observed when the nega-

tive charge on Glu91 of *C. reinhardtii* ferredoxin (the residue that corresponds to Glu94 in *Anabaena* sp. PCC 7120 ferredoxin) was eliminated by site-specific mutagenesis (García-Sánchez et al. 2000). These studies also indicated that a second acidic region of *C. reinhardtii* ferredoxin, which includes Asp25, Glu28 and Glu29, is also likely to be involved in interactions with *C. reinhardtii* Fd-glutamate synthase (García-Sánchez et al. 2000). The corresponding region on spinach ferredoxin, which includes amino acid residues 26–30, has been implicated in the interaction with spinach Fd-glutamate synthase, as has the acidic region on spinach ferredoxin that includes residues 65–70 (Hirasawa et al. 1986).

Although the observations summarized above provide strong support for the involvement of electrostatic interactions in stabilizing the complex formed between ferredoxin and Fd-glutamate synthase, the actual situation may prove to be more complex. A very large body of evidence, involving the same sort of data described above for Fd-glutamate synthase, had been available supporting the role of electrostatic interactions in stabilizing the 1:1 complex formed between ferredoxin and FNR (Knaff 1996). When crystal structures became available recently for two ferredoxin/FNR complexes, one between the maize leaf proteins (Kurisu et al. 2001) and one between the proteins from the cyanobacterium *Anabaena* sp. PCC7119 (Morales et al. 2000), it became clear that, although the interactions between the two proteins do involve some ion pairs and hydrogen bonds, a significant number of contacts between hydrophobic side chains are also involved. In fact, earlier studies of the effects of ionic strength on the kinetics of electron transfer from *Anabaena* sp. PCC 7120 ferredoxin to *Anabaena* sp. PCC 7119 FNR had indicated that hydrophobic, as well as electrostatic, effects were likely to play an important role in this system (Hurley et al. 1996). A microcalorimetry study of the complex formed between spinach ferredoxin and spinach FNR indicated that ΔH, the change in enthalpy associated with complex formation, is essentially zero and that the large, favorable negative ΔG (the free energy change) for complex formation results entirely from the favorable positive ΔS (the entropy change) associated with complex formation (Jelesarov and Bosshard 1994). The favorable positive ΔS appears to arise from the transfer of oriented water molecules, bound to the protein surfaces, from the protein/protein interface region into the bulk solvent (Jelesarov and Bosshard 1994). A similar situation appears to be the case for the complex between the two *Anabaena* sp. PCC 7119 proteins (Morales et al. 2000). Given the likely similarities between ferredoxin binding by FNR and by Fd-glutamate synthase (Knaff 1996), it may well be that hydrophobic interactions and the release of oriented water molecules from the protein surfaces make substantial contributions to stabilizing the complex between ferredoxin and Fd-glutamate synthase.

Less is known about the possible location of ferredoxin-binding domain(s) on Fd-glutamate synthase than is known about regions on ferredoxin that may be involved in binding to Fd-glutamate synthase, although it seems likely that a ferredoxin-binding site on Fd-glutamate synthase lies in the region that contains the [3Fe-4S] cluster and FMN prosthetic groups. It has been proposed that a loop in this region (which encompasses amino acid residues 907–933 of the *Synechocystis* sp. PCC 6803 Fd-glutamate synthase) may be part of (or close to) the site of interaction between Fd and Fd-glutamate synthase (van den Heuvel et al. 2003) and the fact that this region is conserved in all Fd-glutamate synthases is consistent with this proposal. Work on the X-ray crystal structure of spinach Fd-glutamate synthase (J.P. Allen, A. Artigas Camara, M. Hirasawa, D.B. Knaff, unpublished observations) has not yet progressed to the point where it can be stated unambiguously that a similar loop is present in the spinach enzyme and the large size of spinach Fd-glutamate synthase has made it impossible to use peptide-mapping techniques, of the type used successfully with FNR (Jelesarov et al. 1993) and ferredoxin-dependent nitrite reductase (Dose et al. 1997), to identify specific lysine and arginine residues that may be involved in binding ferredoxin.

Evidence from chemical modification studies suggests that a tryptophan residue may be present at the ferredoxin-binding site on spinach Fd-glutamate synthase. Treatment of spinach Fd-glutamate synthase with the tryptophan-modifying reagent N-bromosuccinimide resulted in equal losses of ferredoxin-dependent and methyl viologen-dependent activities. The time course observed for this loss of activity was similar to that observed for the modification of tryptophan residues and both the activity loss and the tryptophan modifi-

cation were eliminated if complex formation with ferredoxin preceded treatment with N-bromosuccinimide (Hirasawa et al. 1998). These results have been interpreted in terms of the presence of at least one tryptophan residue, located at or near the ferredoxin-binding site of the enzyme, that plays a role in electron transfer *per se* rather than in ferredoxin binding (Hirasawa et al. 1998). As proposals for specific roles for aromatic amino acids in electron transfer reactions between ferredoxin and target enzymes exist (see for example Hurley et al. 1993), one might imagine that a series of properly oriented aromatic amino acids could bridge the space separating the electron-donating [2Fe-2S] cluster of ferredoxin and the electron-accepting group on Fd-glutamate synthase. While such an arrangement may perhaps exist, it should be pointed out that at present there is no evidence for any special requirement for an aromatic ferredoxin amino acid in its reactions with Fd-glutamate synthase. In fact, site-directed replacement of Phe65, a highly conserved ferredoxin amino acid that had been implicated in some ferredoxin-dependent electron transfer reactions (Hurley et al. 1993), by the non-aromatic amino acids alanine or isoleucine had only relatively modest effects on the ability of ferredoxin from vegetative cells of the cyanobacterium *Anabaena* sp. PCC 7120 to serve as an electron donor to spinach Fd-glutamate synthase (Hirasawa et al. 1998). Similar results were obtained with Fd-glutamate synthase from *Synechocystis* sp. PCC 6803 and F65 variants of the *Anabaena* sp. PCC 7120 ferredoxin or F63 variants of *Synechocystis* sp. PCC 6803 ferredoxin (the corresponding phenylalanine residue in this ferredoxin) in which phenylalanine was replaced by a non-aromatic amino acid (Schmitz et al. 1996).

