bond formation. Because of the folding of the backbone of the molecule and the placement of the $Asn^5$ side chain over the one surface of the molecule, $N_5$ is effectively shielded from the surroundings and makes no contact of less than 3.5 Å with a neighboring peptide or water molecule.

The overall conformation of the molecule results in a structure with a large hydrophilic surface (Fig. 3, left) consisting of the terminal amino and carboxyl groups, the entire $Asn^5$ residue, portions of $Gln^4$, and the carbonyl oxygen of $Phe^3$. A hydrophobic surface, composed primarily of the $Tyr^2$ and the $Phe^3$ aromatic side chains (Fig. 3, right), lies on an edge of the hormone while the opposite edge of the molecule is occupied by the two sulfur atoms of the disulfide link. The entire molecule assumes a very compact shape because the $Gln^4$ and $Asn^5$ side chains are folded back over the ring moiety. Additional stability for these side chain conformations is provided by hydrogen bonds to the peptide backbone. The orientation of the $Asn^5$ side chain over one face of the pressinoic acid molecule is in contrast to that which has been proposed on the basis of spectral studies for both oxytocin and vasopressin (*2–4*). It has been suggested that not only is the $Asn^5$ side chain directed away from the macrocyclic ring, where it interacts with the COOH-terminal tripeptide, but also that it is one of the "active elements" of both hormones. Thus, pressinoic acid is inactive because the COOH-terminal tripeptide is absent and the $Asn^5$ side chain is in the wrong orientation.

The observed conformation of the $Tyr^2$ side chain, extended away from the center of the macrocyclic ring, is stabilized in this orientation by a nearly perpendicular $Phe^3$ aromatic ring. Similar orientations are the most commonly observed interactions between aromatic rings in proteins (*16*). The $Tyr^2$ side chain has been proposed as an additional "active element" in oxytocin and vasopressin and its orientation determines oxytocic or antidiuretic activity (*3*). Antidiuretic and vasoconstrictor activity has been proposed to result from the orientation of the $Tyr^2$ side chain away from the 20-membered ring where it is stabilized by a parallel $\pi$-$\pi$ interaction with the $Phe^3$. However, in oxytocin, $Phe^3$ is replaced by $Ile^3$, and this interaction can no longer exist. As a result, the $Tyr^2$ side chain may be rotated to a position over the macrocyclic ring, imparting oxytocic activity to the hormone. The results of our crystal structure analysis of pressinoic acid strongly support this proposal, but show that the $Tyr^2$ ring is in the commonly observed perpendicular orientation relative to the $Phe^3$ ring rather than in a parallel orientation.


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# Plant Glutamine Synthetase Complements a *glnA* Mutation in *Escherichia coli*


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**A glutamine synthetase gene from alfalfa *(Medicago sativa)* has been expressed in *Escherichia coli* after fusion of bacterial transcription and translation signals to a complete alfalfa glutamine synthetase coding sequence. Synthesis of the alfalfa glutamine synthetase enzyme in *Escherichia coli* was demonstrated by functional genetic complementation of a glutamine synthetase–deficient mutant and by immunoblotting analysis. These results should facilitate protein engineering and structure-function analysis of the plant enzyme.**


THE APPLICATION OF MOLECULAR genetics to protein engineering and structural analysis has been amply demonstrated, especially for *Escherichia coli* proteins (*1–5*). The demonstration of expression, assembly, and activity of certain foreign proteins in *E. coli* (*6*) suggests that similar molecular genetic studies on plant proteins in bacteria is feasible, particularly when a genetic complementation scheme can be devised. As a step toward the molecular genetic analysis of a plant enzyme, we describe here the complementation of an *E. coli glnA* mutation by glutamine synthetase (GS) from alfalfa.

GS is an interesting model enzyme for protein engineering and structure-function studies. In plants, GS is an enzyme of central importance in nitrogen metabolism that, together with glutamate synthase, carries out the assimilation of ammonia resulting from nitrate reduction, catabolism, and nitrogen fixation (*7, 8*). The plant GS enzyme is octameric and is usually composed of a single polypeptide species. It appears to be regulated both genetically and allosterically.