In the case of the ferredoxin complex with FNR (reviewed in Knaff 1996), the higher plant ferredoxin-dependent nitrite (Mikami and Ida 1989) and sulfite reductases (Hirasawa et al. 1987; Akashi et al. 1999), the ferredoxin:thioredoxin reductases from spinach (Hirasawa et al. 1988) and the cyanobacterium *Synechocystis* sp. PCC 6803 (Glauser et al. 2004), and the ferredoxin-dependent nitrate reductase from the cyanobacterium *Synechococcus* sp. PCC 7942 (Hirasawa et al. 2004) the ferredoxin/enzyme stoichiometry of the complexes has been shown to be 1:1. Immunological experiments support the idea that the ferredoxin-binding site on spinach Fd-glutamate synthase resembles the ferredoxin-binding site on FNR and on nitrite reductase (Hirasawa et al. 1989, 1991) and thus it seemed reasonable to expect that the stoichiometry of the ferredoxin/Fd-glutamate synthase complex would also be 1:1. However, while there is evidence from cross-linking (Schmitz et al. 1996), small angle X-ray scattering (van den Heuvel et al. 2003) and mass spectrometry (van den Heuvel et al. 2004) experiments supporting a 1:1 stoichiometry for the *Synechocystis* sp. PCC 6803 ferredoxin/Fd-glutamate synthase complex, other cross-linking experiments support a stoichiometry of two ferredoxins:one Fd-glutamate synthase for the complex formed by the spinach proteins (Hirasawa et al. 1991) and the proteins from the green algae *C. reinhardtii* (García-Sánchez et al. 2000) and *Monoraphidium braunii* (Vigara et al. 1996). Membrane ultrafiltration experiments with the spinach proteins are also more consistent with a 2:1 ferredoxin:Fd-glutamate synthase stoichiometry for the complex (Hirasawa et al. 1989). As the stoichiometry of the complex has important implications for the enzyme mechanism (i.e., in deciding whether the two electrons required for the reaction arrive separately in two sequential electron transfers from reduced ferredoxin or whether it may be possible that a concerted transfer of two electrons from two simultaneously bound ferredoxins might occur), it is of considerable importance to remove uncertainties as to the correct value.

Formation of the spinach ferredoxin/spinach Fd-glutamate synthase complex is accompanied by changes in the circular dichroism (CD) spectra of one or both proteins. These spectral perturbations have been interpreted as arising from conformational changes in one or both proteins that result from complex formation (Hirasawa et al. 1989). Changes in CD spectra have also been observed as a consequence of complex formation between ferredoxin and both FNR and nitrite reductase (Knaff 1996). In the case of the ferredoxin/FNR complex, the availability of crystal structures for the complex between the maize chloroplast proteins (Kurisu et al. 2001) and the *Anabaena* sp. PCC 7119 proteins (Morales et al. 2000) has provided documentation that protein/protein interactions within the complex do indeed cause significant changes in conformation of both fer-

redoxin and FNR. The structures of these two ferredoxin/FNR complexes have also provided specific information about exactly which amino acids undergo changes in conformation and about the possible implications of these conformational changes for understanding the mechanism of the FNR-catalyzed reaction (Morales et al. 2000; Kurisu et al. 2001). Evidence obtained from structural (van den Heuvel et al. 2003) and biochemical (Ravasio et al. 2002) studies on the *Synechocystis* sp. PCC 6803 Fd-glutamate synthase suggests that conformational changes associated with ferredoxin binding and/or changes of the redox state of ferredoxin may be essential to activate the enzyme for catalysis.

Although the studies described above provide a preliminary picture of some aspects of the site(s) involved in the interaction between ferredoxin and Fd-glutamate synthase and of the possibility of significant conformational changes induced by protein/protein complex formation, many details of the interaction remain to be elucidated. It is to be hoped that current attempts to obtain diffraction-quality crystals of the ferredoxin complexes of *Synechocystis* sp. PCC 6803 Fd-glutamate synthase (van den Heuvel et al. 2003) and of spinach Fd-glutamate synthase (J.P. Allen, A. Artigas Camara, M. Hirasawa, D.B. Knaff, unpublished observations) will eventually lead to three-dimensional structures that will settle the question of ferredoxin/enzyme stoichiometry within the complex, will identify the interaction domains involved in complex formation between ferredoxin and Fd-glutamate synthase and will identify specific conformational changes that arise from the interactions between the proteins.

Regulation of glutamate synthases in higher plants

Light at low fluency is perceived at specific wavelengths by the photoreceptors and signal transduction then triggers chloroplast differentiation, circadian rhythms and a number of physiological and molecular responses in plants (Neff et al. 2000). Studies of gene expression in mutants suggest that plants have sensing and signal transduction mechanisms that respond to the cellular concentrations of carbon and nitrogen (sucrose, NO_3^-, NH_4^+, glutamine, glutamate, 2-oxoglutarate, etc.) (Coruzzi and Zhou 2001). The interaction of light and carbon signals combine to affect expression of several genes involved in nitrogen assimilation (Thum et al. 2003). In higher plants, Fd-glutamate synthase and NADH-glutamate synthase genes are regulated by light, and by carbon and nitrogen metabolites. Exposure of dark-grown *Arabidopsis* seedlings to white light increases the level of *GLU1* Fd-glutamate synthase mRNA two-fold in leaves after 72 h, while *GLU2* mRNA remains constant at low levels (Suzuki and Rothstein 1997; Coschigano et al. 1998). Promoter-*GUS* expression studies revealed that the *GLU1* promoter of *Arabidopsis* Fd-glutamate synthase induces *GUS* reporter gene expression after exposure of the dark-grown transgenic tobacco seedlings to red light (Ziegler et al. 2003). Red light typically enhances the levels of *GLU1* mRNA in dark-grown *Arabidopsis* leaves, and mRNA induction is reversibly suppressed by subsequent exposure to far-red light in a typical phytochrome-mediated response. In contrast, phytochrome-treatments do not affect the levels of *GLU2* mRNA in *Arabidopsis* leaves (unpublished data). *GUS* expression is induced by sucrose in the dark albeit to a lower extent than the increase produced by red light or white light, ranging between 2.5- and 3.5-fold (Ziegler et al. 2003). The accumulation of the corresponding *GLU1* mRNA produced by sucrose addition in the dark is also lower than that produced by light in *Arabidopsis* (Coschigano et al. 1998; unpublished results). Sucrose also mimics the phytochrome effects by inducing high levels of *GLN2* mRNA (16–17- fold increases) in *Arabidopsis* or repressing *ASN1* mRNA in maize (Lam et al. 1994; Chevalier et al. 1996; Oliveira and Coruzzi 1999; Thum et al. 2003). Light or sucrose induces a two- to three-fold increase in *GLN1* mRNA expression in *Arabidopsis* leaves (Oliveira and Coruzzi 1999). However, supplying sucrose or glucose to excised dark-grown barley leaves does not induce Fd-glutamate synthase mRNA or produce detectable enzyme activity (Pajuelo et al. 1997). In etiolated maize leaves, Fd-glutamate synthase mRNA increases as early as 6 h after the onset of illumination, and it accumulates four-fold, reaching a level equivalent to that found in green leaves (Sakakibara et al. 1992a). Induction of *GLU* mRNA in maize leaves is at least in part mediated by phytochrome (Suzuki et al. 2001). Fd-glutamate synthase mRNA accumulation produced by

the light correlates with *de novo* synthesis of the enzyme protein (Sakakibara et al. 1992b; Pajuelo et al. 1997; Suzuki et al. 2001). Induction of Fd-glutamate synthase by a phytochrome-mediated pathway is reflected in an increased ratio of glutamate to glutamine, which is close to the value observed in leaves exposed to white light (Suzuki et al. 2001). In contrast, light has no effect on NADH-glutamate synthase levels in leaves (Suzuki and Rothstein 1997; Turano and Muhitch 1999; Suzuki et al. 2001). As a result of these differential light effects, Fd-glutamate synthase accounts for more than 95% of the total glutamate synthase activity in photosynthetic plant tissues, and NADH-glutamate synthase accounts for only a small residual activity. The induction of Fd-glutamate synthase mRNA via phytochrome signal transduction may include blue/UV-A light receptors in Scot pine (Elminger and Mohr 1991) and in *Spirodela polyrhiza* (Teller et al. 1996). However, *Pinus pinaster*, another pine species, accumulates mRNA and protein for Fd-glutamate synthase, as well as mRNA for the photosynthetic genes of Rubisco large and small subunits and chlorophyll a/b binding proteins in a light-independent manner (García-Gutiérrez et al. 1995). In addition to the phytochrome-mediated induction, Fd-glutamate synthase mRNA expression in green leaves changes diurnally, with induction early in the morning and slight repression at the middle of the day (Suzuki et al. 2001; Ferrario-Méry et al. 2002). Both the *GLU1* promoter and *GLU1* mRNA are up-regulated by light and sucrose, suggesting that *GLU1*-encoded Fd-glutamate synthase functions under conditions of high sucrose availability produced by the photosynthesis. Transcript levels for nitrate reductase (NAR) and nitrite reductase (NII) are also high at the end of the night and decreased during the light period (Scheible et al. 2000). Several lines of evidence indicate that the light- and circadian-responsive elements provide the binding sites for transcription factors, which are diurnally induced (Borello et al. 1993; Anderson et al. 1994; Staigner and Apel 1998). Both phytochromes and cryptochromes are involved in maintaining the rhythm close to 25 h under red and blue light in *Arabidopsis* (Millar et al. 1995; Somers et al. 1998). Also mRNA is post-transcriptionally light-regulated by coupling to photosynthetic electron transport at the level of mRNA stability (Petracek et al. 1998).

The control of glutamate synthase expression by NO_3^- and the downstream products of NO_3^- assimilation has also been extensively investigated. In the leaves of detached maize seedlings, a supply of NO_3^- results in increases in the mRNA levels of both Fd-glutamate synthase (l.5-fold) and NADH-glutamate synthase (2.5-fold) within 4 h (Sakakibara et al. 1997). In these plants, the kinetics observed for the increases in NAR, NII and GS2 mRNA levels are all quite similar (Sakakibara et al. 1997). Three- to five fold increases in Fd-glutamate synthase mRNA have also been observed in illuminated etiolated maize leaves in response to NO_3^- or NH_4^+. In the absence of light, both NO_3^- and NH_4^+ induce Fd-glutamate synthase gene expression to much lower levels (Suzuki et al. 1996; Pajuelo et al. 1997). A similar time course has been observed for labeling of the Fd-glutamate synthase with [^{35}S] methionine. Synthesis of the enzyme protein is blocked by cycloheximide (Suzuki et al. 1996). Nitrate induction of Fd-glutamate synthase mRNA is not affected by the phosphatase inhibitors okadaic acid and calyculin A in maize leaves, while the inhibitors repress the mRNA levels of NAR, NII and GS2 (16–69%) (Sakakibara et al. 1997). The protein kinase inhibitors W-7 and W-5 slightly reduce the nitrate-dependent accumulation of Fd-glutamate synthase mRNA (8–29%), implying a possible involvement of calmodulin-regulated process or Ca^{2+}-dependent but calmodulin-independent protein kinases in the nitrate-signal transduction pathway for regulation of *GLU* expression (Sakakibara et al. 1997). Nitrate also induces mRNA for Fd-glutamate synthase, GS1 and GS2 in tobacco leaves 4 h after exposure to NO_3^- (Scheible et al. 1997). Multiple nitrogen metabolites partially counteract the low mRNA levels produced by nitrogen starvation, i.e., NO_3^- or glutamine induces GS2 mRNA and Fd-glutamate synthase mRNA, and NH_4^+ increases Fd-glutamate synthase mRNA. A specific effect of these nitrogen compounds can thus be excluded (Migge and Becker 1996). However, in suspensions of cultured rice cells deprived of nitrogen, NO_3^- induced Fd-glutamate synthase mRNA levels whereas no induction is produced when NH_4^+ or the amino acids glutamine, glutamate, asparagine or aspartate are supplied (Watanabe et al. 1996; Hirose and Yamaya 1999). In nitrogen-starved rice leaves, neither Fd-glutamate synthase nor NADH-glutamate synthase are affected by the application of NH_4^+