Our interest in GS resulted from the isolation of alfalfa suspension cell lines resistant to a herbicide, L-phosphinothricin, which is an irreversible inhibitor of GS (*9–11*). The mode of resistance in these lines is by increased GS enzyme activity resulting from the amplification of a GS gene. Recently, we have cloned and sequenced this amplified GS gene and the corresponding complementary DNA (cDNA) (*10*). The gene is about 4 kb long, contains 11 introns, and encodes a 1400-nucleotide-long transcript and a 39,000 molecular weight polypeptide.

For the synthesis of alfalfa GS in *E. coli*, fusion of appropriate bacterial transcription and translation signals to a complete and uninterrupted GS coding DNA sequence is required. The longest GS cDNA clone isolated in our previous work was 1.3 kb and lacked 44 nucleotides of coding sequence at the 5′ end (*10*). To reconstruct a complete GS coding sequence devoid of introns, we joined the 5′-terminal 70 bp of GS coding sequence present in a clone of a GS nuclear gene to the 3′-terminal 1000 bp of GS coding sequence present in the cDNA. The resulting construct, pGS100, was cleaved


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with Bgl II and Stu I to produce a 1.2-kb DNA fragment containing an uninterrupted and nearly complete GS coding region missing only 14 nucleotides at the 5′ end (Fig. 1). The 1.2-kb Bgl II–Stu I fragment was cloned into the *E. coli* expression vector ptac1212, which regenerated the entire GS coding sequence and placed it downstream from the bacterial tac promoter and *lacZ* ribosome binding site (Fig. 1) (*12–14*). The resulting GS expression plasmid is pCGS2 (Fig. 1).

Expression in *E. coli* of the alfalfa GS gene as constructed in pCGS2 was initially demonstrated by genetic complementation of a GS-deficient (Δ*glnA*) bacterial mutant (*15*). This strain, FDB213, was transformed with pCGS2 and both the parental and plasmid-containing strains were tested for growth on M9 minimal medium containing or lacking glutamine. The results (Fig. 2) show that the parental FDB213 strain requires glutamine, but the FDB213(pCGS2) derivative is prototrophic. Thus, alfalfa GS must be synthesized in *E. coli* and, furthermore, must at least partially fold and assemble into a catalytically active enzyme. Consistent with this conclusion, GS enzyme activity was detected in FDB213(pCGS2) but not in FDB213(ptac1212) or FDB213 using the hemibiosynthetic enzyme assay (*16*).

Complementation of FDB213 by the alfalfa GS gene in pCGS2 (Fig. 2) was tested at a high ammonium chloride concentration (18 m*M*), requiring that alfalfa GS catalyze the synthesis of glutamine necessary for protein synthesis, but not necessarily function as part of the primary pathway for assimilation of ammonia. Bacterial glutamate dehydrogenase can carry out the assimilation of ammonia at high ammonium concentrations. However, in medium containing low ammonium concentrations (≤0.5 m*M*) or an alternative nitrogen source (L-arginine), assimilation of ammonia requires the GS/glutamate synthase cycle. This is due to the relatively higher $K_m$ for ammonia and lower reaction equilibrium constant in the case of glutamate dehydrogenase compared to GS (*7, 17*). Thus, to determine if alfalfa GS can function in the assimilation of ammonia in *E. coli*, we tested the ability of FDB213(pCGS2) to grow in minimal medium containing either a low concentration of ammonium chloride (0.5 m*M* and 0.1 m*M*) or L-arginine (0.2 percent) as the sole nitrogen source. The FDB213(pCGS2) strain could grow at 0.5 m*M* ammonium chloride concentration (the generation time being four times longer than at 5 m*M*), but could not grow at 0.1 m*M* ammonium chloride concentration or using L-arginine as the sole nitrogen source. These results suggest that assimilation of ammonia cannot be carried out efficiently by alfalfa GS in *E. coli*, assuming that the $K_m$ for ammonia for the plant enzyme is similar to that for the bacterial enzyme.