(Yamaya et al. 1995). *Arabidopsis* plants starved for nitrogen and then exposed to NO_3^- or NH_4^+-treatment were studied using microarrays representing about 21,000 genes (Meyer et al. 2003). The addition of NO_3^- induced mRNA levels for *GLU1* and *GLT* in leaves (Figure 2). The addition of NH_4^+ only slightly affected the mRNA levels for *GLU1*, *GLU2* and *GLT*. In contrast, neither NO_3^- nor NH_4^+ affects Fd-glutamate synthase gene expression after exposure of etiolated soybean leaves to light (Turano and Muhitch 1999). The lack of induction by NO_3^- is also observed for Fd-glutamate synthase and GS2 genes in maize leaves (Redinbaugh and Campbell 1993). The decrease in Fd-glutamate synthase mRNA at the middle of the day is reminiscent of a repression of some of nitrogen assimilatory genes during the light period (Scheible et al. 2000; Ferrario-Méry et al. 2002). Following the diurnal changes in the levels of NO_3^- and NH_4^+ (Scheible et al. 2000; Matt et al. 2001; Ferrario-Méry et al. 2002; Stitt et al. 2002), glutamine and 2-oxoglutatate (2-OG) may regulate the NH_4^+ assimilation by acting antagonistically. It has been suggested that an allosteric effector of the PII-like protein present in chloroplasts serves as a sensor of glutamine by changing uridylylation and adenylation states or is involved in glutamine metabolisms in *Arabidopsis* (Hsieh et al. 1998; Jiang and Ninfa 1999).

In roots, the levels of Fd-glutamate synthase mRNA increases rapidly, showing a 6.5-fold increase within 30 min after exposure to NO_3^-, while the mRNA levels for NAR and GS2 peak after 4 h (Redinbaugh and Campbell 1993). A transient four-fold accumulation of Fd-glutamate synthase mRNA is produced in etiolated coleoptiles of rice only 30 min after addition of NO_3^-, synchronous with the labeling of Fd-glutamate synthase peptide with [^{35}S]methionine (Mattana et al. 1996). In rice roots, NADH-glutamate synthase mRNA is detected in sclerenchyma cells in the inner cell-layer as early as 3–6 h after NH_4^+ is supplied and the level subsequently declined (Ishiyama et al. 1998, 2003). In contrast, little change is observed in the levels of Fd-glutamate synthase and GS isoproteins in roots (Yamaya et al. 1995). This transient expression correlates with the appearance of GUS activity in the

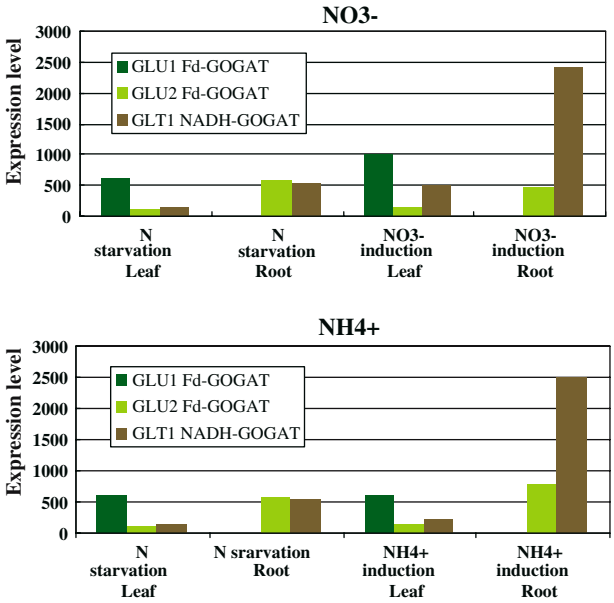

Figure 2. Histograms of the expression of genes for ferredoxin-glutamate synthase (*GLU1* and *GLU2*) and NADH-glutamate synthase (*GLT*) in leaves and roots of *Arabidopsis thaliana*. Thirty-five-day-old *Arabidopsis thaliana* plants were subjected to nitrogen starvation for 10 days, and nitrogen was then supplied in the form of 10 mM NO_3^- or 5 mM NH_4^+ for 6h. Using high-density oligonucleotide probe microarray (Affymetrix GeneChip) analysis (Meyer et al. 2003), expression of 21 000 genes was examined in the leaves and roots. Nitrate induced 1.6- and 3.7-fold increases in *GLU1* mRNA and *GLT* mRNA, respectively in leaves, and a 4.4-fold-increase in *GLT* mRNA in roots. Ammonium induced l.4- and 4.5-fold increases in *GLU2* mRNA and *GLT* mRNA, respectively in roots.

sclerenchyma cells of transgenic plants containing a NADH-glutamate synthase *GLT* promoter-*GUS* fusion 3 h after NH_4^+ treatment (Ishiyama et al. 2003). In the absence of cycloheximide, NADH-glutamate synthase is detected later in the outer cell-layers including epidermal and exodermal cells (Ishiyama et al. 1998, 2003). In nitrogen-starved *Arabidopsis*, NO_3^- induced *GLT* mRNA for NADH-glutamate synthase in roots (Figure 2). The addition of NH_4^+ also induced *GLT* mRNA in roots, while *GLU1* mRNA was barely detected (Figure 2). Ammonium and glutamine act as metabolic signals for the induction of the NADH-glutamate synthase gene, while other amino acids, i.e. glutamate, asparagine, aspartate, alanine or serine, have little effect in rice roots (Ishiyama et al. 1998). Okadaic acid caused the continuous accumulation of NADH-glutamate synthase mRNA, and an okadaic acid-sensitive reversible protein phosphorylation appears to be involved in the signal transduction pathway (Ishiyama et al. 1998; Hirose and Yamaya 1999).

Mutants and transgenic plants affected in glutamate synthase reveal the roles of the enzyme in nitrogen metabolism in higher plants

In most tropical and subtropical plants, primary nitrate assimilation into amino acids occurs predominantly in leaf chloroplasts. In some plant species, such as temperate legumes and maize, efficient nitrate assimilation also occurs in root plastids (Pate 1980). In addition to primary nitrate reduction, NH_4^+ is released within mitochondria of mesophyll cells in C3 plants at rates 5–10-fold higher than rates of nitrate reduction (Somerville and Ogren 1980). As NH_4^+ assimilation into amino acids via glutamate synthase is coupled to the GS-catalyzed reaction, the physiological role of Fd-glutamate synthase and NADH-glutamate synthase are tightly linked to GS2 and GS1, which play tissue- and organ-specific roles (Figure 3) (Lam et al. 1996). The *in vivo* function of different forms of GS and glutamate synthase has been investigated by the characterization of mutants and

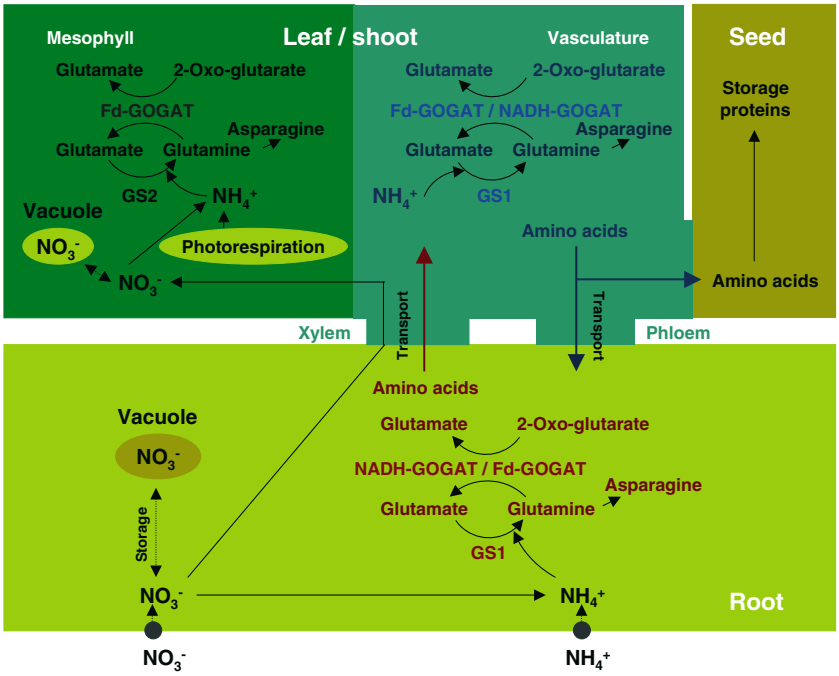

Figure 3. A model for the role of Fd-glutamate synthase and NADH-glutamate synthase in nitrogen assimilation and nitrogen transport. Plants import nitrate and ammonium ion into the roots when available in the soil. Nitrate storage in the vacuoles and nitrate reduction to nitrite and, subsequently into ammonium occur in the roots and in the leaves. Ammonium ion issued from different pathways is assimilated into glutamine and glutamate by the sequential reaction of glutamine synthetase (cytosolic GSI and chloroplastic GS2) and glutamate synthase (Fd-glutamate synthase and NADH-glutamate synthase). Glutamine and glutamate are used for amino acid, nucleic acid and protein biosynthesis. Amino acids are in part mobilized into the xylem for transport to the shoots or into the phloem for transport to other organs.