Synthesis of the alfalfa GS enzyme in *E. coli* was confirmed by immunoblotting analysis (*18*). *Escherichia coli* W3110*lacI*$^q$ containing pCGS2 was grown in the presence or absence of inducer, isopropylthio-β-D-galactoside (IPTG), harvested, lysed, and fractionated on a denaturing 10 percent polyacrylamide–sodium dodecyl sulfate (SDS) gel or a nondenaturing 5 percent polyacrylamide gel. The proteins were transferred to nitrocellulose, and reacted first with antiserum against alfalfa GS, followed by staphylococcal protein A–horseradish peroxidase conjugate. The hybridization was detected colorimetrically (*19*). On the denaturing gel (Fig. 3A) a single band is visible for the *E. coli* extracts (lanes 1 and 3) and this band comigrates with authentic alfalfa GS (lane 4). Similarly, on the native gel (Fig. 3B) a single band is visible for the *E. coli* extracts (lanes 5 and 6) and this band comigrates with the upper band in the plant extract (lane 7). Thus, pCGS2 directs the synthesis in *E. coli* of a protein that cross-reacts with alfalfa GS antiserum and comigrates with alfalfa GS on denaturing and nondenaturing gels. The GS enzyme bands detected in extracts from the *E. coli* W3110*lacI*$^q$(pCGS2) strain were not present in extracts from the parental strain.

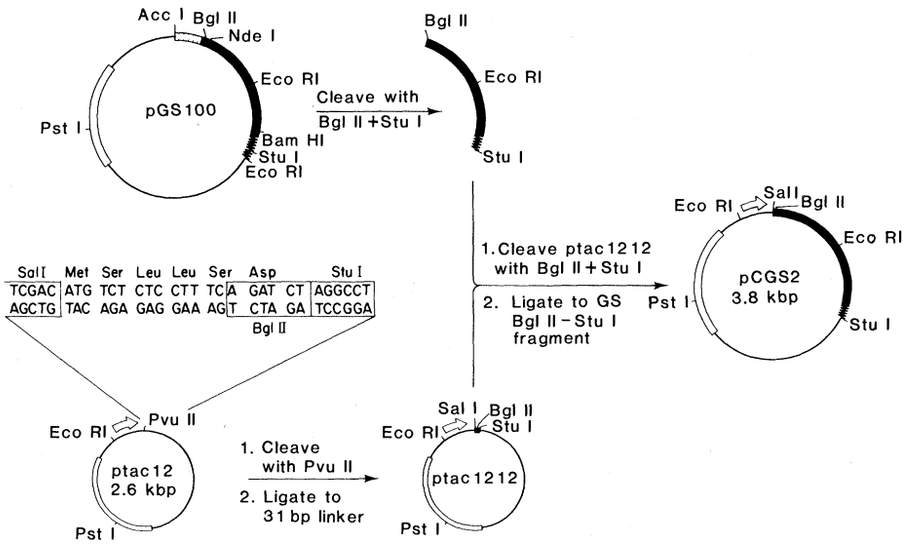


Fig. 1. Construction of the alfalfa glutamine synthetase expression plasmid pCGS2 and intermediate plasmids pGS100 and ptac1212. (pGS100) Construction of the complete alfalfa cDNA in pGS100 was accomplished by cloning two cDNA restriction fragments and a genomic DNA restriction fragment into pSP65 (*24*) in two steps. First, the 270-bp Bam HI–Eco RI cDNA fragment encoding the 3′ end of the GS coding region and the 3′ noncoding region was cloned into pSP65 at the polylinker Bam HI and Eco RI sites [see (*10*) for sequences and sites]. Second, this construct was cleaved with Bam HI and Acc I in the polylinker site and ligated simultaneously with the 950-bp Nde I–Bam HI internal cDNA fragment and the 310-bp Acc I–Nde I genomic DNA fragment encoding the 5′ end of the GS coding region. The resulting plasmid, pGS100, contains a 1.6-kb Acc I–Eco RI fragment insert (5′ Acc I–Nde I 310-bp genomic DNA—Nde I–Bam HI 950-bp cDNA—3′ Bam HI–Eco RI 300-bp cDNA). (ptac1212) Plasmid ptac1212 is a derivative of ptac12 (*12*). Plasmid ptac12 contains the *E. coli* tac promoter (*14*) and the *lacZ* ribosome binding site followed by a unique Pvu II site. For construction of ptac1212, a 31-bp double-stranded synthetic oligonucleotide was cloned into the Pvu II site of ptac12 (*12*). This resulted in the introduction just downstream of the tac promoter of (i) a Sal I site (GTCGAC) by ligation of the vector Pvu II site (. . . CAG-3′) to the oligonucleotide (5′-*TCGAC* . . .), (ii) the 5′-terminal 20 nucleotides of the GS coding sequence containing the translation initiation codon and a Bgl II site, and (iii) a Stu I site. The sequence of the oligonucleotide is indicated above ptac12. (pCGS2) For construction of pCGS2, the 1.2-kb Bgl II–Stu I fragment from pGS100 was cloned into the Bgl II and Stu I sites of ptac1212. Standard procedures were followed for all constructions (*13*). In general, restriction fragments used for construction of plasmids were isolated after electrophoresis on low-melting agarose gels. Ligations were performed using T4 DNA ligase, the ligation reaction products used to transform *E. coli* W3110*lacI*$^q$ (*12*), and ampicillin-resistant colonies selected. Small-scale plasmid preparations from transformants were analyzed by restriction mapping and plasmids meeting restriction mapping criteria were used for subsequent constructions. The accuracy of the final construction (pCGS2) was confirmed by restriction mapping using Eco RI, Sal I, Bgl II, Nde I, Bam HI, and Stu I. In addition, the DNA sequence of the tac promoter and 5′-terminal coding region of GS was determined by the chain termination method (*25*). The sequence of the oligonucleotide used for sequencing was 5′-GGGTCAGTAACTGGT-3′. It hybridizes to the third exon of the GS gene (*10*). The plasmids are drawn roughly to scale. The filled boxes represent the coding region of GS; the hatched box and wavy line represent the 5′ and 3′ noncoding regions of the GS gene, respectively; the open boxes represent the vector ampicillin resistance gene; the arrow indicates the approximate position of the tac promoter and *lacZ* ribosome binding site.

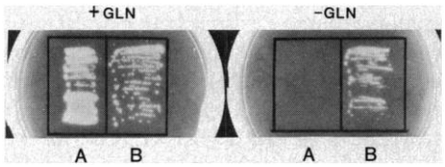


Fig. 2. Genetic complementation of an *E. coli* mutant by an alfalfa glutamine synthetase gene. (A) *Escherichia coli* FDB213 *(endoA, thi-1, hsdR17, supE44, pro, ΔglnAG)*, a derivative of 294ΔAG (*15*), was tested for growth in the presence of 8 m*M* L-glutamine (+GLN) or in the absence of L-glutamine (−GLN). (B) *Escherichia coli* FDB213(pCGS2) was tested for growth in the presence (+GLN) or absence (−GLN) of L-glutamine. The medium used in this experiment was M9 that had been supplemented with 50 μ*M* thiamine and 2 m*M* L-proline. No inducer (IPTG) is present in the medium.

The results in Fig. 3 also confirm the expected subunit molecular weight and native structure for the alfalfa GS enzyme synthesized in *E. coli*. The subunit molecular weight of alfalfa GS, 39,000, based on polyacrylamide-SDS gel analysis, is consistent with the molecular weight predicted from the DNA sequence of the GS gene (*10*). *Escherichia coli* GS has a subunit molecular weight of 55,000 (*7*) and does not visibly cross-react with the antiserum against the plant enzyme in these experiments. The relatively slow rate of migration of GS on the native gel is consistent with the known octameric quaternary structure of eukaryotic GS enzymes (*7*). As indicated by the distribution of protein in the gel, essentially all of the soluble plant GS enzyme in *E. coli* appears to be in the native conformation, indicating that it is efficiently folded and assembled. An additional conclusion from this work is that a single alfalfa GS polypeptide species is sufficient to form a catalytically active enzyme (*20*).