transgenic plants. Photorespiratory mutants defective in Fd-glutamate synthase and/or GS are isolated by conditional lethal phenotype screenings in *Arabidopsis* and barley (Somerville and Ogren 1980; Kendall et al. 1986; Blackwell et al. 1988). The *Arabidopsis gls* mutants contain 2–4% of the wild-type Fd-glutamate synthase activity in green leaves, and NADH- or NADPH-dependent reactions account for 4% of the total glutamate synthase activity in leaves (Somerville and Ogren 1980). There are two expressed genes *GLU1* and *GLU2* for Fd-glutamate synthase, and a single *GLT* for NADH-glutamate synthase in *Arabidopsis* (Figure 1). *GLU1* mRNA for the major Fd-g1utamate synthase is primarily expressed in green leaves, similar to *GLN2* gene for chloroplastic GS2 that is highly expressed in leaves (Peterman and Goodman 1991; Suzuki and Rothstein 1997; Coschigano et al. 1998; Lancien et al. 2002). Presumably, the lethal chlorosis is caused by the mutation of highly expressed *GLU1* gene, which is mapped at the same region as a *gls* allele on chromosome 5 while the *GLU2* gene on chromosome 2 is not affected (Coschigano et al. 1998). The *Arabidopsis gls* mutants typically have decreased levels of *GLU1* mRNA and the corresponding low Fd-glutamate synthase activity in leaves (1.7%) and roots (25%) (Somerville and Ogren 1980; Suzuki and Rothstein 1997). The photorespiratory mutant phenotype has also been demonstrated in transgenic tobacco lines expressing a 936-bp partial Fd-glutamate synthase cDNA in the antisense orientation (Ferrario-Méry et al. 2000). These transgenic tobacco plants exhibit Fd-glutamate synthase activity that has been decreased by 10–81% in leaves and 17–65% in roots in three transgenic lines. Following a shift from CO_2-enriched air (0.4%) to ambient air for 48 h, NH_4^+ accumulates in amounts as high as 50–300 $nmol^{-1}$ mg^{-1} leaf dry weight in a manner that increases in proportion to the inhibition of Fd-glutamate synthase activity. The accumulation of glutamine and 2-OG corresponds approximately to the increase in NH_4^+ in these plants. This is expected because NH_4^+ must be refixed by GS and 2-OG is the substrate for Fd-glutamate synthase (Ferrario-Méry et al. 2000). Following the initial decrease, the levels of glutamate stabilize as further synthesis by Fd-glutamate synthase is prevented. The decrease in Fd-glutamate synthase activity by even 20% in leaves limits photorespiratory NH_4^+ cycling (Ferrario-Méry et al. 2000), and the strict correlation between the mutant phenotype and enzyme deficiency provides evidence that the GS/Fd-glutamate synthase cycle plays the indispensable role for the reassimilation of photorespiratory NH_4^+. In *Arabidopsis*, the loss of the *GLU1* isoform of Fd-glutamate synthase cannot be compensated for by the second *GLU2* isoform of Fd-glutamate synthase or by NADH-glutamate synthase, both of which are constitutive and present at low levels in leaves. Promoter-*GUS* fusion studies with transgenic tobacco plants demonstrated that the *Arabidopsis GLU1* promoter directs expression of the *uidA* reporter gene in the chloroplasts of palisade and spongy parenchyma of the mesophyll (Ziegler et al. 2003; unpublished results), the major site for photorespiration. Immunolocalization studies detected Fd-glutamate synthase protein in the chloroplast stroma of mesophyll in tomato (Botella et al. 1988). These results provide evidence for the efficient *in vivo* function of Fd-glutamate synthase in photorespiratory nitrogen cycling. In the absence of the major Fd-glutamate synthase in barley mutants (0.5–1.3% of the wild-type leaf activity), the impairment of reassimilation of photorespiratory NH_4^+ leads to the accumulation of NH_4^+, even though NADH-glutamate synthase remains at the wild-type level (2.2–3.2% of the wild-type Fd-glutamate synthase activity) (Blackwell et al. 1988). In addition to the decrease in glutamate, the rate of CO_2 fixation declines to 25–30% of the initial rate at high light intensity (Blackwell et al. 1988). The inhibition of photosynthetic CO_2 fixation in the chlorotic plants might be caused by the inhibition of CO_2 exchange arising from the inactivation of CO_2 assimilating enzymes, the lack of amino donors or by high concentrations of NH_4^+, which uncouples photophosphorylation.

Analysis of Fd-glutamate synthase mutants pulsed with $^{14}CO_2$ after a short period of photosynthesis in ambient air revealed that the mutants are affected in the levels of organic acids, sugar phosphates and amino acids within 20 min. The mutants partially recover the ability to fix CO_2 and assimilate NH_4^+ during subsequent exposure to non-photorespiratory conditions, i.e., either in high CO_2 or in darkness (Somerville and Ogren 1980; Kendall et al. 1986). In high CO_2, *Arabidopsis gls* mutants exhibit wild-type levels of total protein and of chlorophyll (Grumbles 1989). In the dark, barley Fd-glutamate synthase mutants,

which exhibit Fd-glutamate synthase activity at a level only 66% of that characteristic of control plants, maintain glutamate contents and glutamate/glutamine ratios close to those of the wild-type plants (Häusler et al. 1994). This implies that the GS/glutamate synthase cycle functions *in vivo* in the dark, while in *Arabidopsis* Fd-glutamate synthase mutants, the impairment of nitrogen assimilation results in an increase in glutamine/glutamate ratios relative to those typical of the wild-type plants (Lancien et al. 2002). Analysis of the kinetics of labeling with ^{15}N revealed that the labeling of [5-^{15}N]glutamine, [2-^{15}N]glutamate, [2-^{15}N]glutamine and double labeling of [2,5-^{15}N]glutamine occurs by the sequential reactions of GS and glutamate synthase, albeit at lower rates in the dark than in the light in the leaves of some plant species. These studies support the view that the GS/glutamate synthase cycle can operate in the dark, even in the absence of energy generated by photosynthesis. Further evidence for NH_4^+ assimilation in the dark was provided by experiments utilizing antisense Fd-glutamate synthase tobacco plants, in which NH_4^+ assimilation occurs with the concomitant decrease in glutamine and 2-OG and constant level of glutamate during the dark phase of dark to light transitions (Ferrario-Méry et al. 2002). The ^{15}N labeling and inhibitor studies on the NADH-GDH null mutants suggest that NADH-GDH does not act in the assimilatory direction (Magalhães et al. 1990; Aubert et al. 2001). Therefore, these results imply that NH_4^+ is assimilated in the reaction of GS via a residual Fd-glutamate synthase and/or NADH-glutamate synthase in the dark, although at lower rates than in the light.

The observation that the Fd-glutamate synthase mutants and antisense Fd-glutamate synthase transgenic plants are fully viable when photorespiration is suppressed in high CO_2 suggests that GS and a low Fd-glutamate synthase and/or NADH-glutamate synthase remaining in the affected plants can provide sufficient amino acids during primary NO_3^- reduction, which occurs primarily in leaves. The antisense Fd-glutamate synthase transgenic tobacco plants lack NADH-glutamate synthase activity in leaves (Ferrario-Méry et al. 2000). Consequently, the residual Fd-glutamate synthase is expected to be involved in primary NH_4^+ assimilation in the leaf mesophyll cells, and the GS/glutamate synthase cycle operates at least partly during the dark to light transition in the antisense transgenic plants. In support of a role of the *GLU1*-encoded Fd-glutamate synthase in primary nitrogen assimilation, genetic studies showed that a *gls1* mutant is impaired in primary nitrogen assimilation: unlike the case of wild-type *Arabidopsis*, the *gls1* mutant (NA60) was unable to respond to increasing concentrations of exogenous NO_3^- and NH_4^+ in the increase of chlorophyll accumulation when photorespiration was suppressed in high CO_2 (Coschigano et al. 1998). However, this does not eliminate the possibility that the residual NADH-glutamate synthase in the *gls1* mutant plays a physiological function in the leaf primary nitrogen assimilation in place of *GLU1*-encoded Fd-glutamate synthase. Similarly, it is not clear whether the second *GLU2* isoform of Fd-glutamate synthase is involved in the leaf primary NH_4^+ assimilation, or whether this enzyme is responsible for supplying basal levels of glutamate for protein synthesis in leaves.

In higher plants, including Fd-glutamate synthase mutants and antisense Fd-glutamate synthase transgenic plants, Fd-glutamate synthase mRNA is expressed at high levels in leaves and low levels in roots. In contrast, NADH-glutamate synthase gene expression predominates in roots, along with a concomitant higher expresssion of the *GLN1* genes encoding cytosolic GS1 (Peterman and Goodman 1991; Suzuki and Rothstein 1997; Coschigano et al. 1998). These organ-specific expression patterns of the distinct glutamate synthase genes imply a specific role of the Fd-glutamate synthase isoforms and NADH-glutamate synthase. When ^{15}N-nitrate was supplied to barley Fd-glutamate synthase mutants in high CO_2, there was little difference in the amino acid levels in roots compared with the wild-type plants, other than a three-fold increase in glutamine and one-third decrease in glutamate (Joy et al. 1992). It thus appears that the major part of labeled nitrogen is transported as glutamine (86%) in the xylem to leaves (Joy et al. 1992). In spite of the high glutamine/glutamate ratio, which is indicative of the lower levels of glutamate synthase, both Fd-glutamate synthase and NADH-glutamate synthase activities in roots remain at wild-type levels accounting for 3% and 4% of the leaf Fd-glutamate synthase activity, respectively (Joy et al. 1992). Using a promoter-*GUS* transgene, rice NADH-glutamate synthase promoter expression was localized in the vascular bundles of the developing

leaf blades and in the dorsal and lateral bundles of developing grains (Kojima et al. 2000). This promoter expression pattern overlaps with the location of immuno-detected NADH-glutamate synthase protein (Hayakawa et al. 1994; Ishiyama et al. 2003). Because NADH-glutamate synthase and cytosolic GS1 genes share a similar organ-specific expression pattern, it has been proposed that the GS1/NADH-glutamate synthase cycle supplies the amino acids for nitrogen transport from roots to leaves or from sources to sinks through the vascular tissues (Edwards et al. 1990; Carvalho et al. 2000; Tobin and Yamaya 2001). Moreover, a role of NADH-glutamate synthase in nitrogen transport was proposed using an *Arabidopsis glt*-T knock-out mutant which lacks NADH-glutamate synthase mRNA and activity, while the activity of *GLU1* isoform of Fd-glutamate synthase remains at the wild-type level in leaves (Lancien et al. 2002). The *glt*-T mutant exhibits a deleterious growth phenotype, and fresh weight and chlorophyll contents are reduced by 20% and 30%, respectively, in the air-grown leaves. The mutant contains a 2.2-fold higher level of glutamine, and glutamate and aspartate decreased by two-thirds relative to the wild-type levels. These effects have been interpreted as arising from the impairment of glutamate synthesis in leaf veins caused by NADH-glutamate synthase deficiency (Lancien et al. 2002). Therefore, it is hypothesized that Fd-glutamate synthase does not compensate for NADH-glutamate synthase in its role in nitrogen translocation in the vascular tissues (Lancien et al. 2002). A substantial portion of the nitrogen arriving at the leaf veins through the xylem is stored, metabolized and redistributed through the phloem sieve tube elements to heterotrophic sink tissues (Andrew 1986; Pate and Layzell 1990). In addition, ^{14}C- and ^{15}N-labeling experiments showed that the bulk of nitrogen in the leaves is transported to roots, and a portion of the reduced nitrogen in roots is in turn transported to shoots as glutamine, asparagine, glutamate and aspartate (Cooper and Clarkson 1989; Jeschke and Pate 1991). In spite of the essential role of NADH-glutamate synthase for amino acid cycling in vascular tissues, the antisense Fd-glutamate synthase transgenic tobacco lines provide sufficient levels of glutamate without NADH-glutamate synthase, which is missing in both leaves and roots in high CO_2 (Ferrario-Méry et al. 2000). This implies that Fd-glutamate synthase presumably plays a redundant role with NADH-glutamate synthase in nitrogen translocation in the antisense Fd-glutamate synthase transgenic tobacco plants. Histochemical studies, which revealed that the *Arabidopsis GLU1* promoter for Fd-glutamate synthase directs *GUS* reporter gene expression in the vascular cells and in the root meristems of transgenic tobacco plants, support this hypothesis (Ziegler et al. 2003, unpublished data). This observation reinforces the view that the GS/Fd-glutamate synthase cycle controls the assimilation of nitrogen that is subsequently transported, with amino acids serving as the nitrogen carriers. The kinetics of $^{15}NH_4^+$ labeling into [5-^{15}N]glutamine, [2-^{15}N]glutamate, [2-^{15}N]glutamine and [2,5-^{15}N]-glutamine correlate with the operation of GS and glutamate synthase, not only in sink leaves but also in source leaves of tobacco (unpublished results). These data imply that GS and even a low amount of Fd-glutamate synthase and/or NADH-glutamate synthase located in the mesophyll and vascular cells provide sufficient glutamine and glutamate for transport to maintain the partitioning of nitrogen between leaves and roots. In contrast to the barley Fd-glutamate synthase mutants, which have wild-type levels of root NADH-glutamate synthase activity, NADH-glutamate synthase is missing in the roots of *Arabidopsis glt*-T knock-out mutant and in the roots of antisense Fd-glutamate synthase transgenic tobacco plants (Ferrario-Méry et al. 2000; Lancien et al. 2002). It is possible that, instead of NADH-glutamate synthase supporting the translocation of amino acids, glutamine and glutamate are formed in the roots by *GLU2*-encoded Fd-glutamate synthase isoform, which is preferentially expressed in the roots (Lancien et al. 2002) or by Fd-glutamate synthase, which is slightly enhanced in the roots of the antisense Fd-glutamate synthase transgenic tobacco under elevated CO_2 (Ferrario-Méry et al. 2000). Glutamate can also be provided by transaminase reactions. In spite of differences in the availability of the energy between the roots and photosynthetic leaves, NADPH can serve as a source of electrons for the Fd-glutimate synthase reaction by reducing a root specific Fd isoform in a reaction catalyzed by a root specific isoform of ferredoxin-$NADP^+$ reductase (Suzuki et al. 1985; Bowsher et al. 1992; Yonekura-Sakakibara et al. 2000). In alfalfa root nodules, NADH-glutamate synthase transcripts accumulate predominantly in the N_2-fixing zone of infected cells