The results described above indicate that the tac promoter efficiently directs synthesis of alfalfa GS in *E. coli*. GS protein is synthesized in detectable amounts even under repressed conditions (Fig. 3). Consistent with this result, complementation of an *E. coli ΔglnA* mutant requires only repressed levels of alfalfa GS (Fig. 2). Induction with IPTG results in a three- to fivefold increase in the levels of GS (Fig. 3, lanes 1 and 3, 5 and 6). At the induced level GS constitutes approximately 1 percent of total cell protein. Fusion of the tac promoter to the *E. coli malPQ* operon also resulted in qualitatively similar results (*21*). This presumably reflects properties of the tac promoter.

We have demonstrated that a plant gene encoding glutamine synthetase, when fused to bacterial transcription and translation signals, can genetically complement a bacterial mutant. Although expression of bacterial genes in plant cells is common [for example, drug resistance genes (*22*)], to our knowledge this report constitutes the first example of complementation of a bacterium by a plant gene. Our results suggest that the plant enzyme is folded and assembled into an essentially native conformation in the bacterium. Hence we have generated a system where the methods of random and directed mutagenesis (*23*) and bacterial genetics can be used for structure-function studies on a plant enzyme. Furthermore, our results make it possible to select directly in *E. coli* for an L-phosphinothricin–resistant alfalfa GS gene, which could be useful in the development of herbicide-resistant plants.

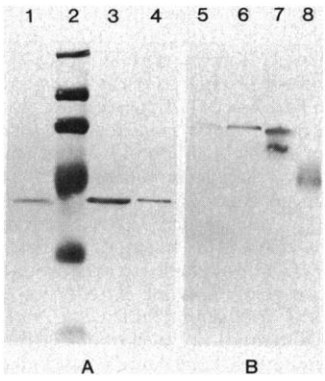


Fig. 3. Immunoblotting analysis of alfalfa glutamine synthetase expressed in *E. coli*. (A) *Escherichia coli* W3110*lacI*$^q$ containing pCGS2 was grown in LB medium to $OD_{600}$ = 0.7 and one-half of the culture was induced by addition of 1 m*M* IPTG. The cells were collected by centrifugation and lysed by addition of a solution containing 125 m*M* tris-HCl (*p*H 7), 0.5 percent SDS, 10 percent glycerol, 0.7*M* 2-mercaptoethanol, and heating at 100°C for 5 minutes. Total cell protein was fractionated on a 10 percent polyacrylamide-SDS gel (*26*) alongside prestained molecular weight markers (Bethesda Research Laboratories) and partially purified alfalfa GS (*9*). The proteins were transferred to nitrocellulose by electroblotting, reacted first with alfalfa GS antiserum followed by staphylococcal protein A–horseradish peroxidase conjugate, and developed by a colorimetric assay (according to the protocol provided by Bio-Rad, the manufacturer of the protein A–horseradish peroxidase conjugate) (*18, 19*). The GS antiserum had been prepared from rabbits injected with purified alfalfa GS (*9*). (Lane 1) *Escherichia coli*(pCGS2) uninduced; (lane 2) prestained protein markers (myosin H-chain, 200,000; phosphorylase B, 97,400; bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen, 25,700; β-lactoglobulin, 18,400); (lane 3) *E. coli*(pCGS2) induced with IPTG; and (lane 4) purified alfalfa GS enzyme. (B) *Escherichia coli* W3110*lacI*$^q$ containing pCGS2 grown as in (A) was collected and treated with 10 mg/ml lysozyme at high cell density. The cells were lysed by sonication, clarified by centrifugation, and the cell lysates were fractionated on a native 5 percent polyacrylamide gel (*27*) alongside crude extracts from the 3 m*M* L-phosphinothricin–resistant alfalfa suspension cell line (*9*). Immunoblotting analysis was performed as in (A). (Lane 5) *Escherichia coli*(pCGS2) uninduced; (lane 6) *E. coli*(pCGS2) induced with IPTG; (lane 7) crude extract from alfalfa; and (lane 8) denatured alfalfa GS. The lower band in lane 7 is likely to be a partially denatured form of GS (possibly a tetramer) that was present in variable levels depending on the extraction procedure.

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