(Vance et al. 1995; Trepp et al. 1999a). Antisense expression of a 2.5-kb NADH-glutamate synthase cDNA decreased the levels of NADH-glutamate synthase transcript and protein in root nodules to 40–50% of those found in control plants. As a result, the transgenic lines exhibit impairment in NH_4^+ assimilation and a moderate chlorotic phenotype, with pale green leaves and stems under symbiotic N_2 fixation conditions (Schoenbeck et al. 2000; Cordoba et al. 2003). Cytosolic GS1, encoded by two genes in alfalfa root nodules, is located in the transfer cells of vasculature pericycles, and it is likely that GS1 plays a role in transporting amino acids away from the infected cells of amido-transporting nodules (Carvalho et al. 2000). NADH-glutamate synthase protein can be immuno-chemically detected in the proximal part of older alfalfa root nodules (33 days old) where N_2-fixation is inefficient, and it is hypothesized that the GS1/NADH-glutamate synthase pathway is involved, at least to some extent, in the nitrogen remobilization in senescing root nodules (Trepp et al. 1999b). Apparently, Fd-glutamate synthase cannot compensate for the reduced activity of NADH-glutamate synthase in the primary NH_4^+ assimilation, even though the levels of Fd-glutamate synthase transcript increase in the transgenic root nodules (Schoenbeck et al. 2000). This could be explained by the tissue-specific expression of Fd-glutamate synthase and NADH-glutamate synthase, although the exact cell types specific to Fd-glutamate synthase are not defined in root nodules. Moreover, transformed tobacco plants over-expressing root NADH-glutamate synthase (10–40% above the wild-type levels) exhibit 10–20% increases in shoot biomass (expressed as carbon and nitrogen contents) when using NO_3^- or NH_4^+ as the sole nitrogen source (Chichkova et al. 2001). This could indicate a significant contribution of NADH-glutamate synthase during primary NH_4^+ assimilation in roots.

Conclusion

Inorganic nitrogen assimilation into glutamine, glutamate, asparagine and aspartate and the amino acid metabolism are the essential processes for plant growth and development. Biochemical and molecular studies have demonstrated that glutamate synthase occurs in three distinct forms: NADPH-glutamate synthase, NADH-glutamate synthase and Fd-glutamate synthase. The three forms are present in a wide variety of organisms and exhibit both shared and unique structural properties. Fd-glutamate synthase and NADH-glutamate synthase serve to synthesize the amino acids within the different compartments of the plant cells, which are located in the photosynthetic and non-photosynthetic organs, in tight concert with GS and with other enzymes of nitrogen and carbon metabolism. Genetic and molecular analyses using transgenic plants and mutants deficient in Fd-glutamate synthase or NADH-glutamate synthase have provided an excellent tool for studying differences in the expression of Fd-glutamate synthase and NADH-glutamate synthase in response to environmental stimuli such as light, metabolic regulation, cell- and organ-specific control and developmental regulation. Molecular analyses and biochemical and ^{15}N labeling approaches have led to a better understanding of the important regulatory mechanisms that affect ammonium assimilation into glutamine and glutamate catalyzed by the GS/Fd-glutamate synthase and/or GS/NADH-glutamate synthase cycles and the amino acid metabolism. Both of these GS/glutamate synthase cycles occur, in both light-dependent and light-independent versions, throughout all stages of plant life starting from the germination and extending through senescence. It is certainly not unreasonable to hope that these basic studies in the nitrogen assimilation and nitrogen metabolism in plants, reinforced by structural and mechanistic studies of the enzymes involved, will ultimately provide tools for improving nitrogen use efficiency under field conditions.

Acknowledgements

The authors would like to thank Dr Masakazu Hirasawa and Dr Maria Vanoni for helpful discussions. Work in DBK's laboratory was supported by a grant from the United States Department of Energy (DE-FG02-99ER20346).

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